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SCREENING FOR LIGNIN DEGRADING MICROORGANISMS

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Abstract

Kraft lignin, a prominent by-product of the paper pulping process, presents notable environmental challenges because of its complex and resistant structure. Microbial degradation of kraft lignin through ligninolytic enzymes (such as lignin peroxidases, manganese peroxidases, or laccases) offers an environmentally friendly biotechnological solution for its utilisation. This study outlines a comprehensive plate screening method aimed at identifying microorganisms capable of degrading kraft lignin. The screening process resulted in the discovery of several microbial strains that showed prominent oxidative discoloration zones, indicating ligninolytic enzyme activity. The microbial strain with the highest ligninolytic activity was determined to be Hericium erinaceus. Cultivating this mushroom on liquid medium with kraft lignin as a substrate provided an insight on the activities of the main oxidative enzymes produced, such as: lignin peroxidase, manganese peroxidase, and laccase. These findings highlight that this screening method is an effective step in identifying microorganisms suitable for kraft lignin valorisation in biotechnological applications.

Key words*: kraft lignin, laccase, lignin peroxidase, manganese peroxidase.*

INTRODUCTION

Annually, approximately 181.5-200 billion tons of lignocellulosic biomass are produced globally, but only a small fraction (8-20 billion tons) is currently utilised (Ashokkumar et al., 2022; Devi et al., 2022; He et al., 2023). The main sectors generating lignocellulosic biomass are agriculture (crops, residues like straw, corn stover, bagasse, etc.), forestry (wood, residues), dedicated energy crops, and some industrial and municipal wastes. Among these, the agricultural sector is typically the largest contributor to global lignocellulosic biomass due to massive volumes of crop residues and by-products (Ashokkumar et al., 2022; Devi et al., 2022).

Current valorisation approaches mainly involves physical, chemical, biological, and physicochemical pretreatments to breakdown lignocellulosic biomass's recalcitrant structure. First and second-generation biorefineries convert cellulose and hemicellulose to biofuels, biochemicals, and materials, while lignin is often underutilised and mostly burnt for energy (Ashokkumar et al., 2022; He et al., 2023; Brienza et al., 2024).

Lignocellulosic biomass consists primarily of cellulose (35-50%), hemicellulose (25-35%), and lignin (10-25%). Cellulose and hemicellulose are usually converted to biofuels, biodegradable materials, and chemicals, while lignin can be converted to aromatic chemicals, adhesives, carbon fibres, phenolics, and more advanced polymeric materials (Devi et al., 2022; He et al., 2023; Ingle et al., 2025).

The main problem in converting lignocellulosic by-products into valuable compounds lies in the structural recalcitrance of the biomass, particularly in depolymerising lignin, which protects cellulose and hemicellulose and undergoes condensation during harsh pretreatments. Traditional methods often degrade lignin into non-valuable products or render it less reactive, hindering its conversion into high-value chemicals (Ashokkumar et al., 2022; Brienza et al., 2024).

The kraft pulping process produces high amounts of lignin-rich waste known as kraft lignin, a highly resistant and aromatic polymer. Although it holds promise as a renewable source for valuable chemicals, most kraft lignin remains unutilised and is mainly incinerated for energy, raising environmental issues and resulting in inefficient resource use (Argyropoulos et al., 2023).

"Lignin-first" processes aim to preserve lignin structure and yield higher-value aromatic compounds – potentially producing bio-based chemicals, resins, carbon materials, and specialty polymers. However, challenges remain in efficiently and selectively breaking lignin bonds without undesirable side reactions, scaling up technologies, and competing economically with petroleum-derived counterparts (Brienza et al., 2024; Ingle et al., 2025).

Biological lignin degradation, especially by fungi and bacteria, has gained attention as a sustainable strategy for lignin valorisation. The process depends on key enzymes such as lignin peroxidases (E.C. 1.11.1.14), manganese peroxidases (E.C. 1.11.1.13), and laccases (E.C. 1.10.3.2.), which synergistically oxidise various lignin structures. Microbial strains that produce these enzymes are especially important for applications in biorefining, bioremediation, and green chemistry (Singhania et al., 2022).

