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INVESTIGATION OF NOVEL LACCASE PRODUCING FUNGAL SPECIES AND ITS SUBSTRATE SPECIFICITY BY WET AND DRY LAB

INVESTIGACIÓN DE UNA LACASA NOVEDOSA PRODUCTORA DE UNA ESPECIE FÚNGICA Y SU ESPECIFICIDAD SOBRE EL SUBSTRATO POR MÉTODOS HÚMEDOS Y SECOS

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Abstract

New and efficient laccases are needed for degrading lignin in various applications. Therefore, present study aimed at the screening and identification of effective laccase producing fungal strain. A total of 30 fungal strains were isolated from air, soil and decaying wood of *Mangifera indica* and screened for their ability to produce laccase on solid medium having tannic acid, guaiacol and syringaldazine as indicator compounds. For quantitative enzyme production, laccase producing strains were grown on fermentation medium having sawdust as a solid substrate. The strain which gave maximum enzyme activity was identified using conventional and molecular methods. Wet and dry lab experiments were performed for determination of better substrate for enzyme using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), guaiacol and syringaldazine. Our results showed that out of 30 only 15 strains exhibited laccase producing capability and the strain MFw3 showed highest enzyme activity of 297±1 U/ml. Phylogenetic tree analysis showed the novelty of *Aspergillus flavus* MFw3 (GenBank accession number: MF167358). Molecular docking between enzyme and substrate resulted that ABTS exhibited better affinity for laccase with minimum dock score (12.5 Å) as compared to guaiacol (14.7 Å) and syringaldazine (15.5 Å).

Keywords: Laccase, sequence analysis, ABTS, docking.

Resumen

Existe una necesidad de lacasas novedosas y eficientes para degradar lignina en diversas aplicaciones. En este estudio se analizaron e identificaron lacasas con eficiencia para producir cepas fúngicas. Un total de 30 cepas fúngicas fueron aisladas de aire, suelo y de madera en descomposición de *Mangifera indica* y analizadas por su habilidad depara producir lacasa en medio sólido conteniendo ácido tánico, guayacol y siringaldazina como compuestos indicadores. Para una producción cuantitativa de la enzima, las cepas productoras de lacasa se cultivaron en un medio de fermentación conteniendo aserrín como sustrato sólido. La cepa que produjo la máxima actividad enzimática se identificó a través de métodos convencionales y moleculares. Experimentos en húmedo y seco en el laboratorio se desempeñaron para determinar el mejor substrato para la enzima usando ácido 2,2'-azino-bis (3-etilbenzotiazolina-6-sulfónico), guayacol and siringaldazina. Nuestros resultados mostraron que solo 15 de 30 cepas exhibieron la capacidad de producir lacasa y la cepa MFw3 mostró la mayor actividad enzimática de 297±1 U/ml. Un análisis filogenético de árbol mostró la novedad de *Aspergillus flavus* MFw3 (Número de accesión en GenBank: MF167358). El acoplamiento molecular entre la enzima y el sustrato result en que ABTS exhibió una major afinidad por la lacasa con una calificación mínima de acoplamiento (12.5 Å) comparado al guayacol (14.7 Å) y a la siringaldazina (15.5 Å).

Palabras clave: Lacasa, análisis secuencial, ABTS, acoplamiento.

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1 Introduction

Laccases are blue multicopper oxidases (MCO) which catalyze the oxidation of various phenolic and aromatic compounds with the reduction of molecular oxygen to water (Si *et al*., 2013; Zhuo *et al*., 2017). Laccase have various applications in different fields including medical diagnosis, pharmaceutical industries, cosmetics and textile etc. In the agricultural industry, it is used for the removal of herbicides and pesticides (Couto and Herrera, 2006). In food industry, laccases are used to transform cross linking of biopolymers and also used for color modification (Selinheimo *et al*., 2006), in nano-biotechnology for their application in bio-electrochemistry, bioremediation of polyethylene (Santo *et al*., 2013) and most importantly, in the decolorization of textile dyes (Telke *et al*., 2009; Liang *et al*., 2018).

