Unraveling the efficient cellulolytic and lytic polysaccharide monooxygenases producing microbes from paddy soil for efficient cellulose degradation

Abstract

Paddy straw, an abundant agricultural residue, poses significant environmental challenges due to its improper management and disposal practices, including the prevalent practice of residue burning. To address these issues, there is a growing need to explore sustainable alternatives for paddy straw decomposition. This study aims to unravel the crucial role of microbes in facilitating rapid paddy straw decomposition. In this study, both bacterial and fungal cultures were isolated and screened for cellulolyticenzyme activity. Among the microorganisms, fungi isolates showed significantly higher CMSase and FPase activity compared to bacterial isolates. Fungal isolates exhibiting superior enzymatic activities were subsequently identified using ITS. Among the fungal isolates F-9:*Aspergillus fumigatus* and F-5:*Trichoderma asperellum* exhibited the highest CMCase and FPase activity with 40.14 and 68.02 U mL^{-1} respectively,when inoculated in a Reese's mineral medium containing 1% microcrystalline cellulose. Through spectrophotometric analysis the highest LPMOs activity was recorded in F-8:*Aspergillus aculeatus*with 0.85 U mL^{-1} and F-3:*Phanerodontiachrysosporium*with 0.73 U mL^{-1}. This study highlights the important role of fungi in utilizing cellulose and emphasizes the need to further explore their potential for facilitating paddy straw decomposition.

Keywords: Lignocellulose;LPMO; CMCase; FPase; *Trichoderma asperellum*; *Phanerodontiachrysosporium*; *Aspergillus fumigatus*; *Aspergillus aculeatus*;

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1. Introduction

Residue management is a major challenge in rice-wheat cropping systems (Ladhaet al.,2000). Across the globe, rice is cultivated over 158 million hectares (Mha) yielding around 700 million tonnes (Mt) per year to supply grain demand (Parihar et al., 2023). Asia produces 640 Mt of rice from 143 Mha of land, which accounts for more than 90% of the world's rice

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production(Ministry of Agriculture and Farmers Welfare, 2021). Rice production generates up to 700 million tons of rice straw yearly, which is a leftover byproduct of rice after harvest. (Singh et al., 2023). Uttar Pradesh produces 4.4 Mt of unused rice residue from 1.3 Mha, while Haryana produces 7.5 Mt from 1 Mha, and Punjab generates 22 Mt from 2.9 Mha. Managing this rice straw poses a substantial challenge. Hence farmers often burn to clear the fields(NAAS, 2017;Parihar et al., 2023). West Bengal, Odisha, Andhra Pradesh, Tamil Nadu, and Bihar are the primary cultivators, accounting for over 80% of the rice production in India (Madhu et al., 2023). In Odisha, rice is one of the most widely grown crops occupying nearly 73% of the total cultivated areaand producing 9% of total rice in India (Jena and Mahapatra, 2023). Burning of stubble is less prevalent in Odisha compared to North India but is gradually spreading across this coastal state.In India, the burning of rice straw typically takes place from October to November, indicating the start of winter each year. During this time, dense fog is frequently seen, which combines with substantial smoke from burning straw, leading to smog formation (Singh and Kaskaouits, 2014). In Delhi, atmospheric particulate matter has risen twentyfold beyond the threshold level recommended by the World Health Organization (Manisalidis et al., 2020).

Farmers commonly burn rice straw due to its low value, labour shortages, and perceived benefits for pest management (Dobermann and Witt, 2000; Hays et al., 2005; Parihar et al., 2023). This practice, while addressing storage constraints, contributes to soil degradation, alters plant and soil ecology, and significantly elevates soil temperatures (Thakur et al., 2018; Lohan et al., 2017; Gupta, 2012). Elevated soil temperatures cause the removal of 23–73% of nitrogen, altering the C: N ratio in the topmost soil layers (Gupta et al., 2010; Kumar et al., 2015), while also emitting CO_2 and converting nitrogen to nitrate, resulting in significant loss of major soil nutrients (*i.e.*, N, P, K) from the soil (Gupta et al., 2004; Jat et al., 2013). In addition, residue burning degrades soil health, reduces crop yields, and decreases microbial diversity down to a soil depth of 2.5 cm (Mehta et al., 2013; El-Sobky, 2017; Abdurrahman et al., 2020; Sagarika et al., 2022).Previous studies have highlighted the utilization of crop residues as mulch to improve crop productivity and soil health (Chatterjee et al., 2016, 2017, 2018); nevertheless, the adoption of mulching for cereal crops remains limited in India.