Accurate discovery of such microorganisms requires selective, high-throughput screening techniques. This study designed a plate-screening method able to identify microorganisms capable of degrading kraft lignin through ligninolytic enzymatic activities

MATERIALS AND METHODS

Microorganisms

A total of 31 microbial strains isolated from different sources were used in this study. **Bacteria** (Table 1) (*Bacillus amyloliquefaciens* [OS15, OS17, BW, BN7, and BIR]*, Bacillus subtilis* ATCC 6633*, Bacillus* sp. [1T2, B4, B5, B6, B7.2, and BPA]), **actinomycetes** (*Streptomyces* sp. [SV1, SV2, SV3, SV4, SV5, SV6, SV7, and SV8]), **fungi** (*Penicillium* spp. - isolated from apple [P.a.] and cucumber [P.c] *Trichoderma* spp. [Tdcp, Td2]*, Aspergillus niger* An5*,* and *A. carbonarius*), and **macromycetes** (*Ganoderma applanatum, G. lucidum, Agaricus arvensis, Pleurotus ostreatus,* and *Hericium erinaceus)* were provided by the Faculty of Biotechnology (USAMV of Bucharest) collection.

To carry out the screening and evaluation of ligninolytic capacity, the tested microorganisms were cultivated under standardised conditions specific to each taxonomic group, using nutrient media appropriate for their initial growth, followed by inoculation onto selective media.

Table 1. Isolation source for bacterial strains

Microbial strain	**Isolation source**
Bacillus amyloliquefaciens OS15	onion rhizosphere
B. amyloliquefaciens OS17	onion rhizosphere
B. amyloliquefaciens BW	soil
B. amyloliquefaciens BN7	agricultural wastes
B. amyloliquefaciens BPA	plant pathogen antagonists
B. amyloliquefaciens BIR	plant pathogen antagonists
B. *endophyticus* 1T2	endophyte of various plant species
B. *subtilis* ATCC 6633	reference strain from the American Type Culture Collection
B. subtilis B7.2.	agricultural wastes
B. subtilis B4	compost tea
B. subtilis B5	compost tea
B. subtilis B6	compost tea

Bacterial cultures were obtained by cultivating the isolates on LB (Luria Bertani) medium (Roth, Karlsruhe, Germany) for a period of 4-5 days. These mature cultures were subsequently used to inoculate the selective media.

Actinomycetes cultures were developed on Gauze medium (Table 2) adjusted to pH 7.4 for 4-5 days and were transferred to selective media.

Table 2. Gauze's medium composition

Ingredient	Concentration (g/L)
Soluble starch	20
KNO_3	1
NaCL	0.5
$MgSO_4$ x 7 H_2O	0.5
K_2HPO_4	0.5
$FeSO_4$ x 7 H_2O	0.01
Agar	15

Fungal cultures were initially grown on PDA (Potato Dextrose Agar) medium (Roth, Karlsruhe, Germany) for 7-8 days, after which they were used for inoculation onto selective media.

All incubations were conducted under aerobic conditions and at constant temperature, in order to ensure the optimal development of the colonies.

Screening of microbial strains for ligninolytic activity

The microbial strains were subjected to a plate screening method for their ligninolytic activity using a specific cultivation medium with kraft lignin as a substrate. The medium composition (g/L) was: agar - 20, glucose - 5, ammonium tartrate - 5, malt extract - 1, $MgSO_4$ x $7H_2O$ - 0.5, NaCl - 0.1, $CaCl_2$ - 0.01, $FeCl_3$ - 0.01, thiamine - 0.001, and kraft lignin - 0.1.

The Petri dishes containing the medium were inoculated with the microorganism and incubated at 28±2°C for 3-10 days, depending on the type of strain. The plates were analysed at every 24 hours for the formation of discolouration zones (halos) around the colonies. For better visualisation and measurements, the plates were flooded with a solution prepared with 1% $FeCl_3$ and 1% $K_3[Fe(CN)_6]$ (1:1). The solution was a chromogenic system capable of highlighting the oxidative-reductive changes produced by the enzymatic secretions of the cultivated microorganisms. The discolouration around the colonies was used as an indirect indicator of potential ligninolytic oxidative-enzymatic activity. After 5-10 minutes, the solution was discarded.

The microbial strains that exhibited the formation of a decolourisation halo around the colonies were subjected to measurements of their colony diameter and halo diameter.

$$\text{Ligninolytic capacity} = \frac{diameter\ of\ the\ halo\ zone}{diameter\ of\ the\ colony}$$

According to the obtained values, the microbial strains were categorised into three levels of ligninolytic activity:

- Low degradation capacity (+): values = 1.0;
- Moderate degradation capacity (++): values between 1.0-2.0;
- High degradation capacity (+++): values greater than 2.0.