Laccases are widely distributed in various plants, bacterial and fungal species (Qiu *et al*., 2014). In fungi, it plays an important role in various functions such as morphogenesis, pathogenesis, delignification, pigment formation and in transport of metal ions (Strong and Claus, 2011). In comparison to all other sources, extracellular laccases produced by fungi are of major interest due to their ability to degrade lignocellulosic biomass. It is usually present in Ascomycetes, Basidiomycetes and Deuteromycetes.

Fungal laccases have ability to oxidize various organic compounds especially phenolic compound (Cañas and Camarero, 2010) and can be extended to non-phenolic compounds by the addition of mediators. The mediators are group of low molecular weight organic compound such as ABTS (2, 2' azino bis (3 ethyl benzthiazoline-6 sulfonate), 1 hydroxy benzotriazole (HOBT), syringaldehyde etc. They are oxidized by the laccase. These mediators form highly active cations radicals which have ability to oxidize non-phenolic compounds that laccase cannot oxidize alone (Gochev and Krastanov, 2007). So, the better choice of mediators enhances laccase action against recalcitrant compounds.

This study was planned to investigate novel laccase producing fungi. Additionally, *in vitro* and *in silico* studies were performed for selection of better substrate to increase the catalytic efficiency of enzyme. Structure of laccase was obtained by bioinformatics tools to gets molecular information of the protein and its substrates to list it in to versatile biocatalyst and identification of aminoacid residues at the active site of enzyme are important to know the structure function relationship.

2 Materials and methods

2.1 Isolation of Fungi

Laccase producing fungi were isolated from air, soil and decaying wood of *Mangifera indica* near Lahore College for Women University, Lahore, Pakistan. Isolation of fungi from air was carried out by direct exposure of culture medium plates to air. Whereas, soil and wood samples were brought to lab in sealed and sterile plastic bags. 1g of each sample was added to 10 mL autoclaved water and mixed well. Samples were serially diluted up to 10^{-9} dilution factor. 100 μL from last two dilutions (10^{-8} and 10^{-9}) were spread evenly on potato dextrose agar plates containing three different indicator compounds i.e. 0.5% Tannic acid (Merck, USA), 0.01% Guaiacol (sigma, USA) and 0.01% syringaldazine (sigma, USA). These plates were incubated at 25 ºC for 7 d. Presence of laccase producing fungi is indicated by the appearance of coloured zone around fungal growth.

2.2 Screening of cultures

Screening of laccase producing fungi was carried out in 250 mL Erlenmeyer flasks. Each flask having 5g sawdust as solid support and 10 mL mineral medium (peptone 6.0 g/L, Glucose 5.0 g/L, KH_2PO_4 0.025 g/L, $MgSO_4.7H_2O$ 0.25 g/L, KCl 0.5 g/L). Flasks were sterilized at 121ºC for 15 min. Fermentation medium was inoculated with 5 agar plugs (5mm in diameter) per flask from fungal colony grown in potato dextrose agar slants. Flasks were incubated at 25ºC in complete darkness (Salmones and Mata, 2015; Nasreen *et al*., 2015) for 7 days under static conditions.

2.3 Extraction of enzyme

After 7 days of incubation, samples of each flask were mixed with 25 mL of 5mM sodium acetate buffer (pH 5.0) and were stirred on rotary shaker (GFL 3033, Germany) at 150 rpm for 1 h. Samples were then centrifuged at 6000 rpm for 10 min. Solids were discarded and culture supernatants were used for extracellular laccase enzyme activity (Akpinar and Urek, 2012).

2.4 Enzyme assay

Extracellular laccase enzyme activity was determined by monitoring the oxidation of 5mM ABTS (Sigma, USA) in 1M sodium acetate buffer, pH 4.5 at 420nm (Johannes *et al*., 1996; Carmona and Blandon, 2015). One unit of enzymatic activity is defined as the activity of an enzyme that transforms 1 μmol of substrate per minute.

2.5 Native-PAGE

In order to confirm the presence of laccase in isolated enzyme. Native-PAGE was performed using 8% polyacrylamide gel. Gel was run at 80mV for 3.0 h at 4ºC. For activity staining, non-denaturing gel was incubated for 15 minutes in 100mM sodium acetate buffer (pH 4.5) with 5mM ABTS as described by Patel *et al*. (2014).

