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Isolation, characterization and screening of *Pleurotus* species for cellulase enzyme production

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Abstract

The increasing need for sustainable methods of lignocellulosic waste degradation has driven interest in the enzymatic capabilities of fungi, particularly members of the genus Pleurotus. This study aimed to isolate, characterize, and screen Pleurotus species from agro-waste-rich soil for their potential cellulase enzyme production. Ten soil samples were separately collected with sterile containers from different locations from an agro-waste dump site having decaying plant organic matter within a mushroom farm at Ugbene 2, Abakpa-Nike, in Enugu state. The samples were serially diluted before being cultured on potato dextrose agar (PDA) using spread plate technique. After incubation at 37°C for 72 hours, fungal colonies were sub-cultured and characterized both macroscopically, microscopically and molecularly. Cellulase activity screening was carried out using a qualitative assay on carboxymethyl cellulose (CMC) agar stained with Congo red, followed by destaining with NaCl. Enzyme activity was indicated by clear halos around fungal colonies. Molecular identification using Internal Transcribed Spacer (ITS) region amplification confirmed the isolates as *Pleurotus ostreatus* strain 24, *Pleurotus ostreatus* strain ICMP 11679, *Pleurotus ostreatus* isolate ITCC3226, and *Pleurotus pulmonarius* strain DMRP-32. The cellulolytic potential of these strains depicted by the zones of hydrolysis ranged from 14 mm (*P. ostreatus* strain 24 and ICMP 11679) to 16 mm (*P. ostreatus* strain ITCC3226 and *P. pulmonarius* DMRP-32), indicating significant cellulase activity. These findings suggest that the isolated Pleurotus strains are promising candidates for biotechnological applications in biodegradation and biofuel production.

Keywords: *Pleurotus* species; Soil fungi; Molecular identification; Cellulase activity; Agro-waste biodegradation; ITS gene amplification; Fungal enzyme screening

1. Introduction

Mushrooms of the genus *Pleurotus*, commonly known as oyster mushrooms, are widely cultivated edible fungi valued for their nutritional richness, medicinal properties, and environmental benefits. They are saprophytic organisms capable of growing on a wide variety of organic substrates, making them ecologically significant decomposers in forest ecosystems (Sánchez, 2010). *Pleurotus* species are also known for their low-fat content, high protein levels, and bioactive compounds, contributing to their global demand in the food and pharmaceutical industries (Bellettini *et al*., 2019). Mushrooms are a rich source of nutrients, especially proteins and minerals, in addition to vitamins B, C, and D (Panjikkaran and Matthew, 2013). They contain 20–35% protein (dry weight), are low in lipids, and contain all nine essential amino acids (Kalac, 2009). Mushrooms are considered a delicacy, acclaimed for their characteristic texture and enjoyable flavor. They have received significant attention from food and pharmaceutical researchers because of their bioactive constituents (Sheu *et al*., 2007; Mariga *et al*., 2014).

Pleurotus spp. is one of the most extensively studied fungi due to its strong enzymatic activity and versatility in breaking down organic matter (Philippousis *et al*., 2001; Olivieri *et al*., 2006; Li and Shah, 2016). These fungi are known to

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degrade plant cell wall components such as cellulose and hemicellulose, making them suitable for agricultural waste recycling (Machado *et al*., 2016). *Pleurotus* species can produce various hydrolytic enzymes. Thus, by-products from agricultural crops containing organic polysaccharides can be effectively utilized as substrates (Kumla *et al*., 2020). Annually, large amounts of fruits and vegetables are harvested, leading to substantial quantities of agricultural wastes (Fukuda, 2021). After harvesting, these wastes are often burned, contributing to soil depletion and severe air pollution (Rungjindamai *et al*., 2024). This situation demands urgent solutions (Arunral *et al*., 2018; Mihai *et al*., 2022). Concerns about pollution and environmental conservation have triggered the need for a new generation of cleaner industrial production systems that maximize efficiency while minimizing contamination (Illuri *et al*., 2021). Mushrooms are now being exploited as an alternative and safe source of extracellular enzymes, with *Pleurotus* species recognized for their efficiency in utilizing plant-based residues (Zhang *et al*., 2002; Salmones *et al*., 2005; Albores *et al*., 2006).

