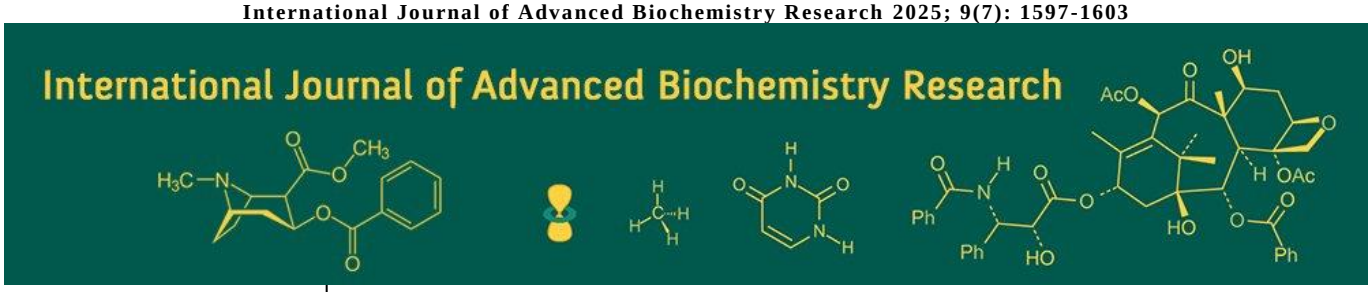

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Isolation and screening of potential ligninolytic fungi from different ecological niches in the Marathwada region

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Abstract
Ligninolytic fungi play a significant role in the biodegradation of lignocellulosic biomass through the production of extracellular oxidative enzymes such as laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP). In the present study, soil samples were collected from various ecological niches in the Marathwada region of Maharashtra-including field soil, compost pits, and sites near decaying woodto isolate potential ligninolytic fungi. A total of 30 fungal isolates (PBNF 1 to PBNF 30) were obtained using serial dilution and spread plate techniques on Potato Dextrose Agar. Primary screening was performed using qualitative plate assays with methylene blue, azure B, guaiacol, tannic acid, and ABTS as indicators. Out of the 30 isolates, five isolates (PBNF 11, 15, 17, 22, and 29) demonstrated positive reactions in at least one assay, indicating ligninolytic activity. These isolates were morphologically and microscopically identified as *Cladosporium sp.*, *Trichoderma harzianum*, *Aspergillus niger*, *Penicillium chrysogenum*, and *Aspergillus awamori*, respectively. This preliminary screening suggests that these fungi hold promising potential for ligninolytic enzyme production and may be applied in diverse biotechnological processes such as lignocellulosic biomass degradation, bioremediation, dye decolorization, and bioethanol production.

Keywords: Ligninolytic fungi, oxidative enzymes, qualitative screening, dye, decolorization, fungal isolates

Introduction
Lignocellulosic biomass, primarily composed of cellulose, hemicellulose, and lignin, represents the most abundant renewable organic resource on Earth (Sanchez, 2009). Among these components, lignin is a complex, non-carbohydrate, polyphenolic polymer that provides structural rigidity and resistance to microbial attack in plant cell walls. It accounts for approximately 15-30% of the dry weight of lignocellulosic biomass and is the second most abundant biopolymer on Earth, after cellulose (Bugg *et al*., 2011) [1]. Its recalcitrant nature is attributed to its high molecular weight and the presence of stable carbon-carbon and ether linkages, making it a major barrier in the efficient bioconversion of lignocellulose into value-added products (de Gonzalo *et al*., 2016) [2].

Ligninolytic fungi are microorganisms capable of degrading lignin through the secretion of extracellular oxidative enzymes. They are primarily found among Basidiomycetes, especially white-rot fungi, which are regarded as the most efficient natural degraders of lignin. Biological degradation of lignin is predominantly carried out by a specific group of microorganisms, with filamentous fungi-particularly basidiomycetes and certain ascomycetes-being the most efficient due to their ability to secrete a battery of extracellular ligninolytic enzymes. These enzymes include laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP), which synergistically depolymerize and mineralize lignin under aerobic conditions. Given their enzymatic potential, ligninolytic fungi have garnered significant attention for their roles in various biotechnological applications such as bioethanol production, bioremediation of xenobiotics, treatment of industrial effluents, and delignification in the paper and pulp industries.