2.6 Strain identification

2.6.1 Morphological identification

The fungus was identified morphologically under light microscope (NIKON ECLIPSE LV100, JAPAN). Lactophenol blue was used as a stain.

2.6.2 Molecular Identification

a) DNA extraction. At molecular level, fungus was identified by the extraction of fungal DNA from mycelium (Talhinhas *et al*., 2002). One milliliter of fungal spore suspension (1×10^6 spores per milliliter) (Velázquez *et al*., 2017) was inoculated in to conical flask having 50 ml YEPD (Yeast extract peptone dextrose) medium and flasks were then kept at 30°C, agitation speed: 150 rpm for 72 h. The mycelia were harvested from the culture broth by filtering the broth through a 10 ml syringe. This glass wool containing syringe allow only culture broth to pass while retaining fungal mycelium. The harvested mycelia were washed with 0.5 M EDTA (ethylene-diamine tetra-acetic acid) and sterile double distilled water. Then freeze-dried it and grounded into fine powder. In sterile tube of 1.5 mL, add 100 mg powder of mycelium, 400 μL lysis buffer and 150 μL of 3 M potassium acetate was added up to microtube and kept at 100°C for 20 min. Suspension was kept for ten minutes at 20°C and then centrifuged for 10 min at 14,000 × g at 4ºC. The supernatant obtained was transferred to an Eppendorf tube and add 250 μL phenol-chloroform-isoamyl alcohol (25:24:1, v/v) in it and vortexed the solution. Then, the solution was centrifuged at 14,000 × g for 10 min. The supernatant was shifted to another 1.5 mL microtube and 250 μL chloroform-isoamyl alcohol (24:1) was added again vortexed it then centrifuged. The supernatant obtained was shifted to new microtube, ice-cold 2-propanol was added in an equal volume in it and kept at -20°C for 10 min. Again, centrifuge it for 10 min. Pellet obtained was washed with 70% ethanol. Air dried the DNA pellet and then dissolved in 50 μL of distilled H_2O.

b) PCR amplification. For molecular studies, universal fungal primer, internal transcribed spacer (ITS) was used for gene amplification (Aamir *et al*., 2015). DNA extracted from *Rigidosporus sp*. as template was used for positive control. The PCR mixtures (50 μL) contained 0.1 μg template DNA, 400 μm dNTPs (dATP, dTTP, dGP and dCTP), 1.5 mM Mg^{2+}, 40 pM primers, 1.5 U Taq DNA polymerase. The PCR reaction was performed on a BIO-RAD S1000 PCR instrument with the following steps:

Table 1: Steps involved in Polymerase chain reaction.

Cycle steps	Temperature (ºC)	Time	Number of cycles
Initial denaturation	98	30s	1
Denaturation	98	30s	
Annealing	65	30s	25
Extension	72	30s	
Final extension	72	5 min	
		hold	1

2.7 Agarose gel electrophoresis

The amplified PCR product was electrophoresed on 1% agarose gel in TBE buffer and stained with ethidium bromide (EtBr) and visualized and photographed by Gel Doc system (BIORAD, USA). The gene fragment sequence was deposited in GenBank (Accession number:MF167358) https://www.ncbi.nlm.nih.gov/nuccore/MF167358 and analyzed using Blast/blastp. NCBI Blast Tree Method was used to generate phylogenetic tree via Neighbor-Joining method.

2.8 Substrate specificity

To determine the substrate specificity of laccase, different substrates were added to the reaction mixture at a concentration of 5mM. These included ABTS (control), Guaiacol and syringaldazine. The oxidation rate of these substrates can be determined at their specific wavelength 420nm (Wang *et al*., 2018), 450nm (Ademakinwa and Agboola, 2016) and 530nm (Perna *et al*., 2018) respectively.