Evaluation of lignin-degrading enzymes from a selected microbial strain

Following the plate screening method, the microbial strain identified with the highest estimated ligninolytic activity was cultivated on liquid medium in order to determine the activity of the main enzymes involved in lignin degradation such as: lignin peroxidase, manganese peroxidase, and laccase.

The composition of the medium (g/L) was: glucose - 10, ammonium tartrate - 0.2, KH_2PO_4 - 2, $MgSO_4$ x $7H_2O$ - 0.5, $CaCl_2$ x $2H_2O$ - 0.1, thiamine - 0.001, $FeCl_3$ - 0.01, kraft lignin - 2.5, and trace elements solution - 1mL.

The trace elements solution (g/ L) contained: $FeSO_4$ x $7H_2O$ - 5, $MnSO_4$ x H_2O - 1.6, $ZnSO_4$ x $7H_2O$ - 1.4, $CuSO_4$ x $5H_2O$ - 0.3, $CoCl_2$ x $6H_2O$ - 0.2, Na_2MoO_4 x $2H_2O$ - 0.2, H_3BO_3 - 0.5, EDTA - 1.

Lignin peroxidase assay

Lignin peroxidase (LiP) assay was determined using a kinetic spectrophotometric assay with veratryl alcohol as the substrate (Vandana et al., 2019). The reaction mixture contained 0.39 mL sodium tartrate buffer 50 mM, pH 2.5, 20 µL veratryl alcohol 2 mM, and 70 µL sample. The reaction was initiated with 20 µL H_2O_2 0.1 mM, and the oxidation of veratryl alcohol to veratraldehyde was monitored by measuring the increase in absorbance at 310 nm using a spectrophotometer. The change in absorbance per minute was recorder, and the enzyme activity was calculated as follows:

$$\text{LiP activity (U/mL)} = \frac{\frac{\Delta A}{\Delta t}\ x\ Vtotal\ x\ 1000}{\varepsilon\ x\ l\ x\ Vsample}$$

where:

$\frac{\Delta A}{\Delta t}$ - slope of the absorbance change per minute during the linear phase;

V_{total} - total reaction volume (mL);

V_{sample} - volume of the sample used (mL);

l - optical path length;

ε - molar extinction coefficient for veratraldehyde (9300 $M^{-1}cm^{-1}$).

One unit (U) is defined as the amount of enzyme that catalyses the formation of 1 µmol of product per minute, under the assay conditions.

Manganese peroxidase assay

Manganese peroxidase (MnP) assay activity was assessed spectrophotometrically using a guaiacol-coupled assay in the presence of Mn^{2+} (Ayla et al., 2018). The assay monitors enzyme-catalysed oxidation of guaiacol that is mediated by H_2O_2 in a Mn^{2+}-dependent reaction cycle. In the presence of an appropriate chelating buffer (50 mM sodium tartrate buffer, pH 4.5), the enzyme oxidises Mn^{2+} to Mn^{3+}, which in turn

oxidises guaiacol, producing a chromogenic product (tetraguaiacol) whose formation is followed as an increase in absorbance at 470nm. The reaction mixture consisted of 25 µL guaiacol 10 mM, 25 µL $MnSO_4$ 10mM, 25 µL sample, and 0.4 mL buffer. In order to initiate the reaction, 25 µL H_2O_2 1 mM was added, and the absorbance associated with guaiacol oxidation was recorded as a function of time at 470 nm. Appropriate controls were included in order to correct for non-enzymatic oxidation of guaiacol and for baseline drift. Enzyme activity was quantified from the initial rate of increase in absorbance using Beer-Lambert relationships and the appropriate extinction coefficient for the guaiacol oxidation product. The MnP activity was calculated using the following formula:

$$\text{MnP activity (U/mL)} = \frac{\frac{\Delta A}{\Delta t} x\ Vtotal\ x\ 1000}{\varepsilon\ x\ l\ x\ Vsample}$$

where:
$\frac{\Delta A}{\Delta t}$ - slope of the absorbance change per minute during the linear phase
V_{total} - total reaction volume (mL);
V_{sample} - volume of the sample used (mL);
l - optical path length;
ε - molar extinction coefficient for tetraguaiacol (26600 $M^{-1}cm^{-1}$).
One unit (U) is defined as the amount of enzyme that catalyses the formation of 1 µmol of product per minute, under the assay conditions.