Agro-industrial wastes—comprising agricultural and industrial residues—are mainly composed of organic polymers such as cellulose and hemicellulose (Kumla *et al*., 2020). These wastes are widely used as substrates in mushroom cultivation. Most agro-industrial wastes are low in nitrogen content, necessitating the addition of organic or inorganic nitrogen supplements (Ragunathan and Swaminathan, 2003; Cueva *et al*., 2017). Cellulose is a homopolymer composed of a linear chain of several hundred to thousands of β-1,4-linked D-glucose units. The composition of these polysaccharides in agro-industrial waste depends on the species, tissue, and maturity of the plant (Anwar *et al*., 2014; Ravindran and Jaiswal, 2016; Zhou *et al*., 2016; Sadh *et al*., 2018). Efficient degradation of these materials requires the synergistic action of various carbohydrate-active enzymes, which act on different chemical bonds (Kumla *et al*., 2020). This degradation is facilitated by cooperative activities of hydrolytic and oxidative enzymes (Lombard *et al*., 2014; López-Mondéjar *et al*., 2016; Madeira *et al*., 2017). These enzymes are involved in the breakdown and assembly of glycosidic bonds (Eichorst and Kuske, 2012; Lombard *et al*., 2014; Andlar *et al*., 2018).

Cellulase enzymes—comprising endoglucanases, exoglucanases, and β-glucosidases—play a critical role in the hydrolysis of cellulose into fermentable sugars. These enzymes have diverse industrial applications, including bioethanol production, textile processing, animal feed improvement, and agricultural waste recycling (Singhania *et al*., 2013). The sustainable production of cellulase from fungi offers an eco-friendly and economically viable approach to converting plant biomass into valuable bioproducts. Mushrooms, especially *Pleurotus* species, are recognized for their robust enzyme-producing systems, including potent cellulolytic activity. This makes them suitable candidates for biotechnological applications in agricultural waste degradation and environmental bioremediation.

The increasing accumulation of agricultural residues poses a significant environmental challenge, particularly in developing countries. Exploring native *Pleurotus* species for cellulase production may provide a sustainable strategy for biomass valorization and waste management. Moreover, identifying high-yield, cellulase-producing strains can enhance industrial enzyme production and contribute to the development of bio-based economies.

This study aims to

- Isolate and characterize *Pleurotus* species from natural substrates;
- Screen the isolated species for cellulase enzyme production;

2. Material and methods

2.1. Sample Collection

Ten soil samples were separately collected with sterile containers from different locations from an agro-waste dump site having decaying plant organic matter within a mushroom farm at Ugbene 2, Abakpa-Nike, Enugu in Enugu state.

2.2. Isolation of Pleurotus Species

2.2.1. Sample Preparation

Each soil sample was air- dried, to reduce moisture content, which could inhibit fungal growth and separately sieved to remove larger particles. Then 1g was taken separately from each soil sample and suspended in 9ml of sterile distilled water in a test tube. Each test tube was thoroughly shaken for 15 minutes, to allow heavier particles to settle at the bottom. The supernatant from each tube was separately decanted into another sterile test tube and was serially diluted (up to 10^{-4}), using ten-fold dilution method to decrease the microbial load of each sample (Aneja, 2003).

2.2.2. Isolation of Organisms Using Potato Dextrose Agar

The spread plate method was employed. From each diluted soil sample, 0.1ml was separately pipetted and placed on the surface of solidified PDA in plates already containing chloramphenicol, to suppress bacterial growth. Each inoculum was evenly spread on the surface of the solidified medium with a glass spreader. The plates were incubated at 30°C for 6 days. Meanwhile the plates were observed daily, looking for characteristics typical of *Pleurotus* species. After incubation, each developed mycelium was sub-cultured into fresh PDA medium to obtain a pure culture. The grown cultures were later subjected to identification (Chang and Miles, 2004).