2.9 Characterization of predicted laccase structure

Crystallographic tertiary structure of fungal laccase from *Trametes hirsuta* (PDB ID: 3FPX) was retrieved from Protein data bank (PDB). Tertiary structure of fungal laccase has been determined at resolution of 1.9Å using X-ray crystallography. For docking analysis, PyMol and Jmol softwares were used. Non-protein ligands associated with that protein were removed using PyMol software. Whereas, chemical structures of substrates were obtained from Jmol. Dock score between ligand and substrates were obtained by PyMol.

3 Results and discussion

3.1 Isolation of Fungal strains

A total of 30 fungal strains were isolated from air, soil and decaying wood of *Mangifera indica*. Out of 30, only 15 strains (MF2, MF3, MF4, MF6, MF7, MF8, LCS4, LCS7, LCS8, LCS10, LCS13, LCS14, MFw1, MFw2 and MFw3) showed positive response to indicator plates as shown in Table 2. Laccase producing strains gave brick red halo in response to guaiacol, dark brown colour for tannic acid and green colour in response to syringaldazine. On the basis of colour intensity strains were differentiated into excellent, very good, good and low laccase producers.

3.2 Screening of cultures

Fungal species with positive response in plate test method were screened for their quantitative production of laccase. A total of 14 strains exhibited enzyme activity in the range of 100±4.9 to 270±1.52 U/ml. Maximum enzyme activity (297±1 U/ml) was obtained by MFw3 strain as shown in Table 3. Therefore, this strain (MFw3) was selected for further experimental work.

3.3 Zymogram analysis

Zymogram of the laccase enzyme was performed to confirm the presence of laccase. Non denaturing gel was incubated with 5mM ABTS and sodium acetate buffer, pH 4.5. Bovine serum albumin (BSA) was used as standard marker and stained with coomassie blue to confirm the presence of protein in monomeric, dimeric, trimeric or multimeric form. Comparison of this native gel with Coomassie blue stained gel resulted that protein is present in monomeric (68KDa) and multimeric form (260 KDa). Molecular mass of *Aspergillus flavus* MFw3 laccase compares well with the laccase of 68KDa of *Pleurotus ostreatus* HP-1 and P. sanguineus as reported by Patel *et al*., 2014 and Gonzalez *et al*., 2008. Castillo *et al* (2011) also reported that major laccase (lacI) is a monomeric protein of 65 KDa isolated from *Trametes hirsuta* Bm-2.

3.4 Macro and microscopic studies

Identification of the effective strain was carried out by macro and microscopic analysis. Macroscopic analysis showed that it has unraised green lawn Figure 2(a). Their conidiophores have yellowish green nodes and conidiophores are round and light green in colour. Morphological analysis was carried out by microscopic observation using lactophenol blue as a stain. Septate hyphae with long conidiophores were observed Fig 2(b). Conidia are round, smooth and form long chains as shown in Figure 2(c).

3.5 Molecular analysis

For accurate identification at specie level molecular studies were performed using universal primer ITS-

Table 2. Comparison of response of isolated fungal strains with different phenolic compounds as laccase substrates on potato dextrose agar plates for qualitative laccase activity.

No.	Strain	Guaiacol	Tannic acid	Syringaldazine
1	MF1	-	-	-
2	MF2	++	++	++
3	MF3	+++	++	++
4	MF4	+++	++	+
5	MF5	-	-	-
6	MF6	+++	+	+
8	MF7	+++	+	+
9	MF8	+++	+++	+++
10	MF9	-	-	-
11	MF10	-	-	-
12	LCS1	-	-	-
13	LCS2	-	-	-
14	LCS3	-	-	-
15	LCS4	+	+	+
16	LCS5	-	-	-
17	LCS6	-	-	-
18	LCS7	++	++	++
19	LCS8	+++	+++	+++
20	LCS9	-	-	-
21	LCS10	+	+	+
22	LCS11	-	-	-
23	LCS12	-	-	-
24	LCS13	+	+	+
25	LCS14	+	+	+
26	MFw1	+	+	+
27	MFw2	++	++	++
28	MFw3	++++	-	-
29	MFw4	-	-	-
30	MFw5	-	-	-

Intensity of colour (++++ Excellent, +++very good, ++good, +low, - no response)

Table 3. Screening of cultures and their Enzyme activity (U/ml).