A potential solution to curb the menace of rice residue burning is leveraging the microbial potential for the production of lignocellulase enzymes (*i.e.*, hydrolytic and non-hydrolytic

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enzymes)that efficiently and rapidly degrade lignocelluose biomass. Fungi and bacteria play a major role in the breakdown of lignocellulosic materials present in paddy straw. Through their enzymatic activities, these microorganisms produce a range of lignocellulolytic enzymes, such as cellulases, hemicellulases, and ligninases, which efficiently degrade the complex organic compounds present in paddy straw (Hu et al., 2023). These enzymes work synergistically to break down the lignocellulosic structure, releasing simple sugars that can be utilized by microorganisms for energy production and growth (Raza et al., 2023). For example, microbes produce hydrolytic enzymes (e.g., CMCase, FPase) and non-hydrolytic enzymes (e.g., lytic polysaccharide monooxygenases) that are mainly responsible for the degradation process (Sagarika et al., 2022). Previous studies emphasized mostly onmicrobes that produce hydrolytic enzymeshowever,the roles of non-hydrolytic enzymes such as lytic polysaccharide monooxygenases(LPMOs) have not been studied wellfor paddy straw decomposition. Hence, the current study was conducted to screen potential fungal and bacterial isolates found in paddy soil environment for their production of hydrolytic and non-hydrolytic enzymes for efficient decomposition of paddy straw in tropical humid climate in eastern India.

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2. Materials and methods

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2.1 Collection and isolation of bacteria and fungi

The bacterial and fungal isolates were screened from paddy soil and compost samples at ICAR-National Rice Research Institute (ICAR-NRRI) farm in Cuttack, India (20.5°N, 86°E) for this study followed by storage at 4 °C for isolation purposes.Serial dilution and plating techniques were used to isolate potential bacterial and fungi cultures following the method of Barnett and Hunter (1972). The nutrient agar (NA), potato dextrose agar (PDA) and Rose-Bengal agar mediumwere used for isolation and were further tested for lignocellulolytic activity (Fig. 1)

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2.2 Identification of fungal isolates

Fungal isolates were cultured from slant agar, transferred to fresh Carboxymethyl cellulose (CMC) agar Petri dishes, and incubated at room temperature for 6 days. Morphological observations were done based on colony pigmentation, and physical appearance (Kim et al., 2011). Genomic DNA extraction from fungi was performed using the (HiPurA^{TM} Fungal DNA purification kit) from 7 day-old fresh cultures. Amplification of ribosomal internal transcribed

spacer (ITS) regions was carried out using primers ITS1 and ITS4. The obtained sequences were compared to related sequences using a BLAST search within the GenBank database NCBI (Alsohaili et al., 2018).

2.3 Screening of potent bacterial cellulolytic activity

CMCase activity was done by taking a loopful of bacterial isolates from the 7 days NA plates which were inoculated in Reese's mineral medium broth containing 1% CMC and Whatman No. 1 filter paper strip (50mg) were incubated at 30°C for 48 hrs. The cell biomass was harvested by centrifugation and supernatant was used for estimation of CMCase assay FPase assay. The absorbance was measured at 540nm as described by Ghose (1987).

2.4 Screening of potent fungi on microcrystalline cellulose medium

Enzyme activity was done by taking a 6 mm disc cut fungal culture from 7 days old PDA plates and was inoculated in Reese's mineral broth containing 1% microcrystalline celluloseand incubated at 30°C for 120 h. After 7 days the mycelial mat were separated through Whatman filter paper No.1 from the culture filtrate. The obtained filtrate was stored at 4°C and assayed for Reducing sugar, CMCase, FPase, and LPMO activities.

