

Crop Residue Degradation by Autochthonous Fungi Isolated from Cropping System Management Scenarios

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In the rice-wheat system dominated belt of India (Indo-Gangetic plains; IGP), paddy leaves, about 8 to 9 t/ha of loose and anchored residue in the field, are mechanically harvested. Farmers prefer to burn this residue to clear the field for the timely preparation of conventional wheat sowing. *In-situ* degradation by autochthonous fungi can be a better option for the disposal of unwanted crop residues. Autochthonous fungi isolated from conservation agriculture-based crop management fields were screened and assessed for their residue degradation potential. Nineteen isolates were selected for detailed enzymatic analysis in submerged fermentation, responsible for lignocellulosic residue degradation. Out of these five fungal isolates RPW 1/3 (*Aspergillus flavus*), RPW 1/6 (*Aspergillus terreus*), RPW 1/9 (*Aspergillus terreus*), RPWM 2/2 (*Penicillium janthinellum*) and RZWM 3/1 (*Aspergillus niger*) showed higher activities of cellobiase, CMCase, FPase, xylanase, and laccase enzymes in solid state fermentation. Further two isolates RPW 1/3 and 1/6 showed approximately 30% degradation of straw residue after 10 days incubation.

Keywords: Autochthonous fungi; Crop residue; Cellobiase; Carboxy methyl cellulase; Filter paperase; Xylanase; Laccase; Dry mass loss; Conservation agriculture

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INTRODUCTION

Rice is the main staple crop in the world; 661.81 million tons of rice were produced from 155.71 million hectares of land in 2008 (USDA 2009). Annually, a large amount of straw is accumulated as a byproduct from rice cultivation, as straw comprises about 50% of the dry weight of the rice plant. In India, over 500 million tons of crop residues (CRs) are produced every year from agricultural fields (MNRE 2009). The rice-wheat system accounts for nearly one-fourth of the total CRs produced in India (Sarkar *et al.* 1999). The surplus rice-wheat residues (44.5 Mt rice straw and 24.5 Mt wheat straw) are typically burned on-farm annually. Rice alone contributes 40% of the total residue (Jain *et al.* 2014), and the major portion of this straw is disposed of through open field burning (Samra *et al.* 2003). Farmers do not incorporate or retain rice straw in the field because of slow degradation rates, inadequate seeding machinery, and insufficient turnaround time for incorporation. Several factors support the burning of CRs, including it being a quick and easy way to manage large quantities of CRs, timely field preparation for the next crop, and fertilization of the field with ash and pest control. Environmental problems associated with CR burning include smoke, trace gases, and micron-sized aerosols that affect the composition of the atmosphere (Saud *et al.* 2011; Awasthi *et al.* 2011) and are responsible for respiratory diseases in human beings. There is a need for sustainable and environmentally friendly techniques for the safe disposal of rice straw. These include: (i)

collecting and exporting the residue to the deficit areas; (ii) the recycling of CRs with fast decomposition rates; and (iii) the use of new machinery prototypes (seeding machinery for residue conditions). For the majority of small and marginal farmers in the region, the first and the third approaches to residue disposal are not very effective, as it costs more to transport the residue and purchase new machinery. Recycling of rice straw in the field promotes sustainable agriculture, protects the environment, and improves the physical, chemical, and biological properties of soil (Perez-Piqueres *et al.* 2006; Rasool *et al.* 2008; Mylavaram and Zinati 2009), which ultimately results in better plant growth and yield.

Micro-organisms play an important role in the efficient disposal of CRs and accelerate nutrient solubilization and mobilization by secreting organic acids. Of all micro-organisms, fungi are quite efficient at composting lignocellulosic waste because they are filamentous and have the ability to produce prolific spores, which can invade the substrates quickly. There is evidence that fungi play a crucial role in the degradation of agricultural wastes such as rice straw (Kausar *et al.* 2010; Chang *et al.* 2012), corn stover (Wan and Li 2010), and sugarcane residue (Maza *et al.* 2014). The present experiment was undertaken with the aim of selecting lignocellulolytic fungi from conservation agriculture fields where three production principals were followed, *i.e.* minimum disturbance of soil, CRs retention for surface cover, and with cropping system diversification/sustainable intensification. Large amounts of CRs were retained/incorporated in the field. The goal was to determine the degradation potential of rice-wheat residue under liquid and solid state fermentation through lignocellulolytic enzyme activities. Exploring fungal enzymatic potential in the agricultural residue degradation may lead to the production of an improved end-product that could be useful in enhancing crop yields.