2.3. Characterization and Identification of Pleurotus Isolates

2.3.1. Phenotypic Identification

The isolates were characterized macroscopically (whitish, creamy to grayishy, fluffy mycelium, spreading radially outwards from the point of inoculation) and microscopically (septate hyphae with clamp connections (typical of Basidiomycetes) and spore structure.

2.3.2. Genomic Identification

The isolates were subjected to genomic identification. The DNA isolation and other procedures were adopted as described by Shen (2025)

2.4. Screening for Cellulase Production

2.4.1. Qualitative Detection of Cellulase Enzymes:

The cellulase enzymes activity was detected by dye staining of carboxymethyl cellulose (CMC). The composition of Cellulose Basic Medium (CBM) (g L^{-1}) was listed below:

Table 1 Basic Cellulose Medium Composition

Composition	Amount (g/L)
$C_6H_{12}N_2O_6$	5
KH_2PO_4	1
$MgSO_4 \cdot 7H_2O$	0.5
Yeast Extract	0.1
$CaCl_2 \cdot 2H_2O$	0.001

The above composition (CBM) was supplemented with 2 % w/v CMC and 1.6 % w/v agar and later autoclaved (Atri and Sharma, 2011). The medium was poured into plates and after solidification, the test organisms were inoculated onto the medium and incubated at 25 degrees in darkness for 6 days. The plates were stained first by flooding with 2 % w/v aqueous Congo red in which they were left to stand undisturbed for 15 minutes. The stain was poured off and then the plates were flooded with 1M NaCl, to destain for 15 minutes. The activity was observed as yellow opaque area against a red colour of undegraded CMC.

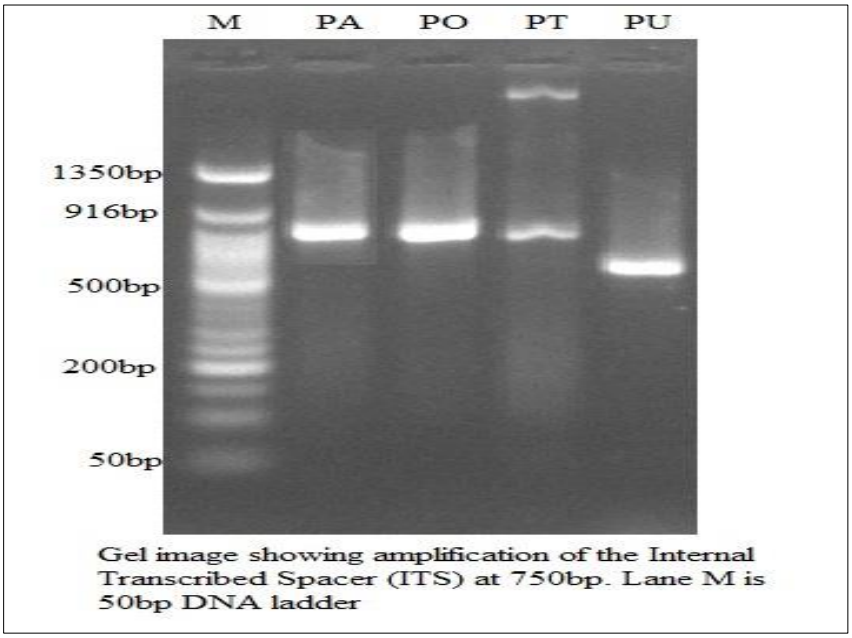
2.4.2. Zone of Hydrolysis Measurement

After destaining, the clear zones of hydrolysis surrounding the fungal colonies were measured. The diameter of the clear zone (including the colony) was recorded in millimeters using a transparent ruler. The actual zone of hydrolysis was calculated by subtracting the diameter of the fungal colony from the total diameter of the halo. The results were recorded and used as a measure of cellulase activity.