Sr.no	Fungal strains	Laccase activity (U/ml)
1.	MF2	147.2±0.45
2.	MF3	252.6±2
3.	MF4	237±0.57
4	. MF6	270±1.52
5.	MF7	215±2
6.	MF8	210±1
7.	LCS4	140±0.57
8.	LCS7	159.3±1
9.	LCS8	150±1
10.	LCS10	100±4.932
11.	LCS13	137.9±1
12.	LCs14	110.2±0.75
13.	MFw1	120.5±2.25
14.	MFw2	132±2
15 .	MFw3	297±1

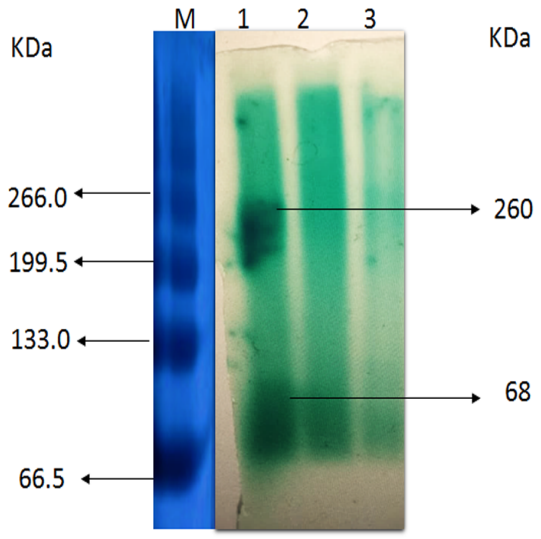

Fig. 1. Zymogram analysis of lacasse performed by native PAGE: Native gel was stained with 5mM ABTS to confirm the presence of laccase only, laccase appeares as monomeric and multimeric forms. M: Marker (bovine serum albumin), stained with Coomasie blue; Lane 1, 2, 3 different concentrations of laccase loaded onto gel. Native PAGE conditions: Temperature 4ºC, Current 80 mV, Time 3h.

700bp (Varga *et al*., 2011). The amplified product was of 608bp long as shown in Figure 3(a) and having sequence shown in Figure 3(b). Our results are in close proximity with Mohankumar *et al*. (2010) who also reported in his work that the DNA was extracted from *Aspergillus flavus* and PCR amplified product was 600 bp long. BLAST search sequence algorithm of NCBI (National Centre of Biological Information) database was conducted to get similarity between regions of most similar sequences and homology analysis. The results showed 100% similarity with *Aspergillus flavus*. The sequence was submitted in GenBank (accession number: MF167358). Construction of phylogenetic tree by Neighbor-Joining method Figure 3(c) showed that *Aspergillus flavus* MFw3 is a novel strain.

3.6 Substrate specificity

Different substrates of enzyme were tested at concentration of 5 mM, in Na-acetate buffer (0.1 M) at pH 4.5. Laccase enzyme strongly oxidized ABTS, moderately oxidized guaiacol and had slight activity with syringaldazine as shown in Table 4. Our results showed similarity to the work reported by Zouari-Mechichi *et al*. (2006) about the substrate specificity of both laccases obtained from *Galerina sp*. and laccases from *Trametes trogii*. Both showed highest activity toward ABTS, whereas the activity with the phenolic substrates was much lower (Ibrahim *et al*., 2011). Substrate specificity of enzyme depend upon the catalytic constants (k_m and k_{cat}) of enzyme. Values of these catalytic constants vary with the source organism of laccase (Xu *et al*., 1996). Baldrian, 2006 reported that k_{cat} for *Agaricus blazei* laccase was 21 s^{-1}, whereas k_{cat} value of oxidation of ABTS by *Pleurotus ostreatus* was reported as 350,000 s^{-1}. So, substrate specificity depends upon the source of laccase. ABTS is mostly recruited for assaying laccase activity due to its intrinsic advantages of high molar extinction coefficient (ε_{420nm} =36,000 M^{-1} cm^{-1}) of its oxidized product ($ABTS^{+\bullet}$) and pH independence (Baldrian, 2006; Majeau *et al*., 2010).