Laccase assay

Laccase (Lac) activity was determined spectrophotometrically using guaiacol as the chromogenic substrate (Albu et al., 2019; Mohammed et al., 2024). The reaction mixture contained 0.1 mL sample, 0.3 mL sodium acetate buffer 0.01M pH 5.0, and 0.1 mL guaiacol 2 mM. The assay was performed at 30°C. Oxidation of guaiacol by laccase produced coloured reaction products that absorb in the visible region, and the product formation was followed by measuring the increase in absorbance at 450 nm. Initial reaction rates were obtained from the linear portion of the absorbance versus time trace at 450 nm. Lac activity was calculated from the slope of absorbance change per minute (ΔA/min) using Beer-Lambert relationship and expressed as U/mL, where one unit is defined as the amount of enzyme that catalyses formation of 1 µmol of tetraguaiacol per minute, under these experimental conditions.

$$\text{Lac activity (U/mL)} = \frac{\frac{\Delta A}{\Delta t} x\ Vtotal\ x\ 1000}{\varepsilon\ x\ l\ x\ Vsample}$$

where:
$\frac{\Delta A}{\Delta t}$ - slope of the absorbance change per minute during the linear phase
V_{total} - total reaction volume (mL);
V_{sample} - volume of the sample used (mL);
l - optical path length;
ε - molar extinction coefficient for tetraguaiacol (12100 $M^{-1}cm^{-1}$).

RESULTS AND DISCUSSIONS

The discovery of efficient lignin-degrading microorganisms is critical for advancing sustainable biomass valorisation, enabling the conversion of recalcitrant lignin into value-added products and reducing environmental waste from lignocellulosic industries.
In this study, 31 microbial strains were tested for their ability to degrade kraft lignin, a highly complex and recalcitrant aromatic polymer. The isolates were cultivated on specific media and were monitored at 24-hour intervals in order to assess the dynamics of colony development and to determine the optimal time for the application of the chromogenic indicator. The use of this indicator enabled the detection of either the presence or absence of a discolouration halo, which was considered a visual marker of lignin-degrading activity. In addition, the measurements of colony diameter compared to halo diameter allowed to determine the microbial strains with the highest ligninolytic activities.
Among the 12 bacterial strains tested, all the strains belonging to the genus *Bacillus* exhibited growth on the selective medium, however after the use of the dye solution, no discolouration zone was observed (Figure 1), a phenomenon considered an indirect marker of ligninolytic activity. This suggest either the absence of lignin degrading enzymes or its occurrence at levels below the detection limit under the experimental conditions tested.

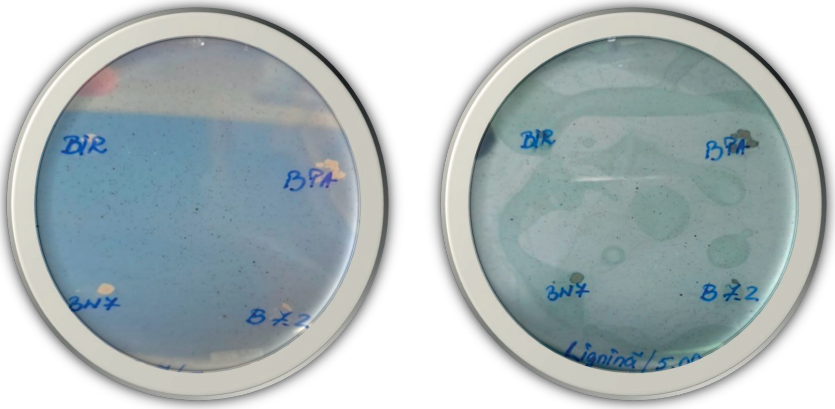

Figure 1. Bacterial isolates aspect before (left) and after (right) application of chromogenic indicator

Among the eight *Streptomyces* sp. strains isolated from soil and cultivated on selective medium all exhibited colonial development. Nevertheless, the chromogenic indicator did not reveal the formation of discolouration halo, suggesting the absence of detectable ligninolytic activity under the experimental conditions employed.

Within fungi, six filamentous strains were tested belonging to the genera *Penicillium*, *Trichoderma*, and *Aspergillus*. Among the *Penicillium* sp. strains examined, the grown colonies did not exhibit a discolouration halo, indicating low levels of ligninolytic activities (Figure 2).