3. Result

Table 2 Physical Morphology and Microscopic Appearance of the Fungal Isolates

Physical Morphology	Microscopic Appearance	Probable Organism
Mycelium whitish initially, later a white spot at the center surrounded by a dark brown patch, which was also surrounded by off- white patch, all fluffy and spreading radially outwards from the point of inoculation. Fast growth denser at the center but lighter towards the margin.	Septate hyphae; clamp connections present and spores smooth, cylindric- ellipsoid and slightly smaller.	*Pleurotus spp. (PA)*
Mycelium whitish initially, became off-white and fluffy, spreading radially outwards from the point of inoculation.	Septate hyphae with clamp connections; smooth, cylindric-ellipsoid spores, slightly bigger.	*Pleurotus spp. (PO)*
Mycelium whitish initially, became golden yellow, fluffy, spreading radially outwards from the point of inoculation.	Septate hyphae with clamp connections; smooth, cylindric-ellipsoid spores, slightly bigger.	*Pleurotus spp (PT)*
Mycelium whitish initially, turned grayish and later became densely dark and fluffy, spreading radially outwards from the point of inoculation.	Septate hyphae with clamp connections; smooth, cylindric-ellipsoid spores, slightly bigger.	*Pleurotus spp (PU)*



Gel image showing amplification of the Internal Transcribed Spacer (ITS) at 750bp. Lane M is 50bp DNA ladder

Figure 1 Gel Image Showing Amplification of the Internal Transcribed Spacer at 750bp of the Isolates

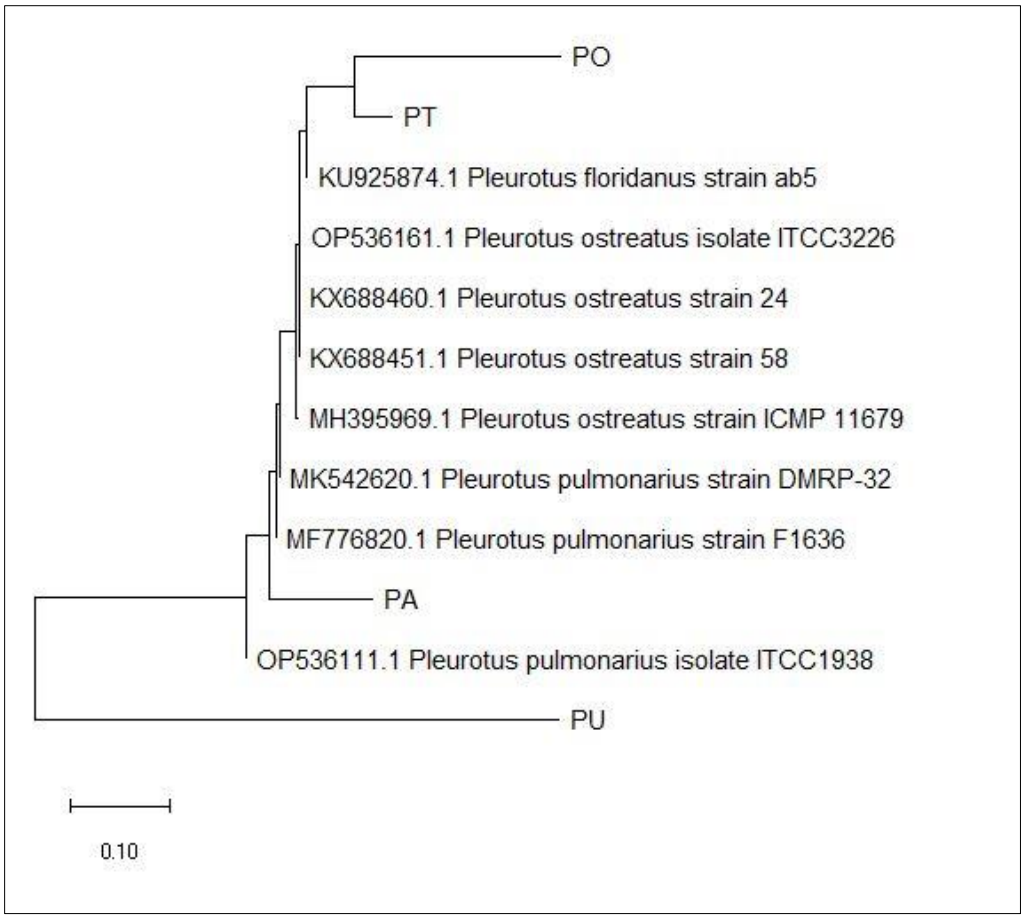


Figure 2 Phylogentic Tree of the Isolates

Table 3 Screening for Cellulase Activity of the Isolates

S/N	Isolate ID	Zone of Hydrolysis (mm)
1	*Pleurotus ostreatus* strain 24 (PO)	14
2	*Pleurotus ostreatus* strain ICMP 11679 (PT)	14
3	*Pleurotus ostreatus* isolate ITCC3226 (PU)	16
4	*Pleurotus pulmonarius* strain DMRP-32 (PA)	16

