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Isolation, Screening and Production of Fungal Laccases

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Abstract: ***Laccase (E.C. 1.10.3.2) are enzyme belonging to the group of blue multi-copper oxidases. Laccases catalyze the oxidation of variety of phenolic compounds, diamines and aromatic amines. Fungal isolate producing laccase was screened on Potato Dextrose Agar medium enriched with 0.02% guaiacol from soil as well as decaying wood samples collected from farm/forest site. Confirmation of laccase production was done by Bavendam test which is a plate assay technique using 3mM guaiacol. The isolate was identified and found to belong genera Aspergillus and was tested for producing laccase enzyme. An attempt was made to isolate, screening and production of laccase enzyme produced from Aspergillus niger. Isolate giving best result was processed further for production of laccase using Submerged fermentation. Guaiacol and sodium citrate buffer were used to assay laccase production. Characterization of fungal isolate was done by morphological analysis. This study showed that enzyme production can be increased by media manipulation in the fungal cultures. Optimization of the media condition can maximize the desired enzyme production. Application or Dye decolorization assay was been performed using 0.1ml crystal violet solution, where dye decolorization efficiency showed the ability of laccase to decolorize dyes. It is present in Ascomycetes, Deuteromycetes and Basidiomycetes and abundant in lignin degrading white / brown rot fungi. In the recent years, these enzymes have gained application in the field of textile, pulp and paper and food industries. Recently, it is also used in the design of biosensors, biofuel cells as a medical diagnostics tool and bioremediation agent to clean up herbicides, pesticides and certain explosive in soil. Laccases have received attention of researchers in the last few decades due to their ability to oxidize both phenolic and non phenolic lignin related compound as well as highly recalcitrant environmental pollutants.***

Keywords: Laccase, Aspergillus niger, Enzyme activity, Enzyme assay, Guaiacol, Optimization, Lactophenol cotton blue, Crystal violet

I. INTRODUCTION

Laccase (Lac, benzenediol: oxygen oxidoreductases; E.C.- 1.10.3.2) In recent years enzymes have gained great importance in industries, laccases are one among them which are widely present in nature. Laccase are the oldest and most studied enzymatic systems. Laccase was first studied by Hikorokuro Yoshida in 1883 and then by Gabriel Bertrand in 1894 in the sap of the Japanese lacquer tree, where it helps to form lacquer, hence the name laccase. These enzymes contain 15-30%Carbohydrates and have a molecular mass of 60-90 kDa. These are copper containing 1,4- benzenediol, oxygen oxidoreductases found in higher plants and microorganisms. These are glycosylated polyphenol oxidases that contain four copper ions per molecules that carry out one electron oxidation of phenolic and it's related compound and reduce oxygen to water. When substrate is oxidized by a laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or non-enzymatic reactions including hydration, disproportionation and polymerization. These enzymes are polymeric and generally contain each type 1, type 2, type 3 copper centres where the type 2, and type 3 are close together forming a trinuclear copper cluster. Laccases are widely distributed in higher plants, bacteria, fungi and insects. In plants, laccase are found in cabbages, potatoes, pears, apples and other vegetables. They have been isolated from Ascomyceteous, Deuteromyceteous and Basidiomycetous fungi to which more than 60 fungal strains belong. The white rot Basidiomycetes fungi efficiently degrade the lignin in comparison to Ascomycetes and Deuteromycetes which oxidize phenolic compounds to give phenoxy radicals and quinines. Fungal laccases are involved in multiple processes such as pathogenesis, detoxification, degradation of lignin and involvement in the development and morphogenesis of higher fungi. Laccase play and important role in food industry, paper and pulp industry, textile, cosmetics, synthetic chemistry, soil bioremediation and biodegradation of environmental phenolic pollutants and removal of endocrine disruptors. These enzymes are used for pulp delignification, pesticides or insecticides degradation, organic synthesis, waste detoxification, textile dye transformation, food technological uses and biosensor and analytical applications. Recently laccases have been efficiently applied to nanobiotechnology due to their ability to catalyze electron transfer reactions without additional cofactor.

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The technique for the immobilization of biomolecule such as layer by layer micro patterning. Self assembled monolayer technique can be used for preserving the enzymatic activity of laccases.

