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Research Article

DISCOVERING MYCOFLORA ASSOCIATION IN RICE STRAW AND POTENTIAL CELLULOLYTIC AND LIGNINOLYTIC ISOLATES

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Abstract: Globally, the habitation of post- harvest residues in crops like paddy, with fungi causes a major loss economically and in terms of sustainability due to pathogenic manifestation and alter of taste. Lignocellulosic biomass produced from the cultivation of rice holds high potential for solving the problems of new generation biofuels in India. This lignocellulosic mass can be utilized for effective production of these sustainable by- products by exploiting and manipulating the pathogenic ability of the inhabiting mycoflora in them. The cellulolytic and ligninolytic property of fungi can help in the extraction of lignin and cellulose.

Keywords: *Lignocellulosic Biomass, Cellulolysis, Ligninolysis*

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Introduction

Diverse array of microscopic organisms is common occurrence in the rice straw like bacteria, yeast and fungi [1,2]. The rice straw stored as fodder generally gets contaminated with the moulds and other fungi, which cause mycotoxin contamination leading to toxicity in ruminants [3]. A wide no. of fungi inhibiting the stored rice- straws are *Alternaria alternata, Aspergillus species, Cladosporium, Fusarium, Penicillium* sp., *Nigrospora oryzae, Ulocladium atrum, Verticillium lecanii, Stemphylium lycopersicon etc*. Most of these enzymes are from the classes of white rot, brown rot and soft rot fungi. Brown rot fungi are responsible for degradation of lignin mainly while the white and soft rots are proficient in both cellulose and lignin hydrolysis, both [4]. Chief fungi from white rot fungi group are *Ceriporiopsis subvermispora, Ceriporia lacerate, Pycnoporus cinnarbarinus, Phanerochaete chrysosporium* and *Pleurotus ostreaus*, which are capable of the biological degradation with high delignification efficiency [5]. Similarly, the fungi of other two categories with degradation ability are *Gleophyllum trabeum, Laetoporeus sulphureus, Meruliporia incrassata, Coniophora puteana and Serpula lacrymans (*Brown rot fungi*)* and *Gleophyllum trabeum, Laetoporeus sulphureus, Meruliporia incrassata, Coniophora puteana* and *Serpula lacrymans* (Soft rot fungi). Of all these classes, most effective is white rot fungi which is active on different kind of lignocellulosic masses [6]. Pre- treatment of rice straw with these microbes can yield a lot of benefits by enzymatic hydrolysis of the biomolecules of straw; leading to improvement of nutritional content and digestibility of animal feed, bioethanol production by sugar generation, efficient bioenergy conversions (as the lignin present in rice straw hampers the digestibility, which reduces the efficiency of bio energy conversions [7]. This approach also solves the problems associated with burning of the post- harvest bulk like emission of greenhouse gases, particulate matters and smog layers [8, 9].

Materials and methods

Rice straw

Rice straw samples (Belonging to four different varieties namely; Gopal Bhog, Kala Namak, Sambha and Malaysia) were obtained directly from local farms of Gorakhpur (region of Eastern Uttar Pradesh), India in the month of December from open fields. Rice straw was turned into stubbles of 5 mm. These rice straw stubbles were placed equidistantly on PDA plates and sterilized wet Blotter papers for the growth of mycoflora present in them. The major biopolymers of the rice straw were: Cellulose, Hemicellulose and Lignin.

Straw decomposition and Mycoflora Isolation

Straw stubbles grown on the PDA and Blotter paper plates were decomposed by the growth of fungal colonies. For identification and further studies of these mycoflora, they were isolated individually on agar slants and after appropriate growth were grown as full- fledged colonies on PDA Plates as triplicates for a period of 3 days.

