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RESEARCH ARTICLE

PRODUCTION AND CHARACTERIZATION OF LACCASE FROM *ASPERGILLUS TERREUS* ISOLATED FROM SAW MILL SOIL OF OSMANABAD

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Abstract

The novel fungal laccase was produced from *Aspergillus terreus* –S19 isolated from saw mill soil of Osmanabad. The enzyme laccase was produced in solid state fermentation by incubating fungal strain up to 96h incubation, and partially purified by the sequential method of Ammonium sulphate precipitation followed by nominal molecular weight limit (NMWL) cut-off ultra filtration using 30K filters. The partially purified enzyme showed 91.08 U/L of enzyme activity. The molecular weight of the enzyme was confirmed about 56.0 kilodalton (kDa) by the SDS-PAGE analysis. The partially purified enzyme showed excellent activity at pH 5.0 and temperature 30^0C. The enzyme activity enhanced in the presence of Cu^{2+}, Ca^{2+}, surfactants and organic acids, whereas; the L-cysteine completely inhibit the enzymatic activity. In conclusion, the novel laccase enzyme obtained from *Aspergillus terreus* –S19 explored the promising candidature for the several potential applications in the decolourization of dyes, degradation of lignin, pigment xenobiotics, pulp and paper industries, food industries, wine clarification, and several other industrial processes.

Introduction:-

Laccase is a family of multicopper oxidases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) which are widely distributed among higher plants, fungi, bacteria and arthropods. White rot fungi are the most significant lignin degraders among the wood inhabiting microorganisms. Among these fungi degrade lignin efficiently by secreting extracellular enzymes like laccase, MnP and LiP that play a key role in lignin biodegradation (Assavanig et al., 1992; Buddolla Viswanath et al., 2008; Sidhu et al., 2014). Aspergillus sp. is a white rot fungus belongs to Ascomycetes family that produces an efficient laccase enzyme and probably involved in degradation of environmental biomass depository lignin in nature (Kiiskinen et al., 2004; Gupte et al., 2007; Winquist et al., 2008; Jhadav et al., 2009; Brijwani et al., 2010; Kumar et al., 2016).

Solid state fermentation (SSF) is the most common and low cast technique for production of many fungal enzymes, especially where the agricultural waste raw materials are used as a main source of nutrients. SSF is more cost effective than the submerged fermentation, as it requires simple set up for the production of fungal enzymes (Fenice et al., 2003; Desai et al., 2011; Elsayed et al., 2012). Many fungal laccase are belong to glycoproteinious in nature

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with covalently-linked carbohydrate moiety (10-45%) and having average molecular weight ranging from 50-100 kDa. The carbohydrate contents of the enzyme may provide stability to the fungal laccase in some circumstances while being used in harse applications (Assavanig et al., 1992; Gianfreda et al., 1999; Machado et al., 2005; Kunamneni et al., 2007; Jeon et al., 2012). The laccase enzyme from Aspergillus sp. are well known to degrade several phenolic aromatic amines and phenolic compounds and it has wide range of applications in decolourization of textile dyes, degradation of xenobiotics, depolymerisation of lignin, pigment degradation, pulp and paper bleaching, biosensor for biofuel cells, and many more (Wilkołazkaa et al., 2002; Kunamneni et al., 2007; Munusamy et al., 2008; Singh D A et al., 2010; Viswanath et al., 2014).

In light of this, we reported the production, purification and characterization of novel laccase from *Aspergillus terreus* –S19 isolated saw mill soil of Osmanabad. The partially purified enzyme was assayed for the broad range pH and temperature stability and compatibility using various metal ions, surfactants, and inhibiting agents. The enzyme also assessed for its application in dye decolorization.

Material and methods:-

Isolation, selection and identification of fungal strain

The saw mill area soil of Osmanabad was screened and the potent S19 fungal strain was selected for laccase production. The isolated fungal strain subcultured on Czapek dox agar slants for further production and strain identification purpose (Shaikh et al., 2020)

Identification of fungal strain for laccase production

The S19 fungal strain was subjected for primary identification by microscopic identification. Finally, the isolated fungal strain was identified by fungal genomic DNA isolation using the InstaGeneTM Matrix Genomic DNA isolation kit – As per the kit instructions and 18S rRNA gene sequencing (J. Castresana, 2000).