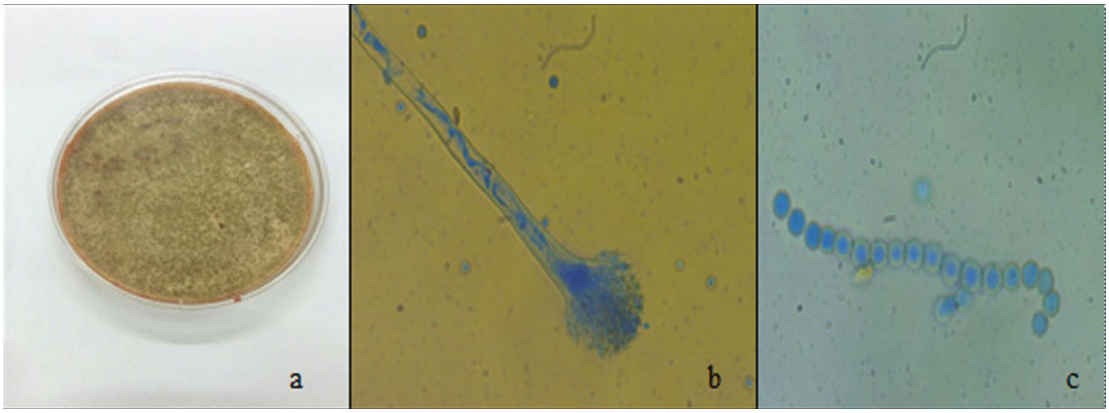

Fig. 2. a) Morphological view of *Aspergillus flavus* (MFw3), b) Mycelial thread and c) Spores under optical microscope (100x).