EXPERIMENTAL

Materials

Sampling site

Soil samples were collected from the Cereal Systems Initiative for South Asia (CSISA) experimental research platform located at Central Soil Salinity Research Institute (CSSRI), Karnal, Haryana, India (29°70'N latitude and 76°96'E longitude). These samples are representative of the trans-Indo-Gangetic plains (IGP) of India. Here, a near production scale long-term cropping systems trial was established in 2009 to assess the performance of different agricultural systems (scenarios) using a wide range of indicators (crop rotation, tillage, crop establishment, crop water, and residue management). The scenarios were designed based on different drivers of agricultural changes and adapted to expected changes in environmental and socioeconomic drivers. The four scenarios were as follows: scenario 1 (farmers' practice or business as usual), rice (CT/TPR; conventional till/transplanted rice)-wheat (conventional till) rotation as in farmers' practice where rice and wheat residues were removed; scenario 2 rice-wheat-mungbean (CT/TPR-ZT; zero-till-ZT) rotation, where full (100%) rice and partial (anchored) wheat residue was retained on the soil surface, while full mungbean residues were incorporated during puddling; scenario 3 (rice-wheat-mungbean (ZT-ZT-ZT)), where full (100%) rice and mungbean, partial (anchored) wheat residue was retained on the soil surface; and scenario 4 (futuristic system) maize-wheat-mungbean (ZT-ZT-ZT), where maize (65%) and full mungbean, partial (anchored) wheat residue was retained on the soil surface. Scenario 2 was based on the partial conservation agriculture (CA), whereas scenarios 3 and 4 were based on the full

CA, all three principles of minimum disturbance, rational surface cover, and crop intensification/diversification were followed. Except for scenario 4 (futuristic), the rice-wheat cropping system is a common practice, where rice and wheat residues were retained on the soil surface in a ratio of 4:1.

Methods

Isolation of fungi

After removing straw residues, samples were taken from the top 15 cm of the soil layer from the different CA-based scenarios and brought to the laboratory in sterilized polythene bags. Samples were stored at 4 °C until processing. Fungi were isolated from these soil samples using the serial dilution technique and plated on potato dextrose agar (PDA), rose bengal agar (RBA), and Czapek-Dox agar (CDA) with 30 µg/mL of chloramphenicol. The plates were then incubated at room temperature (28 ± 2 °C). Isolates were grown on carboxy methyl cellulase (CMC) agar plates. Isolates showing a halo zone upon staining with iodine (Fig. 1) were selected for enzyme production.



Fig. 1. Halo zone around the fungal colony on a CMC agar plate

Lignocellulolytic enzyme production by fungal isolates under submerged fermentation

Selected lignocellulolytic fungi were grown in modified Mandel and Weber media (1969) with 10 g of powdered mixed straw of rice and wheat (4:1) at pH 7.0 under submerged conditions. The secretion of lignocellulolytic enzymes in liquid cultures was evaluated after 10 days of incubation at 30 °C. The content of each flask was filtered through Whatman filter paper No. 1, then centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant was used as crude enzyme preparation.

Identification of fungal isolates

Isolates were identified on the basis of their colony morphology and microscopic examination of the mycelium. Cultural characteristics such as colony color, shape, surface, margin and pigmentation, and size were monitored and recorded during growth. Isolates were stained with lactophenol cotton-blue and were examined under the microscope for morphological characteristics such as structure of reproductive mycelium, septation,

sporulation, *etc.* Fungal isolates were identified by these characteristics using standard manuals (Gilman 2001; Nagamani *et al.* 2006).