Production of fungal laccase by solid state fermentation (SSF)

The SSF was carried out according to Shaikh et al., 2020 for laccase production. The SSF 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). The experiments performed in six flasks and each flask inoculated with 2 ml of spore suspension prepared from a seven days old Czapek dox agar plate of the culture grown at 30°C. After 4 days of incubation, all flasks were added with 25 ml of 0.1M acetate buffer (pH 5.0) and filtered through muslin cloth to obtain clear filtered extract. The obtained extract was centrifuged at 10000 rpm for 10 min and the 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 1.2 ml of sodium acetate buffer (0.1 M, pH 5.0); 0.5mM 500µl of ABTS; 0.5 ml of crude enzyme to obtain 2.2 ml final volume. For blank, 0.5 ml of water was added instead of crude enzyme sample. The mixture was then incubated for 3 min at 30^0C. The absorbance was measured immediately at 420 nm with molar extinction coefficient [Ɛ420] = 36,000M^{-1}.

Laccase activity can be calculated by the formula

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

ΔE= Absorbance at 420 nm

ε = 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

Purification of laccase enzyme

The centrifuged clear supernatant subjected to ammonium sulphate precipitation followed by overnight dialysis in chilled 0.1M acetate buffer (pH 5.0) then the dialysed retentant sample subjected to 30 kDa NMWL cut of ultracentrifugation to concentrate and removal of impurities having molecular weight less than 30 kDa. All the samples subjected for protein and enzyme estimation.

Protein concentration analysis
The protein concentrations in the samples were estimated according to the Lowry et al, 1951. The absorbance measured at 660 nm and BSA used as a standard protein.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)
The protein profile and molecular weights of sample analysed by SDS-PAGE (Shaikh et al, 2018). The crude and partially purified samples were diluted in a native sample buffer solution and loaded on 12% resolving gel. After run, the resolved bands were visualized by Coomassie brilliant blue R-250 staining method. Standard broad range protein marker (Merck, India) values were compared to evaluate protein molecular weights of samples.

Effect of temperature and pH on laccase activity
The effect of temperature on enzyme activity investigated by pre-incubating enzyme at temperatures (20, 30, 40, 50 and 60 ^{o}C) for 10 min before measurement of enzyme activity at respective pH and temperature. The pH range 3–8 [acetate buffer (pH range 3-6) and phosphate buffer (pH range 7-8)] was used to evaluate effect of pH on enzyme activity.

Effect of metal ions on the enzyme activity
The effect of metal ions on the enzyme activity was evaluated by the addition of 1mM concentration of metal ions Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, and Co^{2+} metal to the enzyme solution. The enzyme solution was pre-incubated with metal ions for 10 min before addition to the substrate and allowed to react for 10 min at 30^{o}C. The laccase activity estimated as per previously described enzymatic assay method.

Effect of enzyme inhibitors and surfactants on laccase activity
The enzyme inhibitors Trichloroacetic acid, ethylenediaminetetraacetic acid (EDTA), urea, L-cysteine, and β-mercaptoethanol were used as enzyme inhibitors. The 1mM concentration of each inhibitor was added to the enzyme solution before measuring laccase activity. The influence of 1% surfactants (Tween-20, Tween-80, sodium dodecyl sulphate and Triton-X-100) were studied by the addition these surfactants to the enzyme solution before measuring laccase activity.

Dye decolorization activity of partially purified laccase
Dye decolorization experiments were performed on Bromophenol blue, methylene blue, and Congo red. 1.0 mL of partially purified laccase enzyme solution was added to 10 mL (100 mg/L) of each dye solution prepared in sodium acetate buffer (0.1 M, pH 5.0) and incubated on rotary shaker at 30°C for 3 h. 1 mL samples removed after every hour treatment and measure for the decrease in absorbance compared with the respective control dye samples. The percent (%) dye decolourization was calculated by the formula: (%) dye decolorization $= [(Ai-At)/Ai] \times 100$
Ai = initial absorbance of the dye
At = absorbance of the dye after every time interval
The dye decolorization experiment was performed in triplicate and mean values of decolorization percentage were reported.

Results and discussion:-

Isolation and identification of fungal strain
The S19 fungal strain isolated from saw mill soil was found a potent laccase producer strain and it is identified as *Aspergillus terreus* on the basis of 18s RNA molecular identification. The pure sample of the strain showed best scored homology with *Aspergillus terreus* after phylogenetic tree analysis and the sequence has been deposited in NCBI gene bank with accession number NCBI:txid33178.