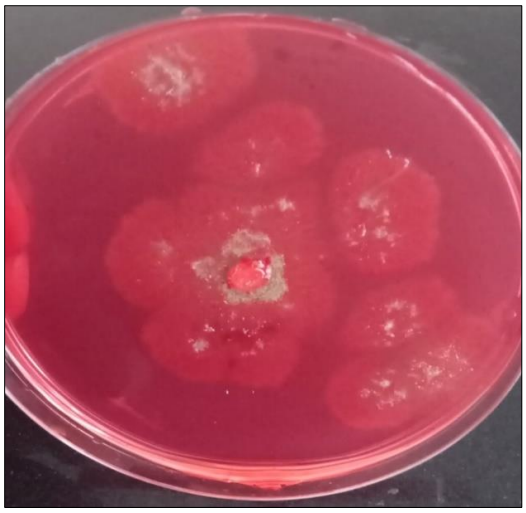


Figure 3 Plate showing the zone of hydrolysis of *P. ostreatus* strain 24

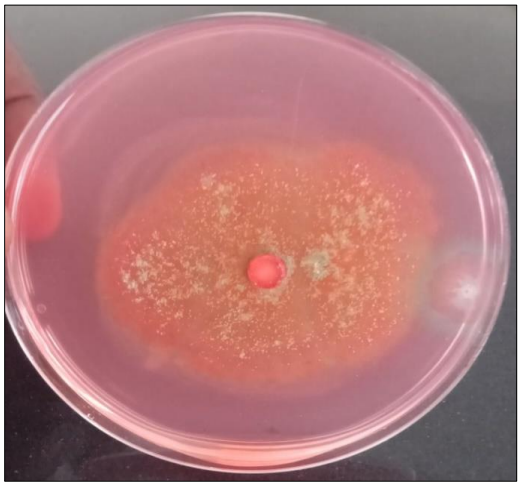


Figure 4 Plate showing the zone of hydrolysis of *P. ostreatus* isolate ITCC3226

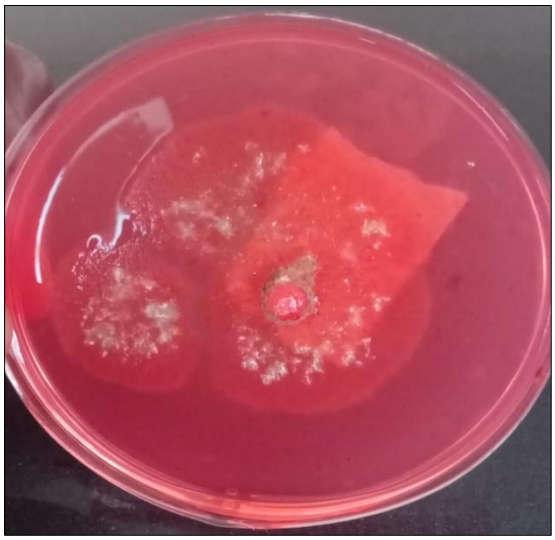


Figure 5 Plate showing the zone of hydrolysis of *P. pulmonarius* strain DMRP-32

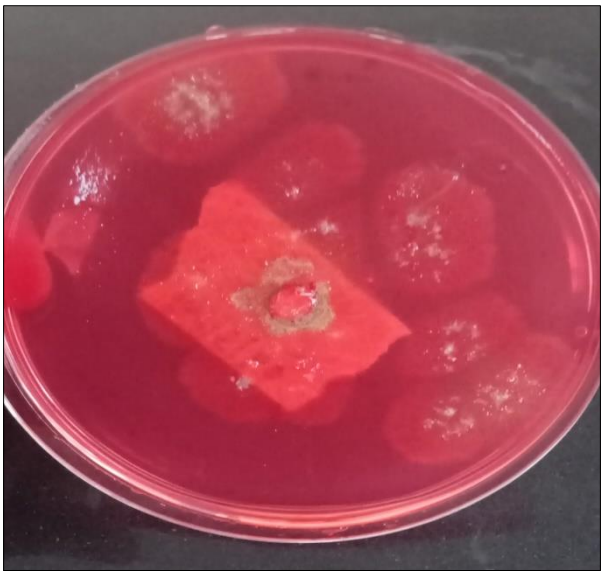


Figure 6 Plate showing the zone of hydrolysis of *P. ostreatus* strain ICMP 11679

4. Discussion

Table 4.1 shows the physical and microscopic features of four fungal isolates identified as *Pleurotus* species. The physical and microscopic examination of the four *Pleurotus* isolates: *Pleurotus pulmonarius* strain DMRP-32 **(**PA), *Pleurotus ostreatus* strain 24 **(**PO), *Pleurotus ostreatus* strain ICMP 11679 **(**PT), and *Pleurotus ostreatus* isolate ITCC3226 **(**PU), revealed typical features of the genus, *Pleurotus*. All isolates initially produced whitish mycelia that spread radially from the inoculation point, with variations in pigmentation and density observed as they matured. For instance, isolate PT turned golden yellow, while PU became densely dark, indicating potential metabolic differences. Microscopically, all isolates showed septate hyphae with clamp connections and smooth, cylindric-ellipsoid spores, consistent with *Pleurotus* characteristics (Jonathan and Fasidi, 2003; Sánchez, 2010). The consistent presence of clamp connections confirms successful isolation of *Pleurotus* species. Differences in colony appearance, especially the intense pigmentation and dense growth of PU, may suggest higher metabolic or enzymatic activity (Zervakis *et al*., 2001; Chang and Wasser, 2017). Such phenotypic variations are often useful for predicting enzyme productivity, especially cellulase. These findings align with earlier reports by Sánchez (2010) and Kumla *et al*. (2020), who emphasized that *Pleurotus* strains vary in growth behavior and enzyme yield depending on genetics and environmental conditions. Isolate PU, in particular, may be a strong candidate for further screening due to its robust growth, which has been linked to enhanced enzyme secretion (Gulati *et al*., 2007). Overall, the morphological traits observed provide a foundational basis for selecting high-performing strains for cellulase production.