2.5 Estimation of CMCase assay

The CMCase assays were performed using 0.5 ml of the enzyme, and 0.5 ml of the substrate solution, which was diluted in citrate buffer thoroughly mixed and incubated at 50°C for 30 minutes. Then 3 ml of dinitrosalicylic acid (DNS) was added to the mixture and boiled for 5 minutes. Then, 20 ml of distilled water was added and the absorbance was measured at 540nm (Ghose, 1987).

2.6 Estimation of FPase assay

The FPase assay wasperformed using 50 mg of filter paper strips, 0.5 ml enzyme solution, and 1 ml of 0.05 M Na-citrate at pH 4.8, incubated at 50°C for 60 minutes. 3 ml of DNS solution was added, and the mixture was boiled for 5 minutes. Later 20 ml of water was added and thoroughly mixed and the resulting colour was measured at A540 nm (Ghose, 1987).

2.7 Estimation of reducing sugar

The reducing sugar was performed by usinga DNS reagent. The formula for calculating reducing sugar in the sample is given below (Miller, 1959).

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$$\text{Reducing sugar (\%)} = \frac{\text{Sugar value from graph (µg)}}{\text{Aliquot sample used (mL)}} \text{X} \frac{\text{Total volume of alcohol free extract}}{\text{Weight of sample}} \text{X } 100$$

2.8 Estimation of LPMOs production

A 116 mM sodium succinate/phosphate buffer at pH 6.0 or 7.5, along with separate stock solutions of 10 mM 2,6-DMP and 5 mMH_2O_2 in pure water, were prepared for immediate use. After centrifuging a 1 mL sample at 6000×g for 3 minutes to remove cells and solids, 500 µL of the supernatant was transferred to a clean vial and kept on ice. Subsequently, a mixture of 860 µL buffer, 100 µL 2,6-DMP stock solution, and 20 µL H_2O_2 stock solution was incubated at 30 °C for 15 minutes before spectrophotometric analysis 20 µL of sample (LPMO)was added and calculation was doneas described by (Breslmayr et al., 2018).

3. Results and discussions

3.1 Identification of fungal strains

In this current study both morphological and molecular techniques were used for the identification of fungal strains isolated from paddy soil and compost samples. Among the 10 fungal isolates, F-4, F-5, and F-6 initially exhibited white to yellow-green pigmentation, transitioning to forest green after 6 days (Fig. 2). The formation of concentric rings denotes that F-5 and F-6 are *Trichoderma* sp.Previous researchers also reported similar findings in the case of *Trichoderma* sp. (Castrillo et al., 2021; Gezgin et al., 2023).The isolate F-1 exhibited a dark green colour with a smooth-walled surface, turned brown with age identified as *Aspergillus* sp. Isolates F-2, F-7, and F-8 exhibited dark brown to black powdered colonies, while F-9 and F-10 displayed greyish to green pigmentation with powdered colonies, all identified as *Aspergillus* sp.The reverse side of the colonies wasfound to be off-white. Previous researchers identified *Aspergillus sp*. based on the "top view" of black colour pigmentation of the mycelium that was due to black conidia growth (Diaz et al., 2021). Diaz et al. (2021) also reported that the colour of conidia varied from greenish brown to dark brown or nearly black on CYA media, with darker

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shades observed as the incubation temperature rose (from 25°C to 37°C). In the current study, F-1, F-2, F-7, F-8, F-9, and F-10 isolates were identified as *Aspergillus sp*. Isolate F-3 appeared completely white, identified as *Phanerochaete* sp.similar findings were reported by Khalil et al., (2021). In this current study, it was found that *Phanerochaete* sp. a white-rot fungus hasa slower growth rate compared to *Trichoderma sp.* and *Aspergillus* sp.The fungal isolates showing superior CMCase, FPase, and LPMO activity were identified by molecular characterization (Table 1). The isolated fungal strains belong to three classes: Eurotiomycetes- F-8: *Aspergillus aculeatus*(PANCOM12), *F-9: Aspergillus fumigatus* (PANCOM13); Sordariomycetes- F-5: *Trichoderma asperellum* (PANCOM5); Agaricomycetes- F-3: *Phanerodontiachrysosporium* (PANCOM3).