In recent years, microbial pretreatment has gained increasing attention as an environmentally sustainable and economically viable strategy to enhance the enzymatic saccharification of lignocellulosic biomass. This approach utilizes various microorganisms-including white-rot and soft-rot fungi, actinomycetes, and certain bacteria-that are capable of breaking down lignin, the most recalcitrant component of plant biomass, through the production of lignin-degrading enzymes (Sharma *et al*.,2017) [12].

India, with its diverse agro-climatic zones, harbors a wide range of fungal species adapted to degrade complex organic matter. The Marathwada region of Maharashtra, characterized by varied ecological conditions and agricultural activities, provides a rich reservoir of indigenous fungal communities with potential ligninolytic capabilities. However, systematic exploration and evaluation of such fungal diversity remain limited.

The present study was undertaken with the objective of isolating and screening ligninolytic fungi from different ecological niches within the Marathwada region. By employing both qualitative plate assays and morphological identification techniques, this work aims to identify promising fungal strains capable of producing ligninolytic enzymes, thereby contributing to the development of sustainable approaches for lignocellulosic waste management.

Material and method

Collection of Samples for Isolation of Ligninolytic fungi

Soil samples were collected from different ecological niches in Marathwada region of Maharashtra, to maximize the diversity of ligninolytic fungi. The sampling sites included field soil, compost pits and soil near decaying wood. The samples were collected in sterile zip-lock plastic bags under aseptic conditions, stored at 4 °C, and labeled according to their source and site. The collected samples were then transported to the laboratory for fungal isolation.

Isolation of Ligninolytic Fungi

The serial dilution and spread plate technique was used to isolate fungi from various sources. One gram of soil was serially diluted in sterilized distilled water to achieve concentrations of 10^{-1} to 10^{-9} in test tubes. 0.1 ml of solution from the dilution concentration 10^{-4} was poured aseptically onto potato dextrose agar plates and was spread thoroughly with the help of a L-shaped cell spreader followed by incubation at 28±1 °C for 2 to 3 days. After observing mixed growth, each morphological different colony was purified on fresh agar plates and was incubated at 28±1 °C for 5 to 7 days.

Pure fungal culture was subculture on Potato Dextrose Agar slant and allowed to grow at 28±1 °C for 7 days. These slants were preserved in the refrigerator at 4 °C and subcultures once in two month. This pure culture was used for future investigations.

Primary Screening

Primary screening of isolates for their ligninolytic potential was done by qualitative plate assay using methylene blue, azure B, guaiacol, tannic acid and ABTS as indicators for ligninolytic potential:

Methylene blue Dye Decolorization Plate Assay

Malt Extract Agar (MEA) supplemented with 0.01% (w/v) methylene blue was prepared and sterilized by autoclaving. Approximately 20 ml of medium was poured into sterile glass Petri plates and allowed to solidify. A 10 mm mycelial disc, excised from the actively growing margin of 7dayold PDA cultures, was aseptically placed at the center of each plate. Inoculated plates were incubated at 28 ± 1 °C for 14 days. Lignin peroxidase activity was assessed based on decolorization of methylene blue:

(+) = Clear zone formation around the colony.

(-) = No decolorization observed.

Guaiacol Plate Assay

Malt Extract Agar (MEA) was supplemented with 1% (w/v) guaiacol after sterilization. Approximately 20 ml of the medium was poured into sterile Petri plates and allowed to solidify. A 10 mm mycelial disc, taken from the actively growing margin of 7 days old PDA cultures, was placed centrally on the agar surface. Plates were incubated in the dark at 28 ± 1 °C for 7 days. Laccase activity was indicated by:

(+) = Formation of a brown halo around the colony.

(-) = No halo formation.