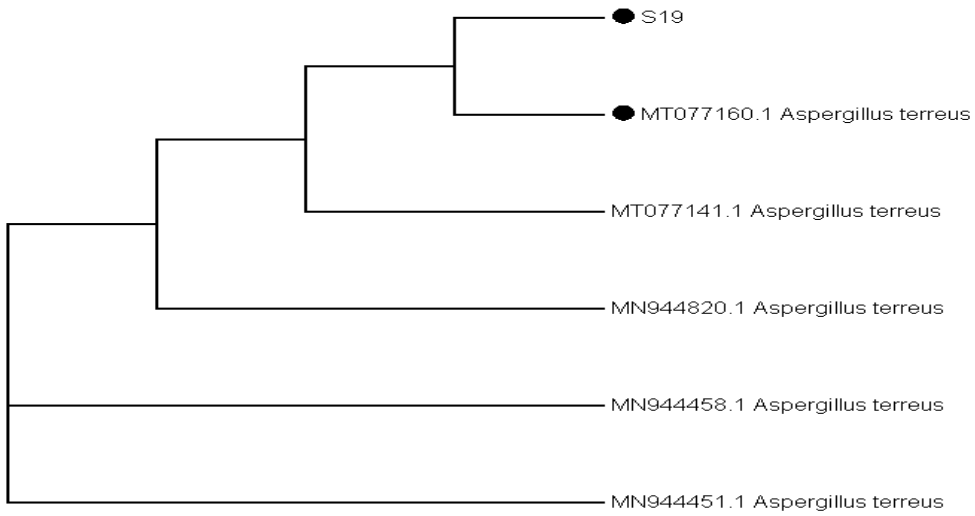

Fig. 1:- Neighbor-joining phylogenetic tree analysis of the isolated strain S19 and its closest fungal strains based on 18S rRNA gene sequences.

Partial purification of laccase enzyme

After 96 hr incubation 150 ml clear supernatant obtained with 15.84 U/L laccase activity. The obtained broth subjected for ammonium sulphate precipitation and centrifuged at 10000 rpm 10 min. The supernatant discarded and pallet dissolved in 0.1M acetate buffer (pH 5.0) and dialysed overnight in the same buffer. The dialysed sample showed 40.92 U/L enzyme activity. Finally the partially purified enzyme subjected to 30 kDa NMWL ultracentrifugation filters. After nominal cut off filtration and concentration the partially purified enzyme showed 91.08 U/L of enzyme activity. The purified laccase sample showed a distinct band at 56.0 kDa on native SDS-PAGE (Fig. 2).

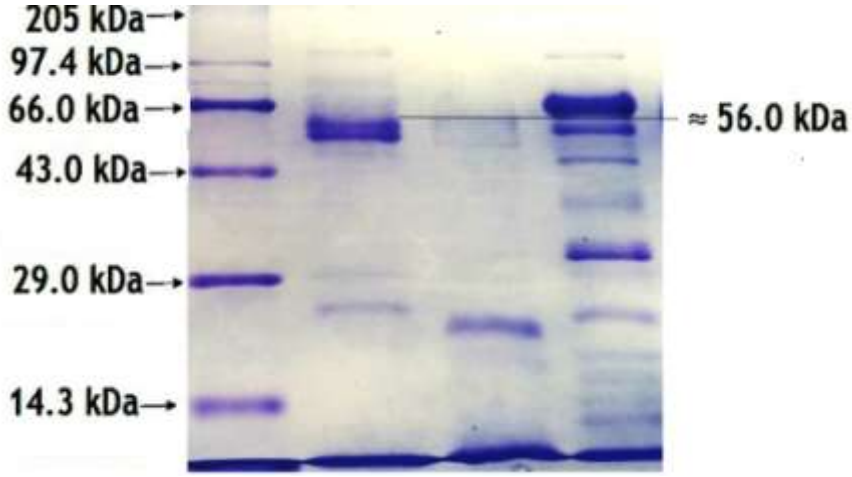

Fig. 2:- SDS-PAGE analysis of laccase enzyme. 12% gel resolved under the native condition and stained with coomassie brilliant blue G-250. Lane1: standard protein marker, lane 2: 30 kDa ultracentrifugation retaintant sample, lane 3: 30 kDa ultracentrifugation filtrate (flow through) sample, and lane 4: cell-free supernatant enzyme sample. The arrow demonstrates molecular weight in kilodalton (kDa).