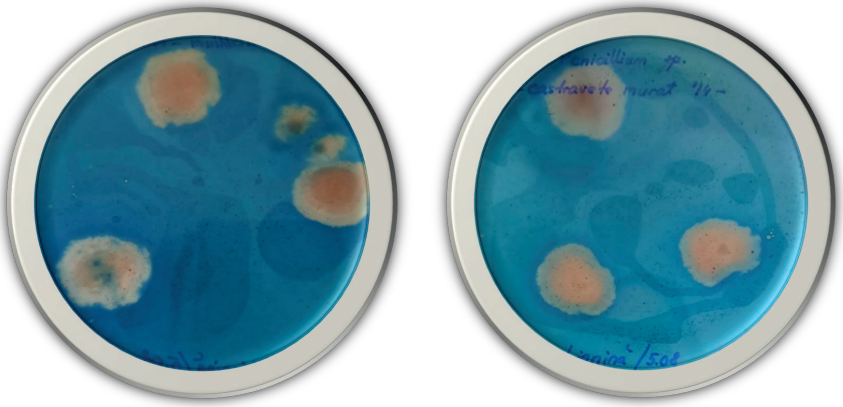

Figure 2. Ligninolytic activity of *Penicillium* spp.: P.a. (left) and P.c. (right)

In the case of *Trichoderma* sp., only one strain (Tdcp) produced a discolouration zone surrounding the microbial colony, suggesting a possible lignin-degrading capacity.

By contrast, both *Aspergillus* strains tested generated visible chromatic halos, highlighting a potential enzymatic capacity for lignin degradation (Figure 3).

Aspergillus niger An5 showed higher ligninolytic activity than *A. carbonarius*, indicating a higher production of oxidative enzymes such as laccases, peroxidases, or other oxidases.

The advantages of *A. niger* are its ability to grow fast on minimal media, and higher tolerance at wider ranges of pH and temperatures.

Figure 3. Ligninolytic activity of *A. niger* An5 (left) and *A. carbonarius* (right)

In the present study, 5 macromycetes strains were also evaluated representing the genera *Ganoderma*, *Pleurotus*, *Agaricus*, and *Hericium.* Both *Ganoderma* sp. strains displayed clear discolouration halos around the colonies beginning on the 5th day post-inoculation, indicating substantial enzymatic activity associated with lignin degradation (Figure 4).

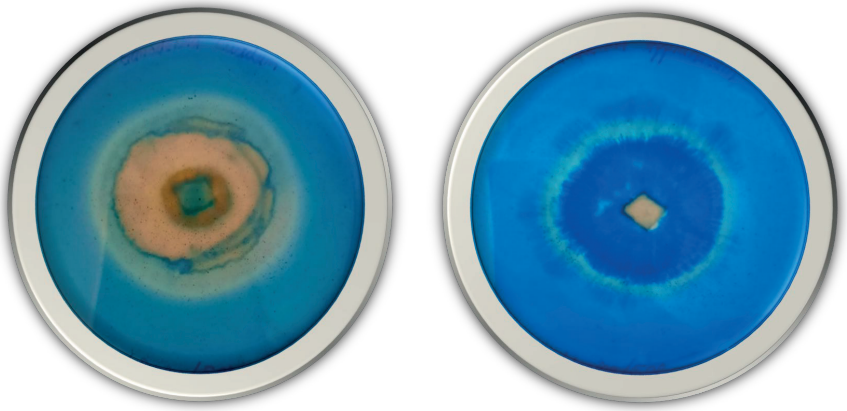

Figure 4. Ligninolytic activity of *G. lucidum* (left) and *G. applanatum* (right)

The *Pleurotus ostreatus* strain likewise produced a distinct discolouration halo after 5 days of incubation, suggesting active ligninolytic potential under the experimental conditions applied (Figure 5).