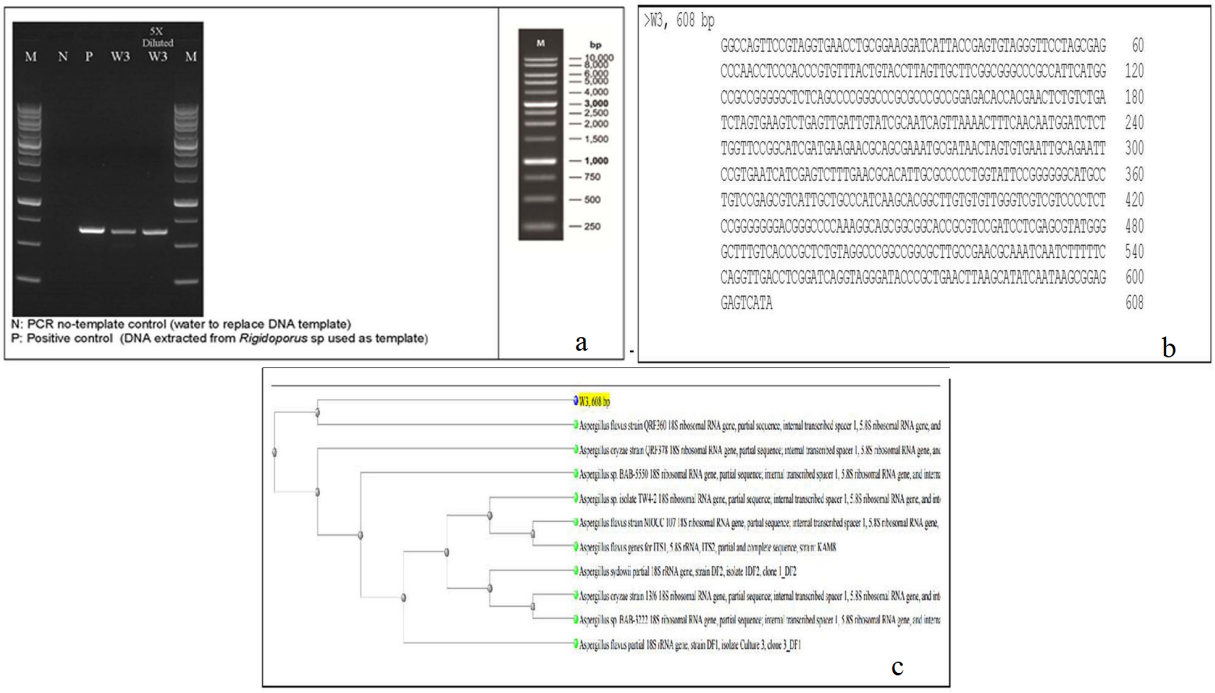

Fig. 3. a) PCR fingerprinting of fungal straints MFw3, M: 250 bp ladder; N: PCR no template control (water to replace DNA template), P: Positive control (DNA extracted from *Rigidosporus* sp. used as template, b) sequence of PCR product of *Aspergillus flavus* MFw3 showing 608 bp, c) Phylogenetic tree analysis of *Aspergillus flavus* MFw3 on using ITS-700 bp sequences and neighbor-joining method.

Table 4. Substrate specificity of laccase enzyme from *Aspergillus flavus* MFw3.

Substrate (5mM)	Relative activity (%)
ABTS (Control)	100
Guaiacol	50
Syringaldazine	20

Guaiacol and syringaldazine oxidation rates are relative to ABTS oxidation

3.7 In-silico modellig and characterization of laccase (Accession code AY081775)

Different bioinformatics tools were used for the characterization of predicted structure. RCSB and PDB server were used to get biological macromolecular structure information and it contains tools that provide information about the relationship between sequence, function and structure of biological macromolecules. Sequence similarity studies revealed that aminoacids are conserved for the laccase. Phylogenetic tree analysis showed the novelty of *Aspergillus* strain so its protein structure cannot be obtained from PDB. Therefore, 3D structure of laccase isolated from *Trametes hirsuta* (PDB ID: 3FPX) helped us to perform the experiment.

3.8 Molecular docking

Laccase enzyme has broad substrate specificity and can oxide different substrates such as ABTS, Syringaldazine and Guaiacol etc. PyMol SOFTWARE was used to get docking results. Low dock score between the substrate and enzyme showed that it has close proximity with the enzyme. It is also based on some chemical features which promote reaction such as hydrogen bonding and ligand torsion strain in enzyme and compound. It was observed that all substrates attach to the same amino acid residue (GLY 341) but dock scores are different. For guaiacol and ABTS, dock scores are 14.7Å and 12.5Å respectively as shown in Figure 4(a) and (b). On the other hand, for syringaldazine docking score is 15.5Å and active site residue is GLY 341 as shown in Figure 4(c). The present study revealed that ABTS is a substrate of choice because it showed minimum dock