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Table 1. Molecular identification of fungal strains isolated from paddy soil and compost.

Isolates	Code Name	Accession Number	Strain Name	Morphological Identification
F-3	PANCOM3	MT007528.1	*Phanerodontiachrysosporium*	White colour
F-5	PANCOM5	MT007530.1	*Trichoderma asperellum*	Yellow-green to forest green
F-8	PANCOM12	MT007537.1	*Aspergillus aculeatus*	Dark brown to black
F-9	PANCOM13	MT007538.1	*Aspergillus fumigatus*	Greyish to green

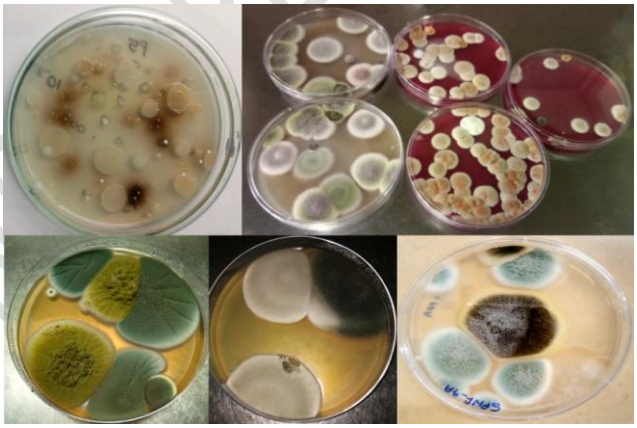

Fig. 1Isolation of fungal isolates on PDA, CMC agar and Rose bengal agar medium.

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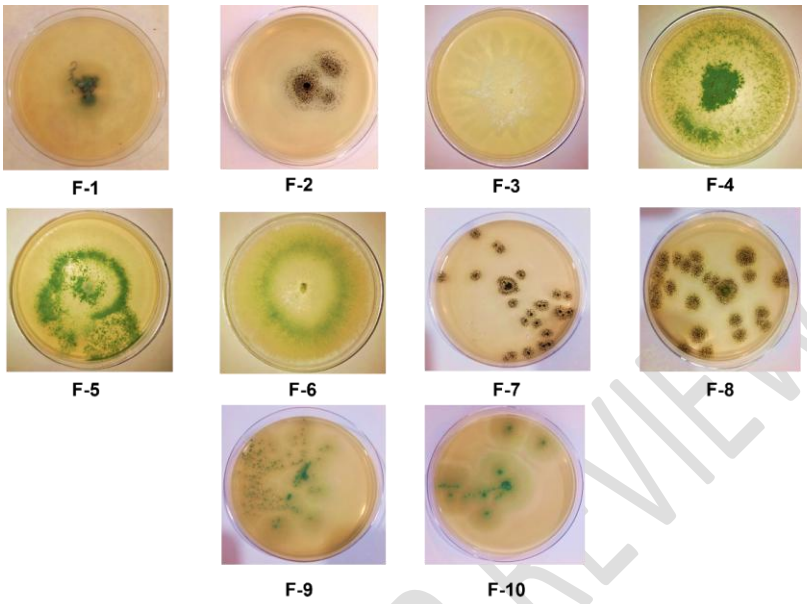

Fig. 2.Growth of fungal isolates on Carboxymethyl cellulose agar medium.