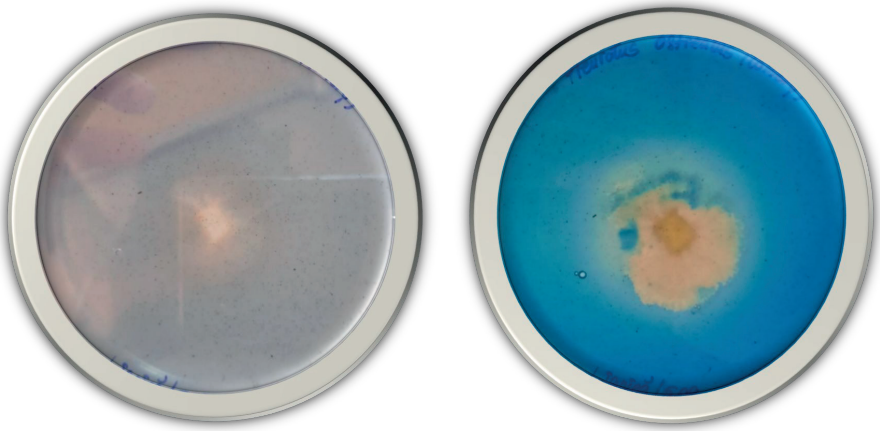

Figure 5. *P. ostreatus* aspect before (left) and after (right) application of chromogenic indicator

Among all the strains tested, *Agaricus arvensis* and *Hericium erinaceus* exhibited the most pronounced lignin–degrading activities, as evidenced by the largest and most well-defined chromatic halos, confirming a remarkable enzymatic potential for delignification. In contrast with other filamentous fungi or macromycetes, the growth of *H. erinaceus* was observed after 7 days post-incubation and the discolouration zone was visible after approximately 11 days (Figure 6).

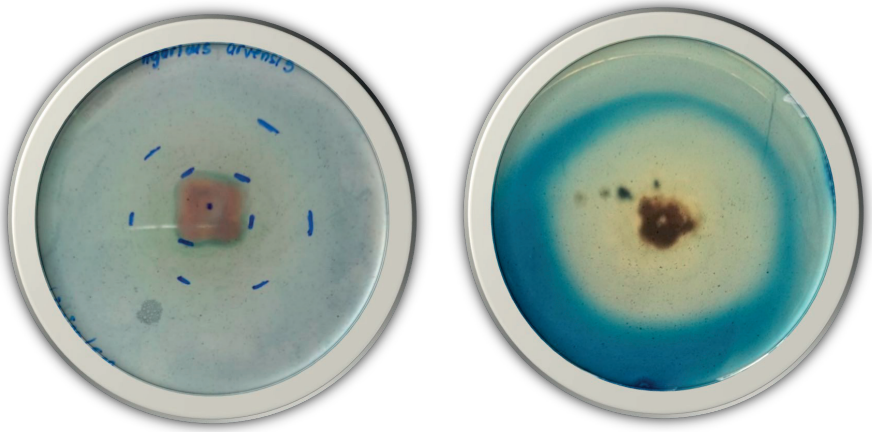

Figure 6. Ligninolytic activity of *A. arvensis* (left) and *H. erinaceus* (right)

After analysing all the plates and measuring the diameters of the colonies and the halos, it was possible to determine that 10 microbial strains showed ligninolytic activity. Low activity was observed for *Penicillium* spp. and *Trichoderma* Tdcp, and moderate for *Aspergillus* spp., *Ganoderma* spp., and *Pleurotus ostreatus*. The highest estimated ligninolytic activities were established for *Agaricus arvensis* and *Hericium erinaceus* (Table 3).

For further research, *Hericium erinaceus* strain was selected as it presented the highest ligninolytic activity, and is not well studied regarding its ability to degrade kraft lignin. Therefore, the strain was cultivated in liquid medium with lignin as a substrate, and tested for its ability to produce lignin peroxidase, manganese peroxidase, and laccase.

Table 3. Ligninolytic activity of tested microbial strains

Nr. Crt.	Microbial strain	Ligninolytic activity
1.	*Penicillium* P.a.	+
2.	*Penicillium* P.c.	+
3.	*Trichoderma* Tdcp	+
4.	*Aspergillus niger* An5	++
5.	*Apergillus carbonnaris*	++
6.	*Ganoderma applanatum*	++
7.	*Ganoderma lucidum*	++
8.	*Pleurotus ostreatus*	++
9.	*Agaricus arvensis*	+++
10.	*Hericium erinaceus*	+++

+ low activity; ++ moderate activity; +++ high activity

LiP activity displays a characteristic curve with a pronounced peak around day 25 (Figure 7). Initially, LiP activity increases gradually, reaching a maximum of approximately 0.043 U/mL. This peak suggests an induction phase during mid-incubation, potentially coinciding with optimal substrate availability or environmental conditions in favor of LiP expression and activity. After reaching maximum value, LiP activity declines steadily, stabilising at a lower level toward the end of the incubation. Other studies (Vrsanska et al., 2016) reported similar behaviour for *Trametes suaveolens* when cultivated on PDB with different inducers. This pattern may reflect enzyme degradation, substrate depletion, or regulatory feedback mechanisms inhibiting LiP production.