Solid state fermentation

Solid state fermentation was carried out in 250-mL Erlenmeyer flasks containing 5.0 g of mixed straw and 15 mL of Reese's mineral medium (Reese and Mandel 1963). After incubation for 7 days at 30 °C, 100 mL of citrate buffer (0.05 M, pH 4.8) was added to the flasks and kept under mild stirring (120 rpm) for 1 h. The slurry was filtered through muslin cloth, followed by Whatman filter paper No. 1, and then centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant was used for enzyme assay and the solid residue was dried for 24 h at 75 °C and weighed. Straw degradation was expressed as the loss of dry mass (%) of used straw.

Enzyme assays

Carboxy methyl cellulase (CMCase) or β -1,4-endo glucanase (EC 3.2.1.4), cellobiase (EC 3.2.1.21), filter paperase (FPase), and endo- β -1,4-xylanase (EC 3.2.1.8) were measured with CMC, cellobiose, Whatman No. 1 filter paper, and beech wood xylan as the substrates, respectively, using standard methods (Ghose 1987; Ghose and Bisaria 1987).

Reducing sugars were determined using the dinitrosalicylic acid (DNS) method (Miller 1959). One unit of activity was defined as the amount of enzyme required to liberate 1 μ mol of reducing sugars per minute under the assay conditions. Laccase or oxygen oxidoreductase (EC 1.10.3.2) was determined using the method of Sandhu and Arora (1985) with slight modifications, and the relative activity was expressed as colorimetric units per mL of the enzyme extract (cu/mL). All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Of 19 total isolates, six, RPW1/1, RPW1/3, RPW1/6, RPW1/8, RPW1/9, and RPW1/10, from scenario 1; three, RPWM2/2, RPWM2/4, and RPWM2/5, from scenario 2; three, RZWM3/1, RZWM3/2, and RZWM3/4, from scenario 3; and seven, MWM4/5, MWM4/6, MWM4/7, MWM4/8, MWM4/9, MWM4/13, and MWM4/14, from scenario 4 produced a zone of hydrolysis on CMC agar plates and were selected for enzyme production under submerged fermentation.

Lignocellulolytic Activity of Isolates under Submerged Fermentation

Fungi have been shown to produce a number of enzymes during the biodegradation of lignocellulosic materials. Effective biodegradation of cellulose to glucose depends on the synergistic action of cellulase enzymes, *i.e.*, endoglucanases, exoglucanases, cellobiohydrolases, and glucosidases (Lynd *et al.* 2002). Selected fungal isolates were grown in broth amended with rice-wheat straw powder in a 4:1 ratio as a carbon source.

Maximum enzyme activity was shown by isolate RPM 1/10 for cellobiase, MWW 4/7 for CMCase, RPWM 2/2 for FPase activity, and MWM 4/7 for xylanase (Table 1). Cellulose is the key component and comprises approximately 35 to 45% of the dry weight of the straw species plant. Hence, the screening of fungal isolates with cellulolytic capability is a critical step for the rapid degradation of crop straw. Laccase activity was either zero or negligible in all isolates under submerged fermentation.

Table 1. Enzyme Activities in Submerged Fermentation (IU/mL)

| Isolate Number | Cellobiase | CMCase | Fpase | Xylanase |
|----------------|------------|----------|-----------|----------|
| RPW 1/1 | 0.328 c | 0.166 g | 0.052 d | 1.216 e |
| RPW 1/3 | 0.354 b | 0.173 g | 0.048 d | 1.389 c |
| RPW 1/6 | 0.202 e | 0.402 c | 0.142 abc | 1.467 b |
| RPW 1/8 | 0.083 h | 0.072 hi | 0.015 f | 1.176 e |
| RPW 1/9 | 0.214 e | 0.475 b | 0.138 bc | 1.409 bc |
| RPW 1/10 | 0.374 a | 0.209 ef | 0.053 d | 1.394 c |
| RPWM 2/2 | 0.236 d | 0.377 cd | 0.167 a | 1.351 cd |
| RPWM 2/4 | 0.118 g | 0.081 h | 0.028 def | 0.755 g |
| RPWM 2/5 | 0.037 i | 0.044 ij | 0.008 f | 0.624 h |
| RZWM 3/1 | 0.351 b | 0.380 cd | 0.140 bc | 1.323 d |
| RZWM 3/2 | 0.129 g | 0.217 e | 0.043 de | 0.401 i |
| RZWM 3/4 | 0.030 i | 0.188 fg | 0.052 d | 0.215 k |
| MWM 4/5 | 0.023 j | 0.043 ij | 0.008 f | 0.316 j |
| MWM 4/6 | 0.166 f | 0.045 ij | 0.011 f | 0.858 f |
| MWM 4/7 | 0.160 f | 0.512 a | 0.159 ab | 1.689 a |
| MWM 4/8 | 0.070 h | 0.005 k | 0.018 ef | 0.098 l |
| MWM 4/9 | 0.370 ab | 0.364 d | 0.163 ab | 1.473 b |
| MWM 4/13 | 0.207 e | 0.402 c | 0.129 c | 1.398 c |
| MWM 4/14 | 0.005 j | 0.042 j | 0.026 def | 0.154 kl |