Azure B Dye Decolorization Plate Assay

Lignin Basal Medium (LBM) medium was used for identification of potential lignin degrading isolates. The medium was supplemented with 0.01% (w/v) Azure B and 1.6% (w/v) agar and autoclaved. After autoclaving, 1 ml of a separately sterilized 20% (w/v) aqueous glucose solution was aseptically added to each 100 ml of the growth medium. Subsequently, 20 ml of the medium was poured into autoclaved petri plates and allowed to solidify. Once solidified, the plates were inoculated by placing 10 mm diameter disc taken from the edges of 7day old culture grown on PDA medium. Each disc was placed on the surface of the Azure B supplemented agar plates and incubated at 28±1 °C in darkness. The plates were examined daily for 10 days and observations for lignin peroxidase and Mn dependent peroxidase activities were recorded as:

(+) = Development of clear zone around fungal colony.

(-) = No change in medium.

Tannic Acid Plate Assay

Lignin Basal Medium (LBM) supplemented with 1.6% (w/v) agar was prepared and autoclaved. After sterilization, 1 ml of sterile 20% (w/v) aqueous glucose solution and 1 ml of sterile 1% (w/v) aqueous tannic acid solution were aseptically added per 100 ml of medium. Approximately 20 ml of the medium was poured into sterile Petri plates and allowed to solidify. Plates were inoculated with pure fungal isolates and incubated in the dark at 28 ± 1 °C. Observations were recorded daily for 10 days. Polyphenol oxidase activity was indicated by:

(+) = Development of a brown oxidation zone around the colony.

(-) = No color change in the medium.

ABTS Plate assay

Lignin Basal Medium (LBM) supplemented with 0.1% (w/v) ABTS and 1.6% (w/v) agar was prepared and autoclaved. After sterilization, 1 ml of sterile 20% (w/v) aqueous glucose solution was aseptically added per 100 ml of medium. Approximately 20 ml of the medium was poured into sterile Petri plates and allowed to solidify. A 10 mm mycelial disc, excised from the actively growing margin of 7dayold PDA cultures, was placed centrally on each plate. Plates were incubated in darkness at 28 ± 1 °C and examined daily for 10 days. Laccase activity was indicated by:

(+) = Development of green coloration in the medium.

(-) = No color change observed.

Identification of fungal isolates

For morphological identification, individual isolates were inoculated on Potato Dextrose Agar (PDA) plates using a sterilized inoculating needle and incubated at 28 ± 1 °C for 7 days. Colony features such as colony colour and texture were assessed visually. The microscopic examination was performed using lactophenol cotton blue staining as described by James and Natalie (2001) [8].

Results and Discussion

Isolation of Ligninolytic Fungi

Soil samples were collected from field soil, soil near decaying matter and compost pit from different ecological niches in Marathwada region of Maharashtra for the isolation of potential ligninolytic enzyme producing fungal strains. Serial dilution technique was used for the isolation of ligninolytic fungi.

From these samples collected, fungal colonies were obtained by serial dilution plating on PDA medium. The colonies were selected based on the colour and texture. Selected isolates were purified on media plates by the single spore isolation technique. A total of 30 fungal isolates, designated as PBNF 1 to PBNF 30 were successfully obtained as pure cultures through repeated sub-culturing based on their distinct colony characteristics. Details of the different isolates and their sources are presented in Table 1, while their morphological characteristics, including colony colour and growth pattern, are given in Table 2.

Table 1: Fungi isolated from field soil, decaying wood and compost pit

Sr. No.	Isolates code	Collection site
1	PBNF 1	Field soil
2	PBNF 2	Field soil
3	PBNF 3	Field soil
4	PBNF 4	Field soil
5	PBNF 5	Decaying wood
6	PBNF 6	Decaying wood
7	PBNF 7	Compost pit
8	PBNF 8	Compost pit
9	PBNF 9	Compost pit
10	PBNF 10	Compost pit
11	PBNF 11	Decaying wood
12	PBNF 12	Field soil
13	PBNF 13	Field soil
14	PBNF 14	Field soil
15	PBNF 15	Field soil
16	PBNF 16	Field soil
17	PBNF 17	Field soil
18	PBNF 18	Field soil
19	PBNF 19	Field soil
20	PBNF 20	Compost pit
21	PBNF 21	Compost pit
22	PBNF 22	Field soil
23	PBNF 23	Compost pit
24	PBNF 24	Compost pit
25	PBNF 25	Field soil
26	PBNF 26	Field soil
27	PBNF 27	Field soil
28	PBNF 28	Decaying wood
29	PBNF 29	Compost pit
30	PBNF 30	Decaying wood