Effect of pH and temperature on laccase activity

The optimum pH for the partially purified laccase activity was determined by incubating the enzyme in acetate buffer (pH 3-6) and phosphate buffer (pH 7-8). The results demonstrated that the partially purified laccase enzyme has the optimum activity at pH 5.0 and temperature at 30^0C. The enzyme activity decreased above and below of the

optimal pH and temperature (Fig. 4). Bagewadi et al. reported laccase from Trichoderma harzianum having average molecular weight about 56 kDa.

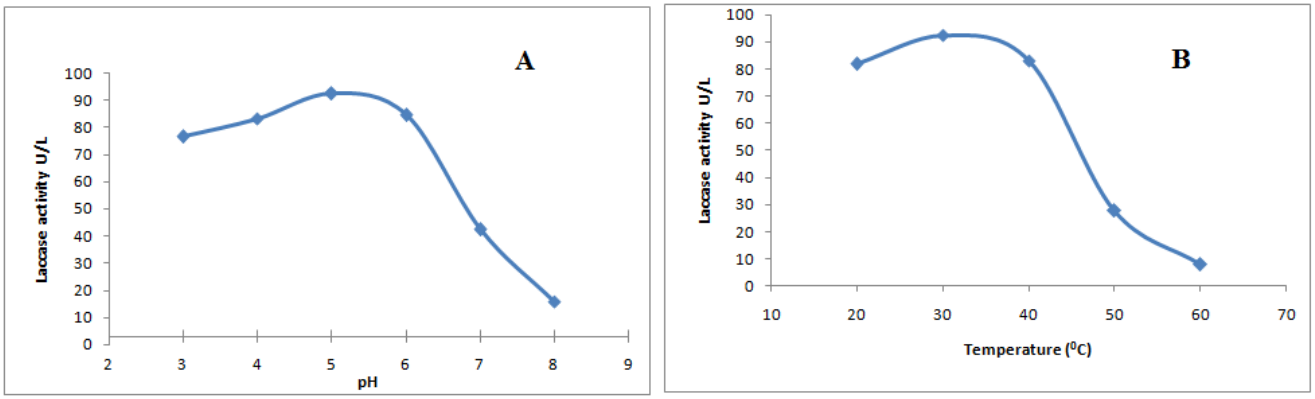

Fig.3:- Effect of pH and temperature on laccase activity. (A) Demonstrate effect of pH on laccase activity, whereas; (B) depicts the effect of temperature on enzyme activity.

Effect of metal ions, enzyme inhibitors and surfactants on laccase activity.

The effect of metal ions on the enzyme activity was evaluated by the addition of 1mM concentration of Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, and Co^{2+} metal ions to the enzyme solution. The enzyme solution was pre-incubated for 10 min before measuring enzyme activity. The results demonstrated that the partially purified laccase enzyme activity significantly enhanced in Cu^{2+} compared to other metal ions (Fig.4).

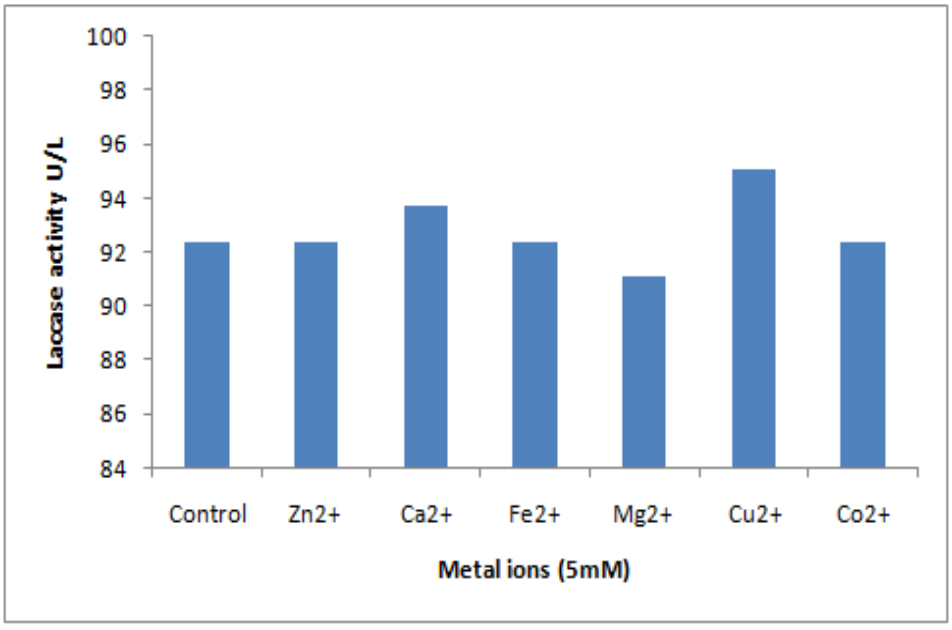

Fig. 4:- Effect of various metal ions on laccase activity.