RPW 1—Scenario 1; RPWM 2—Scenario 2; RZWM 3—Scenario 3; MWM 4—Scenario 4. Different letters followed in a column are significantly different at a 5% level of significance.

Some isolates showed comparatively lower activities of all enzymes than others. Isolates MWM 4/14, MWM 4/5, RZWM 3/4, RPWM 2/5, MWM 4/8, and RPW 1/8 showed less than 0.1 IU/mL cellobiase activity. However, isolates MWM 4/8, MWM 4/14, MWM 4/5, RPWM 2/5, MWM 4/6, RPW 1/8, and RPWM 2/4 showed comparatively lower (<0.1 IU/mL) CMCase activities compared with others. The range of FPase activity was found to be less than all other enzymes. RPWM 2/5, MWM 4/5, MWM 4/6, RPW 1/8, MWM 4/8, MWM 4/14, and RPWM 2/4 recorded lower FPase activity than other isolates. Isolates MWM 4/8, MWM 4/14, RZWM 3/4, MWM 4/5, RZWM 3/2, RPWM 2/5, RPWM 2/4, and MWM 4/6 showed xylanase activity of less than one unit per mL. By comparing all enzyme activities, nine isolates were selected by eliminating the 10 isolates with lower activities for further solid state fermentation experiment.

Identification of Selected Fungal Isolates

Based on morphological and cultural characteristics, out of nine selected isolates, five were found to be *Aspergillus*; isolate RPW 1/3 and RPW 1/10 were found morphologically similar to *Aspergillus flavus*, RPW 1/6 and RPW 1/9 were similar to *Aspergillus terreus*, and RZWM 3/1 as *Aspergillus niger*. Isolates RPWM 2/2, MWM 4/9, and MWM 4/13 were identified as *Penicillium* species. MWM 4/7 was identified as *Alternaria alternata*. All the isolates were ascomycetous fungi. Helal (2005) reported *Aspergillus* as the most frequent genus at 25 °C and 45 °C among other fungal flora found on decomposing rice straw.

Table 2. Identification of Selected Isolates

| Isolate Number | Identified Isolate |
|----------------|---------------------------------|
| RPW 1/3 | <i>Aspergillus flavus</i> |
| RPW 1/6 | <i>Aspergillus terreus</i> |
| RPW 1/9 | <i>Aspergillus terreus</i> |
| RPW 1/10 | <i>Aspergillus flavus</i> |
| RPWM 2/2 | <i>Penicillium janthinellum</i> |
| RZWM 3/1 | <i>Aspergillus niger</i> |
| MWM 4/7 | <i>Alternaria alternata</i> |
| MWM 4/9 | <i>Penicillium oxalicum</i> |
| MWM 4/13 | <i>Penicillium oxalicum</i> |

Lignocellulolytic Activity of Selected Isolates in Solid State Fermentation

Nine isolates selected with higher enzyme activities in submerged fermentation were grown on mixed straw solid media for solid state fermentation. After 10 days of incubation, lignocellulolytic enzymes were assayed in fermented solid media extract. Isolate RPWM 2/2 showed the highest activity of CMCCase (3.79 IU/g), Fpase (1.11 IU/g), and xylanase (17.53 IU/g), whereas the highest cellobiase (1.45 IU/g) and laccase (5.74 CU/g) activity was exhibited by RZWM 3/1 and RPW 1/6, respectively (Table 3).