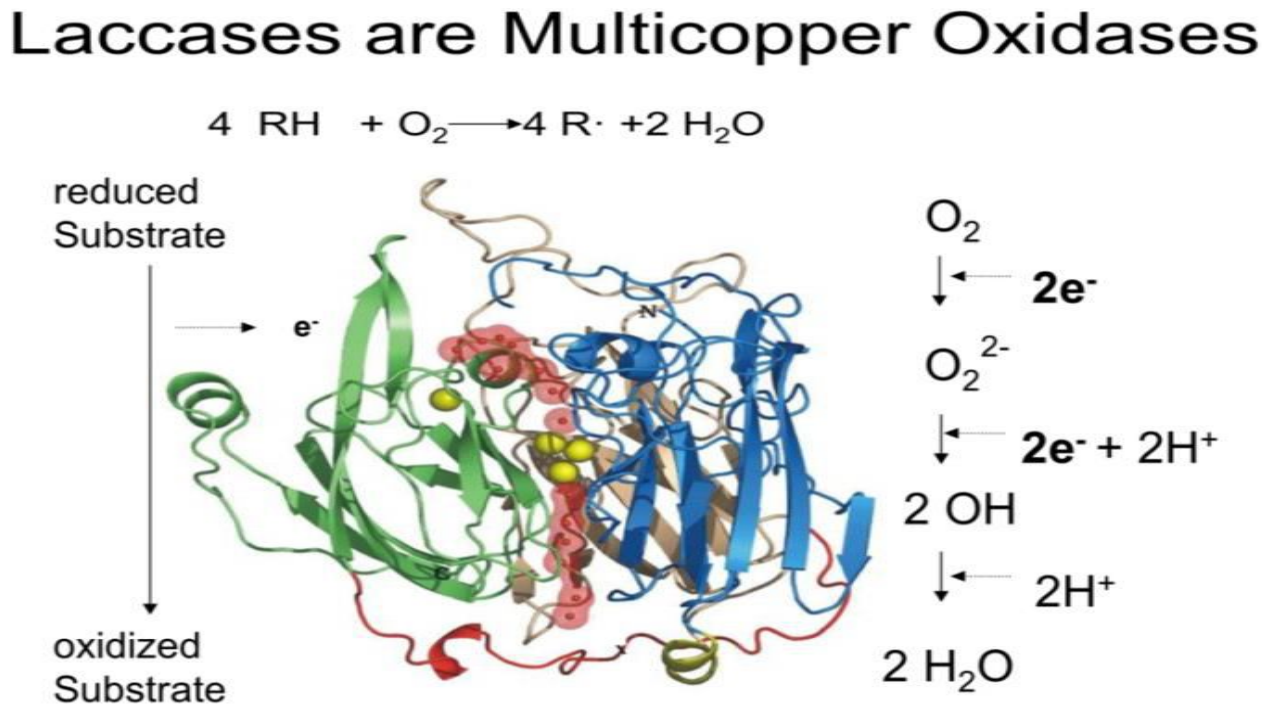
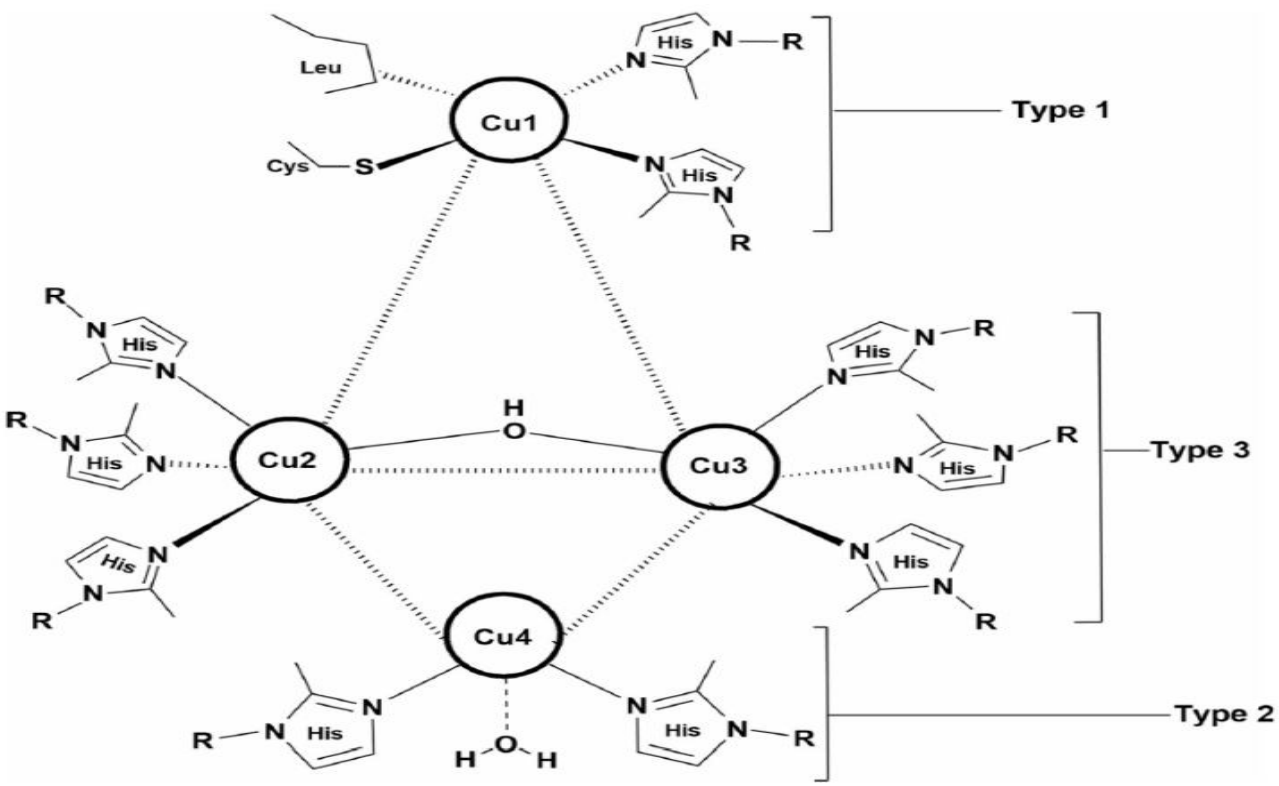