Table 2: Morphological characteristics of fungal isolates

Isolate code	Colony colour	Growth pattern
PBNF 1	Dark grey to bluish grey	Slow growth
PBNF 2	Blue green	Slow growth
PBNF 3	Grey	Rapid growth
PBNF 4	Yellowish brown	Rapid growth
PBNF 5	Grey	Moderate growth
PBNF 6	Yellowish-green	Rapid growth
PBNF 7	Pale to dark green	Rapid growth
PBNF 8	Off white	Rapid growth
PBNF 9	White to grey	Rapid growth
PBNF 10	Grey	Rapid growth
PBNF 11	Olive brown to black	Slow growth
PBNF 12	Blue green	Moderate growth
PBNF 13	White	Moderate growth
PBNF 14	Pale brown	Rapid growth
PBNF 15	Light green	Rapid growth
PBNF 16	White to grey	Moderate growth
PBNF 17	Black	Rapid growth
PBNF 18	white	Moderate growth
PBNF 19	Bluish green	Moderate growth
PBNF 20	Dull green	Moderate growth
PBNF 21	White	Slow growth
PBNF 22	Whitish to blue green	Rapid growth
PBNF 23	Green	Rapid growth
PBNF 24	Light green	Rapid growth
PBNF 25	Black	Moderate growth
PBNF 26	White	Slow growth
PBNF 27	White	Slow growth
PBNF 28	Grey	Moderate growth
PBNF 29	Dark brown to almost black	Rapid growth
PBNF 30	Dark grey to black	Moderate growth

Primary screening

Primary screening was done by qualitative plate assay method using methylene blue, azure b, tannic acid, guaiacol and ABTS as indicators for determining the ligninolytic potential. Table 3 represents the source of isolation and the status of fungal isolates positive for at least one of the indicators. Use of these dyes and indicators using plate assay is preferred during primary screening as it is easy and quick due to the visible change of colour directly on plates.

Methylene blue Dye Decolorization Plate Assay

Among 30 isolates tested three fungal isolates (PBNF 11, PBNF 15 and PBNF 17) produced a clear zone and thus were found to degrade methylene blue and have ligninolytic potential. Ligninolytic enzymes like lignin peroxidaseis known to play a significant role in the decolorization of methylene blue dye decolorization plate assay.

Similar results were reported by researchers who used methylene blue dye as an indicator to identify the decolorization of isolated microbes (Ferreira-Leitão *et al*., 2007 [4]; Sasikumar *et al*., 2014 [11]; Dudhagara and Karetha, 2023) [3].

Guaiacol Plate Assay

Among the 30 isolates tested, isolate PBNF 15 exhibited the formation of a distinct brown zone around the colony, indicating positive ligninolytic activity. Ligninolytic enzymes like laccase known to play a significant role in the brown zone formation of guaiacol plate assay.

Similar results were reported by researchers who used guaiacol as an indicator to identify the ligninolytic fungi (Monssef *et al*., 2016) [9]

Table 3: Screening of isolates on various substrates for ligninolytic activities

Sr. No.	Fungal isolate	Methylene blue	Azure B	Guaiacol	ABTS	Tannic acid
1	PBNF 1	-	-	-	-	-
2	PBNF 2	-	-	-	-	-
3	PBNF 3	-	-	-	-	-
4	PBNF 4	-	-	-	-	-
5	PBNF 5	-	-	-	-	-
6	PBNF 6	-	-	-	-	-
7	PBNF 7	-	-	-	-	-
8	PBNF 8	-	-	-	-	-
9	PBNF 9	-	-	-	-	-
10	PBNF 10	-	-	-	-	-
11	PBNF 11	+	-	-	+	+
12	PBNF 12	-	-	-	-	-
13	PBNF 13	-	-	-	-	-
14	PBNF 14	-	-	-	-	-
15	PBNF 15	+	-	+	+	-
16	PBNF 16	-	-	-	-	-
17	PBNF 17	+	+	-	-	-
18	PBNF 18	-	-	-	-	-
19	PBNF 19	-	-	-	-	-
20	PBNF 20	-	-	-	-	-
21	PBNF 21	-	-	-	-	-
22	PBNF 22	-	+	-	+	+
23	PBNF 23	-	-	-	-	-
24	PBNF 24	-	-	-	-	-
25	PBNF 25	-	-	-	-	-
26	PBNF 26	-	-	-	-	-
27	PBNF 27	-	-	-	-	-
28	PBNF 28	-	-	-	-	-
29	PBNF 29	-	-	-	+	-
30	PBNF 30	-	-	-	-	-