Table 3. Enzyme Activities in Solid State Fermentation by Different Fungal Isolates (IU/g Substrate)

| Isolate | Cellobiase | CMCase | Fpase | Xylanase | Laccase* |
|--|------------|---------|---------|----------|----------|
| <i>Aspergillus flavus</i> RPW 1/3 | 0.95 cd | 3.57 ab | 0.75 b | 16.51 ab | 3.67 c |
| <i>Aspergillus terreus</i> RPW 1/6 | 0.26 e | 3.37 b | 0.72 c | 13.50 d | 5.74 a |
| <i>Aspergillus terreus</i> RPW 1/9 | 0.43 e | 3.56 ab | 0.55 c | 14.82 cd | 4.38 b |
| <i>Aspergillus flavus</i> RPW 1/10 | 0.92 d | 2.36 c | 0.42 d | 16.84 ab | 1.82 e |
| <i>Penicillium janthinellum</i> RPWM 2/2 | 1.05 cd | 3.79 a | 1.11 a | 17.53 a | 3.51 c |
| <i>Aspergillus niger</i> RZWM 3/1 | 1.45 b | 3.51 ab | 0.44 cd | 13.87 d | 3.91 c |
| <i>Alternaria alternata</i> MWM 4/7 | 0.33 e | 1.59 de | 0.20 ef | 13.81 d | 1.29 f |
| <i>Penicillium oxalicum</i> MWM 4/9 | 1.21 bc | 1.49 e | 0.30 e | 8.19 e | 3.53 c |
| <i>Penicillium oxalicum</i> MWM 4/13 | 0.90 d | 1.70 de | 0.16 f | 8.85 e | 1.35 f |

Different letters followed in a column are significantly different at a 5% level of significance.

* CU/g substrate

Species of *Aspergilli* are known to produce all three enzymatic activities of the cellulose complex and exhibit strong hydrolytic activity towards cellulose. *A. niger* (Sharma *et al.* 2001; Adav *et al.* 2010), *A. terreus* (Emtiazi *et al.* 2001), *A. candidus* (Milala *et al.* 2009) and *A. flavus* (Saritha and Maruthi 2010) are known to be effective in the biodegradation of lignocellulosic biomass by producing oxidative and hydrolytic enzymes. *A. terreus* was recorded as highly cellulolytic in producing cellulase activity in both submerged (Mahdi *et al.* 2011) and solid state fermentation (Jahromi *et al.* 2011). Different studies have reported the ability of *A. terreus* for production of FPase, CMCCase and cellobiase (Gao *et al.* 2008) and xylanase (Suvarna *et al.* 2009) on different

types of wastes. Isolate RZWM 3/1 (*A. niger*) was observed to be a good producer of cellulolytic enzymes in both submerged and solid state fermentation (Table 1 and 3) these observation were similar to Kang *et al.* (2004) in solid state fermentation of rice straw.

Isolates RPWM 2/2, MWM 4/9, and MWM 4/13 were identified as *Penicillium* species. Different *Penicillium* species are reported to produce enzyme systems with high performances of lignocellulose degradation. The fungal isolate *P. janthinellum* MTCC10889 was found to be an outstanding producer of endoxylanase, as well as cellulase (Kundu *et al.* 2012). Adsul *et al.* (2014) found that a mutant of *P. janthinellum* showed high production of cellulases in solid state fermentation. Singhania *et al.* (2014) demonstrated that *P. janthinellum* EMS-UV-8 is a fungus with potential for future large-scale production of cellulase. Liao (2014) reported induced high-efficiency lignocellulolytic enzyme production by *Penicillium oxalicum* on complex substrates. Moubasher and Mazen (1991) isolated some fungal species from Egyptian soils and assayed their cellulolytic activities. They found *P. oxalicum* and *A. niger* to be top species in cellulolytic activity among different mesophilic species. MWM 4/7 was identified as *Alternaria alternata*. Sohail *et al.* (2011) found that *Alternaria* sp. MS28 can produce cellulases in the presence of different substrates. A mutant of *A. alternata* can produce more enhanced cellulase and β -Glucosidase than its wild strain (Macris 1984). Overall, the fungal isolates RPW 1/3, RPW 1/6, RPW 1/9, RPWM 2/2, and RZWM 3/1 showed better performances with respect to their enzyme activities, amongst different fungal isolates evaluated.