Figure 1: A] 3D Structure of Laccase



B]: Copper centers of laccase: Type 1, Type 2, Type 3 (Adapted and modified axial ligand as leucine as per fungal

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II. LITERATURE REVIEW

Laccase was first discovered by yoshida (1883) in plants, based on the observation that the latex of the chinese or japanese lacquer trees. Rapidly hardened in the presence of air. Subsequently laccase enzyme have been discovered in numerous other plants tissue (bligny and douce,1983, de marco and roubelakis- angelakis, 1997, ranocha et al., 1999). Despite their aride distribution plant laccase have not been extensively used or characterized. Especially as several isoenzymes of laccase may exists in lignified plant tissues. (gavnholt et al., 2002).fungal laccase were also discovered in late 19TH century (bertrand, 1896) and have been found in most of the basidiomycetes and ascomycetes (baldrian, 2006).

A multicopper enzyme with phenoloxidase activity has even been identified in yeasts (augustine et al. ,2008 and kosman,2003).some phenoloxidases showing laccase like enzymatic properties have been purified from larval and adult cuticles of insects (dittmer et al., 2004, suderman et al., 2006, yamazaki 1969 and 1972).relatively few prokaryotic laccases have been studied so for although rapid progress in genome analysis suggests that their genes are widespread in bacteria (alexander and zhulin 2000, claus 2003, 2004, sharma et al., 2007).convincing experimental data for the existence of a prokaryotic laccase was first presented by givauden et al., (1993) for azospirillum lipoferum , later this laccase was described as a multimeric enzyme composed of a catalytic subunit and one or two larger chain (diamantidis et al.,2000).

A laccase like enzyme was identified in spores of a bacillus sphaericus strain (claus and filip, 1997) and the spore coat protein cota of bacillus subtilis was the first prokaryotic laccase for which the crystal structure was elucidated (enguita et al., 2003; hullo et al. 2001, martins et al. 2002).laccase and laccase like enzymes are abundant and have many diverse natural functions. White rot fungi secrete laccases in association with other extracellular oxidase to degrade complex natural polymers such as lignin (baldrian,2006 ;thurston,1994) or humic acids (claus and filip ,1998).