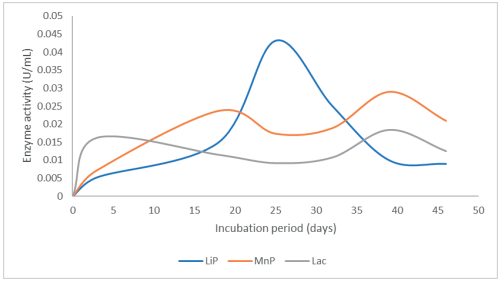

Figure 7. Enzymatic activities (Lip, MnP, Lac) of *Hericium erinaceus* cultivated on kraft lignin

In contrast, MnP activity follows a biphasic pattern, exhibiting two distinct peaks (Figure 7). The first increase is gradual, reaching an initial peak (~0.024 U/mL) around day 17. Following a slight decline, MnP activity exhibits a second higher peak (0.03 U/mL) near day 40. The presence of two peaks may indicate differential regulation or the enzyme's involvement in multiple stages of the substrate degradation process. Similar behaviour was observed in other researches (Vrsanska et al., 2016) with *Fomes fomentarius*. The sustained MnP activity toward the later incubation period suggests its continued importance in lignin degradation processes during prolonged incubation.

The Lac activity profile differs from LiP and MnP, showing relatively moderate and stable activity with minor fluctuations (Figure 7). Lac activity increases rapidly during the initial 5 days, reaching about 0.017 U/ mL, followed by a gradual decline and a slight secondary increase around day 40. Similar fluctuations of Lac activities were also observed for *Trametes versicolor* and *Trametes gibbose* (Vrsanska et al., 2016). The relatively low amplitude and stable pattern suggest laccase may play a consistent, basal role in the enzymatic system, possibly involved in substrate oxidation or electron transfer processes throughout the incubation without dramatic induction or repression.

According to Van La et al. (2023), laccase from *Hericium erinaceus* was considered a highly promising biocatalyst due to its wide substrate range and stability in organic solvents commonly used for converting lignin derivatives. Also, in their study, it was noticed that the enzyme's activity improved substrate solubility, making it more accessible for other enzymes. Similarly, in this study, laccase activity was initially relatively high, but then gradually declined, coinciding with the increased activities of the other two enzymes: lignin peroxidase and manganese peroxidase.

Other studies (Eichlerová and Baldrian, 2020; Kim, 2021; Kang et al., 2024; Ma et al., 2021) have investigated macromycetes abilities in lignin degradation, highlighting the potential of mushroom-derived ligninolytic enzymes (Lac, LiP, MnP, or versatile peroxidases) for converting lignin into valuable bio-based chemicals and materials. However, further investigations are needed in order to explore the enzymatic mechanisms responsible for breaking

down lignin's challenging structure and assess efficiency. Therefore, the fungal ligninolytic enzymes offer a promising environmentally friendly solution for the transformation of lignin into high-value aromatic compounds.

CONCLUSIONS

The plate screening method approach proved effective in isolating potential kraft lignin-degrading microorganisms from various strains tested. Notably, the fungal isolates (*Penicillium* spp., *Trichoderma* Tdcp, *Aspergillus* spp., *Ganoderma* spp., *Pleurotus ostreatus, Agaricus Avensis,* and *Hericium erinaceus*) were the only ones that exhibited ligninolytic activity. The identified microbial strains represent promising candidates for further investigation in biorefinery applications, particularly for the development of enzymatic lignin valorisation platforms.

The enzyme activity profiles of *Hericium erinaceus* cultivated on kraft lignin liquid medium suggest a coordinated and temporally regulated ligninolytic enzymatic system. LiP appears to be predominantly active in the mid-phase of incubation, while MnP demonstrates a more complex, biphasic activity pattern extending into the later stages. Lac maintains relatively constant activity, potentially supporting ongoing oxidative processes. These differential patterns may reflect substrate specificity, enzyme stability, or regulatory mechanisms inherent to the microbial strain or the cultivation conditions employed.

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