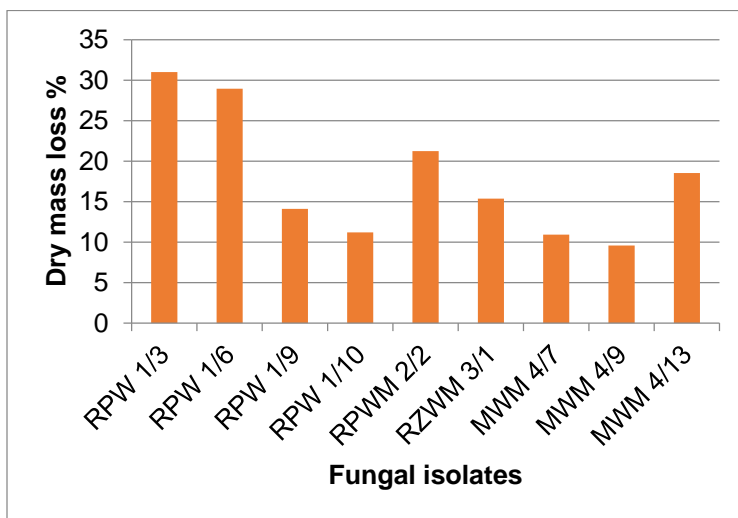


Fig. 2. Ability of selected fungal strains to decompose dry straw residue biomass. The data represent the average of triplicates and the result is reported as the mean \pm SD.

The ability of selected fungi to reduce dry mass of crop residue was evaluated (Fig. 2). Fungi can decompose plant-derived lignin-rich polymers and humus (Ayato *et al.* 2005). Such a process has been shown to result in mineralization of straw (Koutev *et al.* 2001). The maximum dry mass loss was achieved with isolates *A. flavus* RPW 1/3 (31%), followed by *A. terreus* RPW 1/6 (29%) and *P. janthinellum* RPWM 2/2 (21%). These results are in close conformity with the work done by Sinegani *et al.* (2005), who found 21.1% weight loss of rice residue with *A. terreus*. On an average, *Aspergillus* spp. had 15 and 45% more degradation potential with respect to biomass loss compared with

Penicillium spp and *Alternaria alternata*, respectively. *Aspergillus flavus* was reported as one of most efficient holocellulose degrader by Islam and Borthakur (2011) as evidenced by dry weight loss of ~22% in rice stubbles.

By evaluating performances of different isolates in submerged fermentation, solid state fermentation and dry mass loss it can be concluded that the three isolates RPW 1/3 (*A. flavus*), RPW 1/6 (*A. terreus*) and RPWM 2/2 (*P. janthinellum*) have potential to decompose crop residues having major portion of rice residue. These isolates can be used in consortia after observing their interaction and response in consortia. Viji and Neelanarayanan (2015) reported more degradation when paddy straw was inoculated with mixed cultures of *R. oryzae*, *A. oryzae*, and *A. fumigatus* compared to individual application of these cultures. The approach of using autochthonous fungi for CR degradation could help to increase soil organic carbon and crop yields while improving the quality of environment in IGP.

CONCLUSIONS

1. Autochthonous fungi isolated from the rice-wheat cropping system (scenarios 1 and 2) showed higher CR degradation potential than those isolated from the maize-wheat-mungbean system (scenario 4).
2. *Aspergillus* spp showed higher lignocellulosic material (CR) degradation potential compared with *Penicillium* spp and *Alternaria* spp.
3. Looking to the activities of fungi Asocomycota viz., *Aspergillus* spp, *Penicillium* spp, and *Alternaria* spp in CA soils, it can be stated that *Aspergillus* spp has great potential for the degradation of lignocellulosic material.
4. *In-situ* degradation of crop residue by autochthonous fungi is good option for residue management in IGP.

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