Several studies on plants have indicated that laccase like activities are closely correlated with lignification (Dean and Erikson, 1994 The occurance of laccase enzyme in soils and litter underlines their probable role in enzymatic turnover of natural lignin derived polymers (Baldrian,2006). Bola et al. (1988) showed that the addition of laccase reversed the inhibitory effects of a number of phenolic compounds on the growth of *Rhizoctonia praticola* .

The biochemical and catalytic properties of fungal laccases have been comprehensively reviewed by Baldrian (2006). Laccase have high catalytic efficiency and are used for technical applications in various industrial and biotechnological domains (Xenakis et al., 2016) which includes improving properties of fibers , biosynthesis ,energy exploitation, bio-detection, printing and dyeing industry, Bio- pulping in paper industry ,conversion of aromatic compounds (Zheng et al., 2016) and removal of phenols that causes cancer and teratogenicity when present in waste water (Pang et al., 2016).

In addition ,it is also used in fast moving consumer goods (FMCG) as tooth paste ,mouthwash , detergent ,soap, diapers in cosmetics as deodorants, in beverages and food industry for wine and juice stabilization (Piacquadio et al., 1998, Alper and Acar 2004, Surwase et al., 2016) in dough or baked products to increase strength of gluten structures. In pharmaceutical industries as anaesthetic, antiinflammatory drugs, antibiotics and sedative (Nicotra et al.,2004; Surwase et al.,2016) and in nanotechnology as nanoparticles based biosensors. The present review will provide cumulative information on various aspects of fungal laccases, information pertaining to structure, history, categories of laccase, followed by industrial, pharmaceutical and biotechnological applications of laccases.

III. AIMS AND OBJECTIVES

The present study aimed at the production of laccase enzyme from fungus Aspergillus niger and also aimed to investigate the enhancement of microbial production of laccase enzyme and some of its beneficial applications. Morphological characterization was carried out.

The dissertation was carried out with following objectives:

1) Isolation and screening of desired fungi from soil sample and decaying wood. i.e. Aspergillus niger.
2) Identification, morphological characterization and differentiation of the isolate.
3) Preparation and incubation of production media.
4) Qualitative and quantitative analysis of production media.
5) To check the laccase production.
6) Optimization of various growth parameters.

IV. MATERIALS AND METHODES

A. Materials

Chemicals: All the chemicals used during this study were procured from M/S Hi-Media, Mumbai, and Mumbai analytical/guaranteed reagent (AR/GR) grade chemicals and solvents were used.
Glassware preparation: All glassware's were rinsed with ultrapure water and were autoclaved before use.
Microorganism used: Aspergillus niger
Media and reagents:
Potato Dextrose Agar[PDA] (100ml): Potato extract (20 gm potatoes boiled in 100 ml distilled water), Agar 2.5 gm, Dextrose 2.5 gm were mixed and autoclaved for 20 minutes at 121℃ with pH 4 - 5.5 at 25℃
Guaiacol (o-Hydroxyanisole or 2-Methoxyphenol) (mw: 124.14)
Luria Bertani Broth (LB Broth)(g/L): Tryptone 10.0, Yeast extract 5.0, Sodium chloride 10.0, Distilled water 1.0 L, Final pH (at 25°C) 7.5 ±0.2
Sodium Citrate Buffer: Sodium Citrate Dihydrate (mw: 294.10 g/mol) 24.269g, Citric Acid (mw: 192.12 g/mol) 3.358g, Distilled water 1.0 L, pH 6.0
.Lactophenol Cotton Blue: Phenol crystals 20.0 g, Cotton blue(Aniline blue, Methyl blue) 0.05 g, Lactic acid 20.0 ml, Glycerol 20.0 ml, Distilled water 20.0 ml
Crystal Violet Dye: i) Crystal violet solution A - Crystal violet 2.0g, Ethyl alcohol 20.0 ml, ii) Crystal violet solution B - Ammonium oxalate 0.8g, Distilled water 80.0 ml Solution A and B mixed and stored.
HCl , NaOH for pH adjustment

Materials for isolation of Fungi:

1) Soil sample
2) Decaying wood
3) Sterile distilled water
4) Sterile Potato Dextrose Agar plate
5) Alcohol
6) Petri plate
7) Flask
8) Test tube
9) Wire loop
10) Forcep