3.2 Quantitative screening of bacterial enzymes

In this experiment, 10 potential lignocellulolytic bacterial and fungal isolates were evaluated based on their CMCase and FPase activity. Carboxymethylcellulase assay was performed for estimation of the cellulolytic activity of selected bacterial isolates. SB-10 isolate recorded the highest CMCase activity with 4.18 U/mL^{-1} followed by SB-8 (2.94 U/mL^{-1}) (Fig. 3). Bacterial isolates SB-1, SB-3, SB-8, SB-9, and SB-10 showed FPase activity. SB-10 recorded the highest FPase activity with 3.4 U/mL^{-1} followed by SB-3 (2.7 U/mL^{-1}). Cellulose is present in two forms - crystalline and amorphous. The recalcitrance of lignocellulose is primarily due to the crystalline network of cellulose that forms the structural casement of lignocellulose, encompassing an amorphous matrix of cellulose and hemicellulose. Cellulase is the responsible enzyme for cellulose degradation (Akhtar et al., 2016).Microorganisms, including bacteria and fungi, produce a diverse range of enzymes.Previously, researchers considered bacterial cellulases as stronger accelerators in degrading lignocellulose biomass due to their rapid growth rate and adaptability to diverse environments (i.e., pH, temperature) (Bon, 2012; Singhalet al., 2021). The

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majority of *Bacillus*and *Clostridium* strains exhibit increased cellulolytic activity(Mawadza et al., 2000). The maximum CMCase activity observed in different isolates looks much lower than that for common decomposer bacteria. Fungi, on the other hand, have been mainly studied for enzyme production due to their capacity to synthesize a huge number of noncomplex enzymes. For example, *Trichoderma sp.*, a cellulolytic fungus, has been regarded as the highest producer of cellulase enzymes. (Singhal et al., 2021).Therefore, fungal isolates are considered for further analysis in the current study.

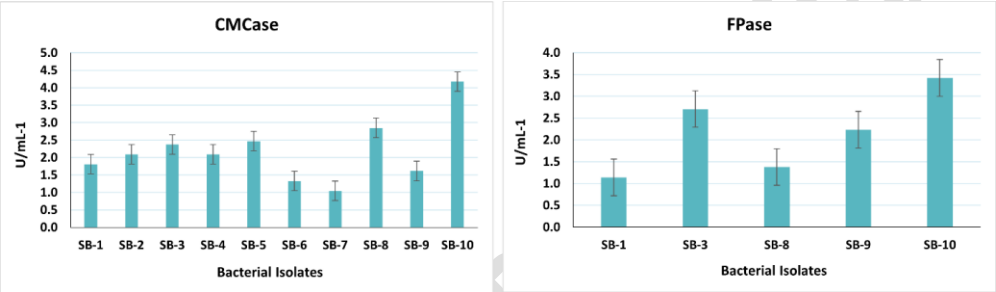

Fig. 3.Hydrolytic enzyme activity of bacterial isolates on paddy straw extract amended medium.

3.3 Quantitative screening of fungal enzymes

Ten fungal isolates from paddy soil and compost sampleswere screened for CMCase, FPase, and LPMO on microcrystalline cellulose as a substrate. Fungal isolates with high CMCase activity were considered to be potential cellulase producers. Table 2 represents the enzymatic activities in fungal species cultivated on microcrystalline cellulose as the only carbon source in Reese minimal media for providing insights into their cellulolytic capabilities for degrading the crystalline portion of cellulose polymers. The obtained results showed distinctive variations in the enzymatic performance among the different fungal species. Among the fungal isolates, F-9, F-7, and F-5 consistently outperformed the other isolates. In terms of CMCase activity, F-9:*Aspergillus fumigatus*exhibited the highest activity (40.14 U mL^{-1}), whereas F-2 recorded the lowest CMCase activity of 6.60 U mL^{-1}. Similarly, F-7 and F-5 demonstrated a CMCase activity of 33.90 and 24.60 U mL^{-1}. Higher FPase activity was recorded in F-5:*Trichoderma asperellum*, and F-4 isolates with 68.02 and 52.71 U mL^{-1}, which was significantly higher as compared to the F-3 (7.92 U mL^{-1}). Previous research showed that*Aspergillus,Trichoderma,and*

*Penicillium*speciespossess all essential components for hydrolytic enzyme production (Khokhar et al. 2012; Yadav, 2017).Similarly, the current study also corroborates these findings. Saroj et al., (2018) reported the maximum CMCase (26.2 IU/mL) and FPase (18.2 IU/mL), activity of lignocellulosic hydrolysis under solid-state fermentation by *Aspergillus fumigatus* strain JCM 10253. Similar researchcarried out on *A. fumigatus* SK1 showed CMCase (54.3 U/g), and FPase (3.35 U/g)activities using oil palm trunk as a substrate (Ang et al., 2013). Soft rot fungi like *Aspergillus niger* and *Trichoderma reesei*, as well as the white rot fungus *Phanerochaete chrysosporium*, are reported to produce significant quantities of cellulose (Xue et al., 2017; Manavalan et al., 2015; Xu et al., 2018).