Azure B Dye Decolorization Plate Assay

Out of the 30 isolates tested, two isolates PBNF 17 and PBNF 22 exhibited clear zones around their colonies, which was considered as positive for ligninolytic activity. Ligninolytic enzymes like lignin peroxidase and manganese peroxidase are known to play a significant role in the decolorization of azure b dye decolorization plate assay.

Tannic Acid Plate Assay

Only two fungal isolates, PBNF 11 and PBNF 22 produced brown coloration around their colonyand were positive for guaiacol plate assay.

The results obtained in the present study are in agreement with those of Sharma *et al*. (2017) [12], who screened fungal isolates on tannic acid supplemented media for ligninolytic activity. Similarly, Geetanjali (2012) reported that fungi particularly *Aspergillus* sp., *Chaetomium* sp., *Trichoderma* sp. and *Penicillium* sp., which exhibited ligninolytic activity were able to grow on tannic acid as the sole carbon source.

ABTS Plate Assay

Among the isolates tested, four isolates PBNF 11, PBNF 15, PBNF 22, and PBNF 29 exhibited positive ABTS oxidation

activity, as evidenced by the formation of distinct green halos on the plates.

In the present study, positive ABTS oxidation was observed in select fungal isolates, indicating extracellular laccase production. These results are consistent with previous findings by Ingle (2020) [7], who screened fungal isolates for lignin-degrading enzyme activity and reported that *Trichoderma* species tested positive for laccase production under plate assay conditions.

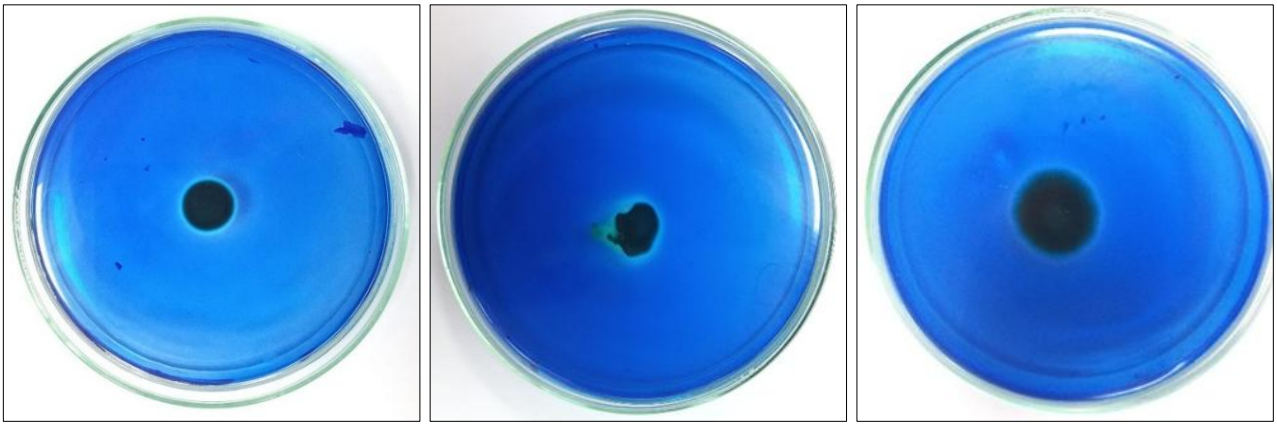
Identification of potential ligninolytic fungal isolates

The selected fungal isolates were initially characterized based on their morphological and cultural traits. For further identification, microscopic examination was performed using lactophenol cotton blue staining.