B. Methods

◆ Sample collection: Decayed wood, bark and tree scraping sample, soil sample for isolation of fungal laccases producers were collected from Agricultural Farm site /forest and then taken to the Laboratory, Department of Microbiology S.S.V.P.S Science College, Dhule. The samples were collected in the sterile plastic bags swapped using alcohol and were sealed and brought to the lab aseptically for further processed inside laminar air flow.
◆ Media for Preservation of fungi:
Potato Dextrose Agar(PDA): Potato extract (20 gm potatoes boiled in 100 ml distilled water), Agar 2.5 gm, Dextrose 2.5 gm were mixed and autoclaved for 20 minutes at 121℃ with pH 4 - 5.5 at 25℃
◆ Isolation and Screening of fungi for laccase production:
1 gm of soil and decaying wood sample were added to 9 ml of sterile distilled water and mixed. The sample was homogenized and 10 ml suspension was prepared in sterile distilled water.
The prepared suspension was used for inoculating onto the plates.
• Primary screening/ Plate assay for laccase positive culture(Primary screening of fungal strain on solid media):
Plate assay method was used for primary screening of fungal laccase using Potato Dextrose Agar(PDA) medium consisting 0.02% guaiacol as substrate and incubated at room temperature (25℃ -27℃) for 5 - 7 days for fungal growth. Other assay method was used for primary screening of fungal laccase using Potato Dextrose Agar medium consisting 4 mM tannic acid as a substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of dark brown color halo around the fungal colonies. The isolates showing positive results were use for the further studies.

One another plate assay method was used for primary screening of fungal laccase using Potato Dextrose Agar medium consisting 3 mM ABTS (2-2'Azino-bis-[3-ethyl benzthiazoline-6-salfonic acid)] as substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of green coloured halo around the fungal colonies.

◆ Bavendam test: Confirmation of laccase production was done by Plate assay the technique using 3 mM guaiacol as a substrate. The colour change from colourless to reddish brown colour was to be observed in fungal culture plate for presence of laccase (Soponsathien,1998).

◆ Production of laccase enzyme: Laccase production was done using submerged fermentation.

● Secondary screening (Secondary screening of fungal strain in liquid medium): All experiment was carried out in 250 ml Erlenmeyer flask containing 100 ml liquid culture medium/ Production medium for submerged fermentation.

C. Components

Wood chip	4.5
Yeast extract	1.5
Glucose	1
Ammonium sulphate	0.5

The mixture was incubated at 30℃ for 15 minutes, after 15 minutes the intense brown color was found to be developed due to oxidation of Guaiacol by laccase and the absorbance was read at 450nm using UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1μmol and Guaiacol per min per ml for the production of colored product .

The laccase activity in U/ml is calculated by this formula:-

$E.A = A \times V/t \times e \times v$

Where,

E.A = Enzyme activity

A = Absorbance

V = Total mixture volume (ml)

v = Enzyme volume (ml)

t = incubation time

e = extinction coefficient for guaiacol (0.6740uM/cm)

D. Optimizations Studies

• Effect of temperature, pH and incubation period on enzyme production :

In order to record the optimum temperature, pH value and incubation period for laccase enzyme production, the productive medium was prepared and inoculated by standard inoculum and tested fungus as a mentioned before. Three different temperatures i.e. 25℃, 30℃, 35℃ were investigated. Also, different levels of initial pH values i.e. 4, 6, 8 were applied. The proper time for the maximum laccase production was detected during 144h (6 days) fermentation period on productive medium.

◆ Characterization and enzyme activity:

Effect of temperature and pH of buffer during the oxidation of Guaiacol reaction was studied. Temperature was studied by incubating the enzyme mixture containing enzyme, Guaiacol and sodium citrate buffer at different temperature 25℃, 30℃ and 35℃ for 15 minutes. pH buffer of enzyme mixture was adjusted at different value for (4,6,8) to record the optimum pH. After incubation for 15 minutes the absorbance of enzyme catalyzed reaction was recorded. Then the optimum temperature and pH of the enzyme activity were detected.

• Cultural and morphological characterization of fungal isolate:

The fungal isolate was further subjected to the colony characteristics and morphological characteristics using staining technique using lactophenol cotton blue(LPCB) as stain and observation under low power and high dry objective lens.