Figure 4.1 shows gel electrophoresis image which confirms successful amplification of the Internal Transcribed Spacer (ITS) region in four *Pleurotus* isolates: *Pleurotus pulmonarius* strain DMRP-32 **(**PA), *Pleurotus ostreatus* strain 24 **(**PO), *Pleurotus ostreatus* strain ICMP 11679 **(**PT), and *Pleurotus ostreatus* isolate ITCC3226 **(**PU). All amplified DNA fragments appear near the 750 bp mark, which is consistent with the expected size for fungal ITS regions. Lane M is the DNA ladder (50 bp ladder) used to estimate fragment sizes. All *Pleurotus* isolates show distinct single bands around 750 bp, confirming that the ITS primers used were specific and effective for amplifying the fungal DNA. The consistency in band position across lanes PA, PO, PT, and PU indicates similar ITS region sizes among the isolates, which is characteristic of the *Pleurotus* genus (Schoch *et al*., 2012). The sharpness and intensity of bands suggest good DNA quality and efficient PCR amplification.

The successful amplification of the ITS region validates the identity of the isolates as fungal species belonging to *Pleurotus*. This molecular confirmation strengthens the morphological and microscopic identification and provides a genetic basis for further phylogenetic or enzymatic studies. ITS-based identification is particularly useful in fungal taxonomy due to its high interspecies variability and has become the standard barcode marker for fungi (Schoch *et al*., 2012). These results are in agreement with previous research demonstrating ITS amplification in *Pleurotus* species using universal fungal primers ITS1 and ITS4. For instance, Kumari and Achal (2008) reported ITS band sizes between 700–800 bp for *Pleurotus* isolates, while Abdullah *et al*. (2022) also obtained ~750 bp bands in their molecular

identification of *Pleurotus ostreatus* and related species. The consistent amplification observed here further supports the use of ITS as a reliable molecular marker for fungal genotyping and identification.