Key morphological characteristics, including the shape and arrangement of conidia, conidiophore structure, and hyphal features, were examined to aid in the taxonomic classification of the fungal isolates. Based on these observations, the isolates PBNF 11, PBNF 15, PBNF 17, PBNF 22, and PBNF 29 were identified as *Cladosporium* sp., *Trichoderma harzianum*, *Aspergillus niger*, *Penicillium chrysogenum* and *Aspergillus awamori*, respectively.The identification of potential fungal isolates based on morphology and microscopic characterization is presented in Table 4.

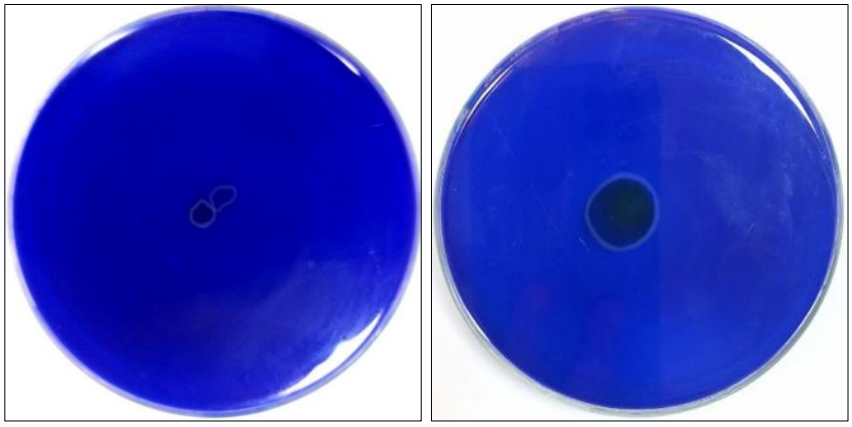
Table 4: Morpho cultural characterization of potential fungal isolates

Sr. No.	Isolates code	Colony characterization	Microscopic characterization	Identified strain
1	PBNF 11	Olive-brown to black and velvety	Branched conidiophores with shield shaped conidia	*Cladosporium* sp
2	PBNF 15	White-green to light green and cottony	Highly branched, aseptate conidiophores with ellipsoidal conidia	*Trichoderma harzianum*
3	PBNF 17	Black and fluffy appearance	Uniseriate conidiophores with round conidia	*Aspergillusniger*
4	PBNF 22	Whitish to blue-green colony and velvety	Branched Conidiophores with chained spherical conidia	*Penicilliumchrysogenum*
5	PBNF 29	Dark brown to almost black colonies	Biseriate conidial heads and smooth to delicately roughened conidia.	*Aspergillusawamori*



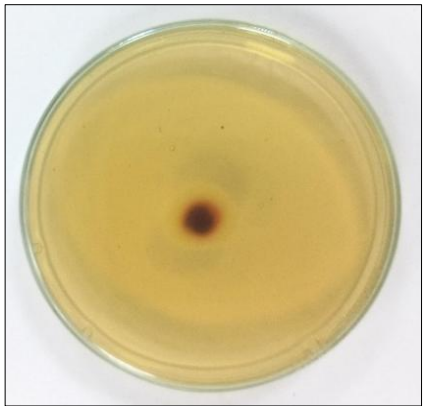
A B C

Fig 1: Clear zone formation on MEA stained with methylene blue A. PBNF 11, B. PBNF 15, C. PBNF 17



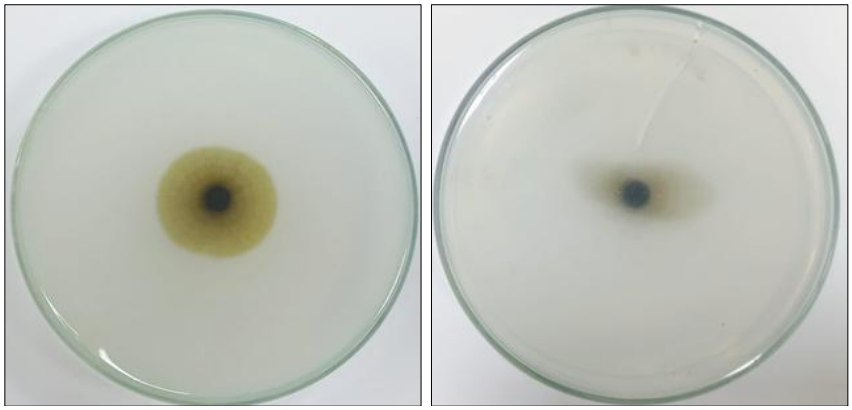
A B

Fig 2: Clear zone formation on LBM medium stained with azure B A. PBNF 17, B. PBNF 22