• Selection of potent fungal strain and identification:

The most potent laccase producing fungal strain was screened according to its capability of strain to grow on Potato Dextrose Agar (PDA) medium supplemented with guaiacol. Guaiacol degrading fungal strain showed considerable growth and reddish brown zone on medium supplemented with different concentration of guaiacol with 0.02%, 0.04% & 0.06 %. Potent fungus was identified on the basis its morphological characteristics i.e. hyphae, sporulating structure, sporocarp, arrangement of conidia and cultural characteristics i.e. colony color, front and bottom view and texture.

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E. Application Of Laccase Enzyme To Decolorize Dye /Dye Decolorization Experiment /Assay

Crystal Violet used for dye decolorization assay. Two-three drops of dye was added in 50 ml Luria-Bertani broth and was sterilized using autoclave. The flask was inoculated by fungal isolate and control was kept uninoculated. Fungal isolate was grown for 5 -6 days. The flasks were incubated at 28°C for 24, 48, 72, & 120 hours. Dye content was monitored photometrically at 590 nm using spectrophotometer, which is maximum visible absorbance of this dye.

Decolorization efficiency was been measured further. Percent decolonization of dye was calculated by considering the optical density of control flask as 100% (0% decolorization)

Decolorization (%) = [(Initial absorbance-observed or Final absorbance)/ (initial absorbance)] ×100

D%= 100× (Aini-Afin)/Aini

Where,

D =Decolorization

Aini =Initial absorbance

Afin =final absorbance of dye after incubation time

Where the initial absorbance indicated absorbance of the untreated dye at the characteristics peak and the final absorbance indicated

V. RESULTS AND DISCUSSION

Isolation and Screening of laccase producing fungi: The laccase producing fungi were isolated from various samples of soil, decayed wood and bark of tree. It was inoculated on Potato Dextrose Agar (PDA) medium supplemented with guaiacol as substrate. The reddish brown oxidation zone was developed around fungal colonies. The fungus showing potential oxidation zone were further screened for laccase production using guaiacol assay method. All fungal isolates were identified depending on their morphological characters according to Gilman (1969) Traute et al. (1980) and Alexopoulas et al. (1985) fungal strain isolated, identified and screened for laccase production was member of fungal family Ascomycetes . The isolated fungi comprised of Aspergillus niger Screening for laccase production by Aspergillus niger has been done using guaiacol as indicator. The screening results confirm that isolated fungal are able to produce laccase enzyme.

A.niger classification: Kingdom -Fungi Division - Ascomycota Class -Eurotiomycetes Order -Eurotiales

Family -Trichocomaceae

Genus -Aspergillus

Species - niger

Binomial name – *Aspergillus niger*

• Rate of growth: Usually rapid. Mature within 3-5 days.

• Colony morphology/Cultural characteristics: Surface at first white then shade of yellow to Brown or black, texture velvety and cottony.

• Morphological characteristics of *Aspergillus niger*:

A. niger is a filamentous fungus, spore forming, forming filamented hyphae that make them appear like small plants.

When observation under microscope, *A.niger* consist of smooth and colourless conidiophores and spores.