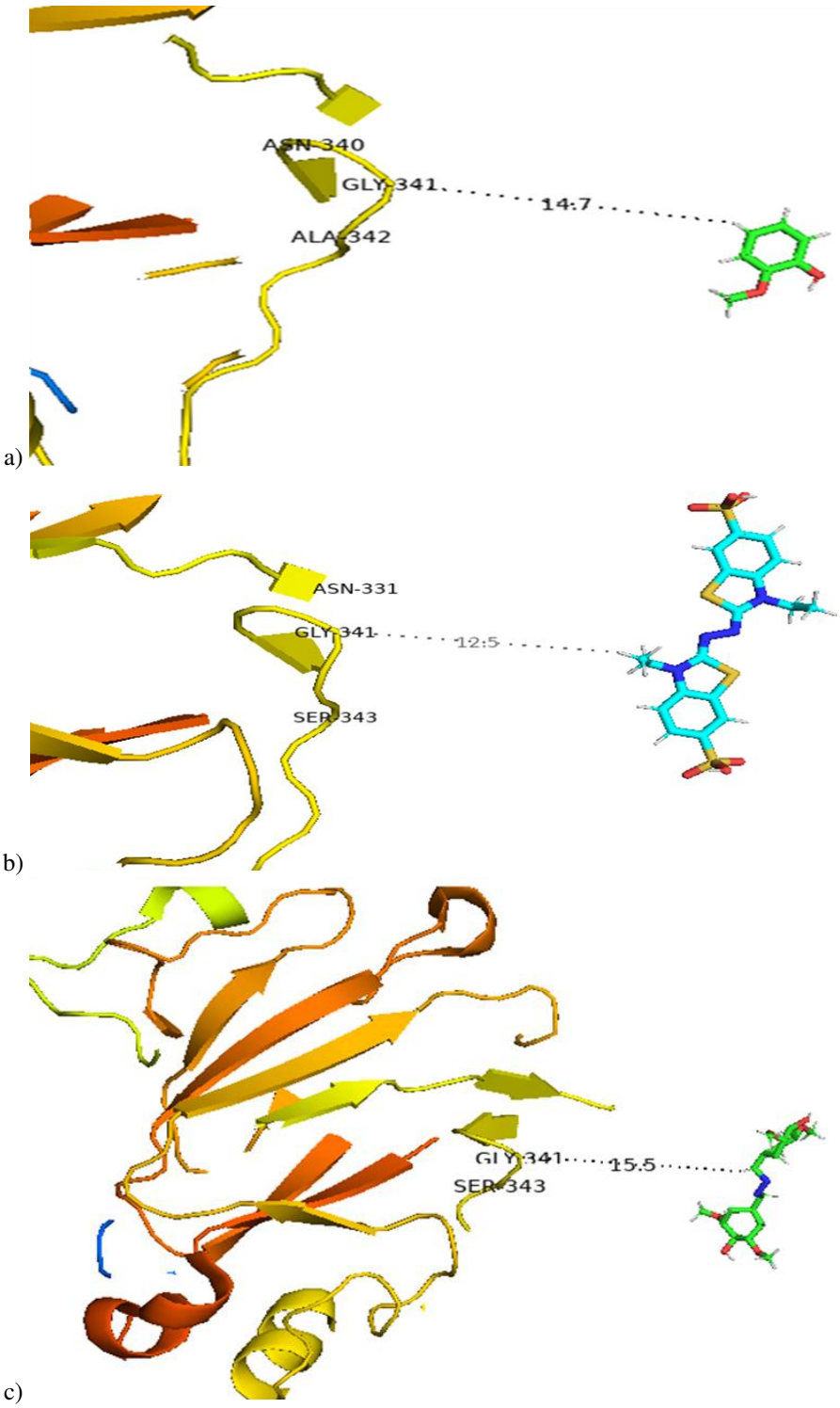

Fig. 4. a) Laccase specificity with guaiacol, active site amino acid residue GLY 341 of protein clearly showed substrate binding affinity with distance 14.7 Å. b) Laccase specificity with ABTS, active site amino acid residue GLY 341 of protein clearly showed substrate binding affinity with distance 12.5 Å. c) Laccase specificity with syringaldazine, active site amino acid residue GLY 341 of protein clearly showed substrate binding affinity with distance 15.5 Å.

Table 4. Table showing different substrates, their docking score and residues to which they attach.

Sr.no	Substrate	Distance from protein (Å)	Residues at active site
1	Guaiacol	14.7	GLY-341
2	ABTS	12.5	GLY-341
3	Syringaldazine	15.5	GLY-341

score as compared to other substrates (guaiacol and syringaldazine) as shown in Table 4. Our results are in close agreement with the work reported by Singh *et al*., 2014. Singh reported in his study that ABTS gave maximum goldscore 91.93 as compared to other substrates (L-tyrosine, ferrous sulfate ammonium, syringaldazine and guaiacol) lignin monomers and two inhibitors i.e., kojic acid and N-hydroxyglycine used for docking analysis with the laccase isolated from different serotypes of *Yersinia enterocolitica biovar* 1A.

In-silico modelling is an environment friendly and time saving approach for selection of an appropriate substrate for enzymes instead of extensive wet lab experiments.

Conclusions

It is concluded from present study that locally isolated *Aspergillus flavus* MFw3 is capable to produce laccase using low cost and environment friendly saw dust as carbon source. The *in vitro* and *in silico* comparison among benchmark substrates showed that the isolated enzyme catalyzes ABTS more efficiently as compared to guaiacol and syringaldazine. Docking analysis revealed that GLY 341 is an active site amino acid involved in the enzyme-substrate interaction.

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