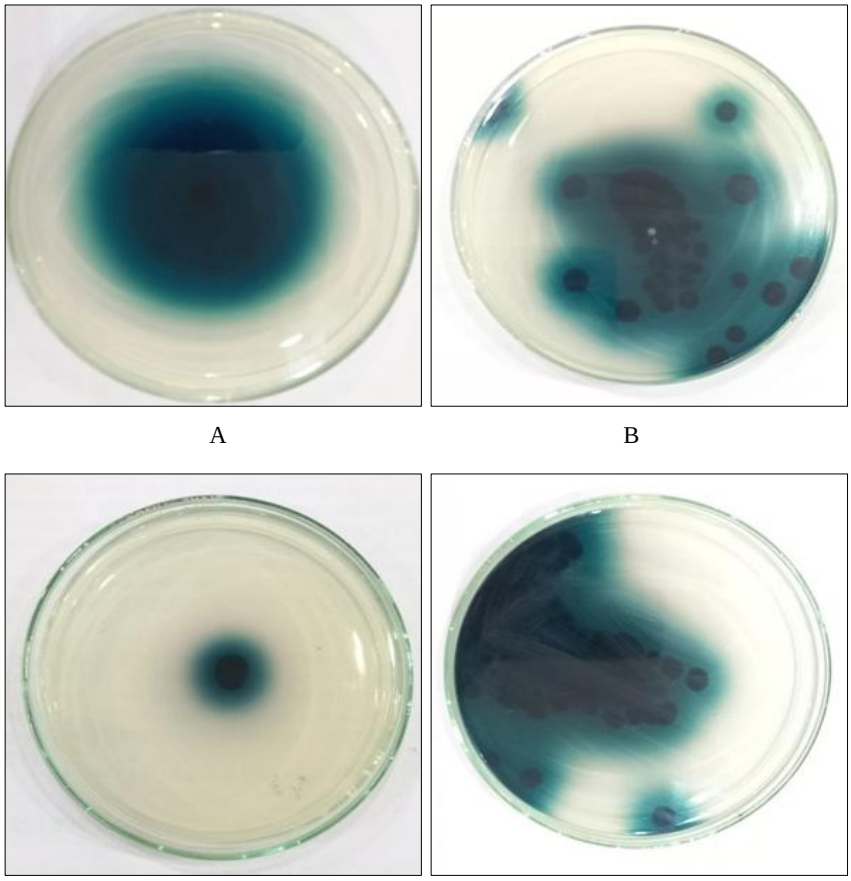
A

Fig 3: Brown colour formation on MEA stained with guaiacol PBNF 29



A B

Fig 4: Brown zone formation on LBM medium stained with tannic acid A. PBNF 11, B. PBNF 22



A B

C D

Fig 5: Green colour formation on LBM medium stained with ABTS A. PBNF 11, B. PBNF 15, C. PBNF 22, D. PBNF 29

Conclusion

The present study was undertaken with the aim of isolation and screening of ligninolytic fungi from various sources. Five fungal strains belonging to four different genera include *Cladosporium* sp., *Aspergillus* sp., *Trichoderma* sp., and *Penicillium* sp., exhibited one of the extracellular ligninolytic enzymes (Laccase, Manganese peroxidase and Lignin peroxidase) activities.

This study serves as a preliminary investigation, and further research is needed to optimize cultural and environmental parameters for improved enzyme yields. However, the fungal isolates examined exhibit considerable potential for the production of ligninolytic enzymes-laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP). These enzymes have wide-ranging industrial and biotechnological applications, including the biodegradation of lignocellulosic biomass for bioethanol production, bioremediation of polluted environments, delignification in the paper and pulp industry, decolorization of industrial dyes, treatment of textile and tannery effluents, and synthesis of value-added bioproducts. The promising enzymatic capabilities of these fungi highlight their relevance in sustainable waste management and green industrial processes.

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