VI. CONCLUSION

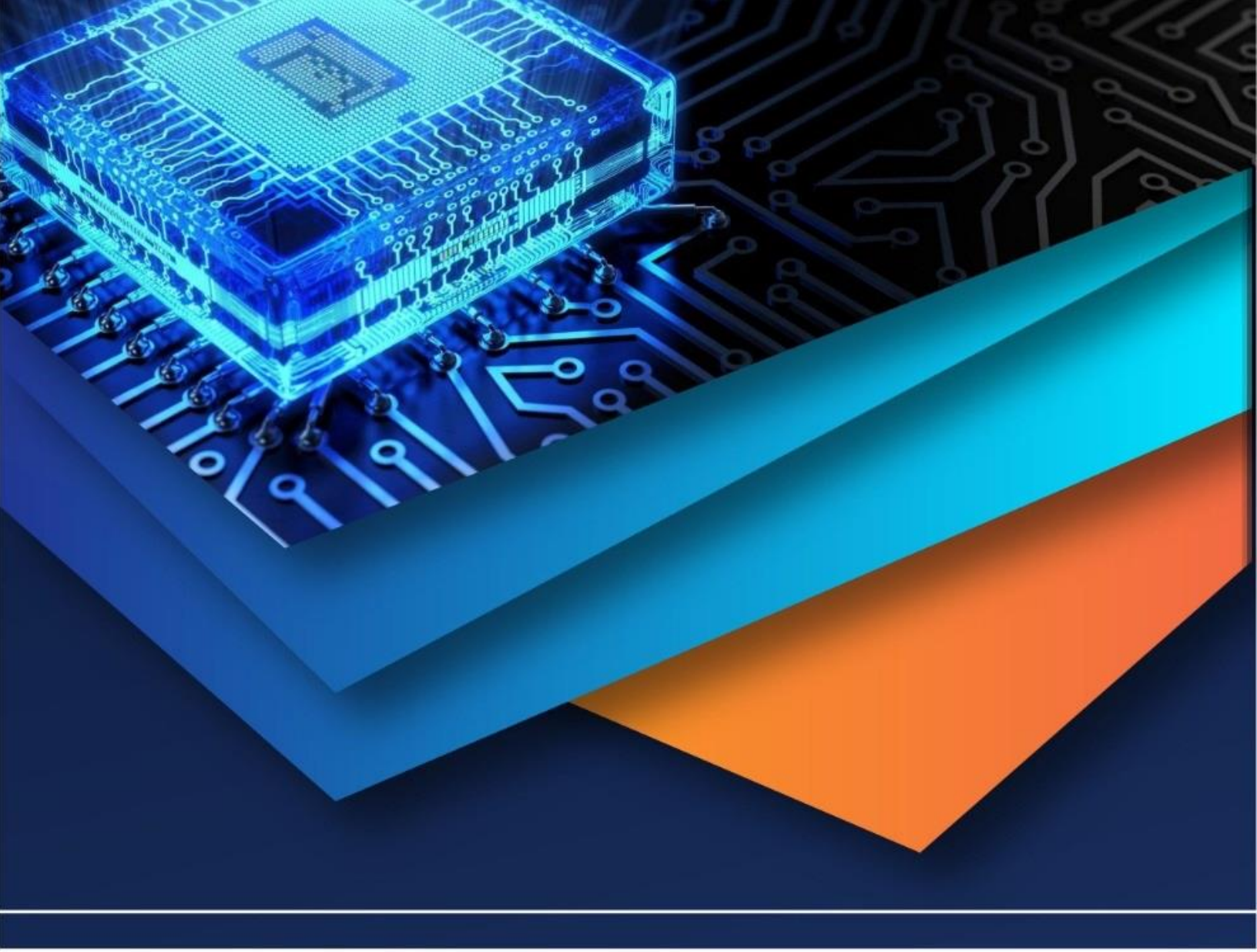
Laccase are the versatile enzymes which catalyze oxidation reactions coupled to four electron reduction of molecule oxygen to water. They are multicopper enzyme which are widely distributed in higher plant and fungi.

They are capable of degradation of lignin and are present abundantly in many white rot fungi. The laccase producing potential isolate was isolated from soil and decaying wood sample and used for enzyme production. Based on the screening results using substrate guaiacol, laccase positive test were confirmed and production was carried out. Fungal laccase production was done using submerged fermentation where maximum enzyme activity was 3.80 µl/ml at 35°C. The optimum laccase activity of 0.113 µl/ml after the incubation of 6 days was obtained by classical method. Laccase exists in decolorization mechanism could be concluded using dye decolorization mechanism as application. They also decolorize and detoxify the industrial effluents and help in wastewater treatment. Results showed that the laccase enzyme was responsible for 80% decolorization of crystal violet in the time interval of 168 hours (6-7 days). The use of extended to other anthraquinone- type textile dyes, suggesting a potential application field for the removal of dyes from industrial effluents.

They act on both phenolic and non phenolic lignin related compounds as well as highly recalcitrant environmental follow turns which health researchers to put them in various biotechnological applications they can be effectively use in paper and pulp industries, textile industries , xenobiotic degradation and bioremediation and act as biosensor. Laccase has been applied to Nano biotechnology which is an increasing research field and catalyzes electron transfer reaction without additional cofactors. Recently several techniques have been developed for the immobilize laccase and preserve their enzymatic activity. Hence laccase is receiving much attention of researchers around the globe.

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