LPMOs are copper-dependent, oxidative, non-hydrolytic enzymes that have gained attention for their ability to effectively degrade resistant polysaccharides, which is critical for biomass conversion to bioethanol production (Meier et al., 2017). It was first discovered in 2010 (Vaaje-Kolstad et al., 2010), and is classified as "auxiliary activities" (AA) which were categorized into seven Carbohydrate-Active Enzyme subgroups (AA9-AA11, AA13-AA16); Sagarika et al., 2022). It exhibits activities on cellulose, chitin, or starch and is utilized in enzymatic cocktails for lignocellulosic substrate degradation (Hemsworth et al., 2015; Ladevèze et al., 2017; Filiatrault-Chastel et al., 2019). AA9, a common fungal LPMO, significantly contributes to cellulose degradation and transformation through oxidative mechanisms (Passoth and Sandgren, 2019). In this study, LPMO activity was lower in magnitude compared to the cellulase activities, it's important to note that F-8 exhibited the highest LPMO activity with 0.85 U mL^{-1} followed by F-3 with 0.73 U mL^{-1}. This highlights that F-8 has potential in oxidative cellulose cleavage. These findings hold promise for biotechnological applications, such as biofuel production and bioremediation for efficient cellulose degradation. The results were comparable to those reported in the literature. The LPMO produced by F-8:*Aspergillus aculeatus*and F-3:*Phanerodontiachrysosporium*in the current study was lower in concentration (0.85 and 0.73 U/mL^{-1}, respectively) to that produced by *Neurosporacrassa*LPMO9F (2.2 U/g) that was observed by Guo et al. (2020).In this study, spectrophotometric analysis employing 2,6-DMP was conducted to detect the novel peroxidase activity of LPMO.However, further confirmation of this assay is need to be validated through gene expression analysis etc.

Table 2.Enzyme activities of fungal isolates by using microcrystalline cellulose as a substrate

Fungal Species	CMCase (U mL^{-1})	FPase (U mL^{-1})	LPMO (U mL^{-1})
F-1	7.98 ± 0.02	9.84 ± 0.02	0.22 ± 0.01
F-2	6.60 ± 0.03	11.14 ± 0.02	0.18 ± 0.01
F-3	6.80 ± 0.02	7.92 ± 0.01	0.73 ± 0.01
F-4	20.38 ± 0.01	52.71 ± 0.14	0.19 ± 0.01
F-5	24.60 ± 0.02	68.02 ± 0.14	0.30 ± 0.01
F-6	23.78 ± 0.03	47.90 ± 0.02	0.70 ± 0.02
F-7	33.90 ± 0.02	49.27 ± 0.05	0.72 ± 0.01
F-8	14.75 ± 0.03	19.98 ± 0.05	0.85 ± 0.02
F-9	40.14 ± 0.11	17.32 ± 0.03	0.35 ± 0.01
F-10	6.85 ± 0.03	13.79 ± 0.03	0.30 ± 0.01

F-3:*Phanerodontiachrysosporium*, F-5: *Trichoderma asperellum*,F-8: *Aspergillus aculeatus*,F-9: *Aspergillus fumigatus*,F-1, F-2, F-7, F-10: *Aspergillus* sp., F-4 and F-6: *Trichoderma* sp.

4. Conclusions

The findings of this study is to emphasize the vital role and presence of microorganisms, particularly fungi in paddy soil for decomposition of cellulose. Four fungal strains *A. aculeatus*, *A. fumigatus*, *T. asperellum* and *P. chrysosporium*from paddy soils showed promising cellulolytic and LPMO enzyme activities, which can be utilized for developing compatible microbial consortia for paddy straw residue management.

Data availability

All data generated or analyzed during this study are included in the manuscript.

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