The effect of inhibitors [Trichloroacetic acid, ethylenediaminetetraacetic acid (EDTA), urea, L-cysteine, and β-mercaptoethanol] and surfactants [Tween-20, Tween-80, Sodium Dodecyl Sulphate and Triton-X-100] on laccase activity was evaluated by adding substances at 1% concentration before measurement of laccase activity. The results demonstrated that L-cysteine completely inhibit the enzyme activity, whereas surfactants has a modest effect on enzyme activity (Fig. 5).

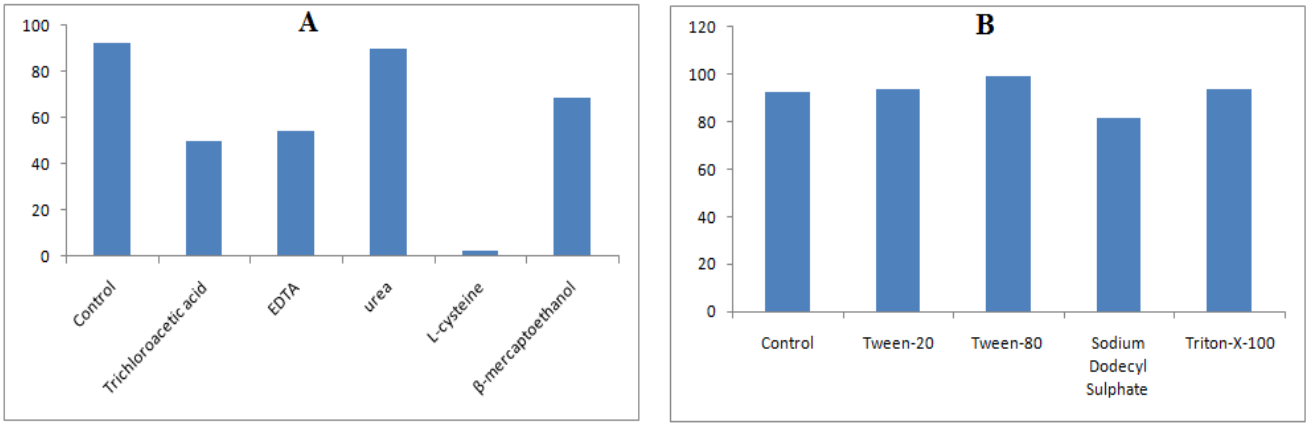

Fig.5:- Effect of enzyme inhibitors and surfactants on laccase activity. (A) Demonstrate Effect of enzyme inhibitors on laccase activity, whereas; (B) depicts the effect of surfactants on enzyme activity.

Dye decolorization activity by laccase enzyme:

The dye decolourization ability of partially purified laccase was assayed using bromophenol blue methylene blue and Congo red. The results demonstrated that the purified enzyme showed excellent dye decolorization ability at 30^{o}C (Table 1).

Table 1:- Percent dye decolorization of enzyme after 3h treatment.

Name of Dye	Concentration of dye (mg/L)	(λmax)	% dye decolorization			
			0 hours	1 hours	2 hours	3 hours
Bromophenol blue	100	592	0	26.7	56.9	66.9
Methylene blue	100	610	0	27.1	55.5	63.1
Congo red	100	514	0	23.7	49.9	71

Conclusion:-

In the present study, we isolated *Aspergillus terreus* –S19 fungal strain from saw mill soil of Osmanabad. After 96 h incubation, a partially purified 56.0 kDa laccase enzyme was obtained that showed optimal enzyme activity at pH 5.0 and 30^{o}C. The enzyme has good range of compatibility with various surfactants and inhibiting agents. The partially purified enzyme may find potential applications in various decolourization of textile dyes, biotechnological processes, depolymerisation of lignin, pulp and paper bleaching industry and several other industrial processes.

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