The phylogenetic tree in figure 4.2 illustrates the genetic relationships among *Pleurotus* species based on ITS sequences. Isolates PO and PT cluster closely with *Pleurotus ostreatus*, while PA and PU group with *Pleurotus pulmonarius*, with PU forming a slightly distinct branch. This pattern supports the molecular and morphological identification of multiple *Pleurotus* species in the study. The results align with previous studies showing the effectiveness of ITS markers in distinguishing closely related *Pleurotus* species and highlight the genetic diversity valuable for various biotechnological applications.

Table 4.2 shows the results of a cellulase activity assay conducted on four *Pleurotus* strains. The ability of each isolate to produce cellulase enzymes was assessed using carboxymethyl cellulose (CMC) agar, where the formation of a clear zone of hydrolysis indicates enzymatic degradation of cellulose. The values observed range from 14 mm to 16 mm, indicating significant cellulolytic potential across all tested isolates. Specifically, *Pleurotus ostreatus* strain 24 **(**PO) and *Pleurotus ostreatus* strain ICMP 11679 **(**PT) each showed a zone diameter of 14 mm, while *Pleurotus ostreatus* isolate ITCC3226 **(**PU) and *Pleurotus pulmonarius* strain DMRP-32 **(**PA) demonstrated slightly higher activity, both with a zone of hydrolysis of 16 mm. This range in hydrolytic activity suggests some degree of variation in cellulase production, even among strains of the same species. The differences could be attributed to intrinsic genetic variation or differential adaptation to substrates. According to Sánchez (2010), such strain-level variability is common in *Pleurotus* spp. and is influenced by both environmental and physiological factors. Importantly, the strains exhibiting the larger hydrolysis zones which are *P. ostreatus* isolate ITCC3226 **(**PU) and *P. pulmonarius* DMRP-32, may possess higher enzymatic efficiency or greater capacity to secrete cellulases into their environment. These traits make them strong candidates for biotechnological applications requiring effective lignocellulose degradation.

From a bioconversion standpoint, all the isolates are promising for industrial use. However, the superior cellulolytic activity demonstrated by *P. pulmonarius* DMRP-32 (PA) and *P. ostreatus* isolate ITCC3226 **(**PU) suggests they could be more effective for processes like biofuel production, composting, and agricultural waste recycling. These findings align with the observations of Baldrian and Valášková (2008), who reported that *Pleurotus* species are among the most efficient lignocellulose-degrading fungi due to their robust enzymatic arsenal, which includes cellulases, hemicellulases, and lignin-degrading oxidases. In comparison to other documented studies, the hydrolysis zone diameters of 14–16 mm fall within the expected range for *Pleurotus* spp. For instance, Jonathan and Adeoyo (2011) reported hydrolytic zones ranging from 13 mm to 18 mm in Nigerian *Pleurotus* isolates, while Ahmad *et al*., (2010) observed similar values between 12 mm and 19 mm across several strains. These comparisons reinforce the validity of the current results and confirm that the isolates under investigation possess adequate cellulase-producing capabilities consistent with those reported in the literature.

5. Conclusion

The study demonstrated that soil enriched with agro-waste serves as a viable source for isolating cellulolytic fungi, particularly species within the Pleurotus genus. Through morphological and molecular characterization, four distinct strains—*Pleurotus ostreatus* strain 24, *P. ostreatus* strain ICMP 11679, *P. ostreatus* isolate ITCC3226, and *Pleurotus pulmonarius* strain DMRP-32—were identified. Screening for cellulase activity revealed measurable enzymatic potential in all isolates, with hydrolysis zones ranging between 14 mm and 16 mm. These results confirm the relevance of Pleurotus species as efficient cellulose degraders and highlight their applicability in eco-friendly waste conversion technologies and industrial enzyme production.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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