Degradation analysis

Fungal colonies obtained were subjected to different assays to determine the capability of degradation for the major components of rice straw (Cellulose and Lignin). Cellulolytic activity was determined via CMC (Carboxymethyl Cellulose) and sterilized Filter Paper strips as substrate while Ligninolytic activity was determined by the use of Methylene Blue (a structural analogue of Lignin) as substrate in the place of lignin. Degradation capability among the various isolated species was analysed under UV light (in the case of CMC and Methylene Blue) by determining the physical degradation of sterilized filter paper.

Cellulolytic Assays

Two different assay methods were adopted to determine the cellulolytic activity of the fungal isolates. In the first method, agar beads containing fungal mycelium were allowed to grow in the presence of sterilized Whatman Filter Paper strips (Cellulose substrate) in 2% Potato Dextrose Broth [10]. In the second method however, PDA plates containing 1% CMC (Cellulose substrate) were inoculated with aseptic culture of fungi and allowed to grow for an incubation period of 3 days (25°± 2 ºC) [10].

Ligninolytic Assay

In the determination of ligninolytic activity, various dyes which are structurally similar to Lignin are used in the place of lignin. In this activity, Methylene Blue (0.2% i.e., 0.2g/ 100 mL) was added to 2% PDA containing plates which were inoculated later with fungal isolates and allowed to form colonies for an appropriate incubation period of 3 days (25°± 2 ºC) [11].

Table-1 *Substrates used in the assays and their particulars*

Carbon Source	Amount	Medium	Test
CMC	1%	Potato Dextrose Agar	Cellulolysis
Filter Paper	5 cm X 5mm width	Potato Dextrose Broth	Cellulolysis
Methylene Blue	0.20%	Potato Dextrose Agar	Ligninolysis

Result and Discussion

Mycoflora Isolation

Rice straw was degraded due to colonisation by the already present mycoflora on the samples. Four different kind of rice straw varieties showed presence of 13 different species of fungi among which 7 were high in frequency. These 13 species were *Achlya americana, A. bisexualis, Alternaria padwickii, Aspergillus flavus, A. fumigatus, Bipolaris oryzae, Curvularia lunata, Fusarium fujikuroi, F. graminearum, Penicillium expansum, P. notatum, Rhizoctonia solani* and *Saprolegnia* sp., identified based on the morphological and colony characteristics. Highest no. of mycoflora were seen in the Kala Namak variety, a total of 7 and the least no. of mycoflora were obtained in Malaysia variety, only 4 species. In Goapl Bhog (6 species) and Sambha (5 species) varieties, most frequent and dominant fungal species were *Penicilium expansum* and *Curvularia lunata*, respectively. Of the 13 species in all four varieties, the highly frequent and most common species were *Aspergillus flavus*, *A. fumigatus, Curvularia lunata, Fusarium graminearum, Penicillium notatum, Rhizocotnia solani* and *Saprolegnia* sp. The rarest were *Achlya americana, A. bisexualis, Alternaria padwickii* and *Bipolaris oryzae*, each being present in only one variety and in least frequency.

Degradation Capability

Separate results were obtained for the two different methods used to determine the cellulolytic capability.

Filter Paper Assay

Among the 7 fungal isolates, degradation of the dipped filter papers was observed in *Fusarium graminearum, Penicillium notatum and Rhizoctonia solani* after 5 days of incubation at 25± 2 ºC, in the aqueous medium, after the fungal mycelium colonized the paper strips. *Aspergillus flavus, A. fumigatus, Curvularia lunata* and *Saprolegnia* sp. failed to show cellulolytic activity over this substrate [Fig-1] and [Table-3].

Fig-1 Filter Paper Assay

Carboxymethylcellulose Assay

In the 7 fungal isolates under testing; *Aspergillus flavus, A. fumigatus, Fusarium graminearum, Penicillium notatum* and *Rhizocotnia solani* were found to be positive for the degradation of 1% CMC in PDA media, after an incubation period of 3 days at 25°± 2 ºC. Degradation activity was confirmed by the development of clear halo around the periphery of growing fungal colonies (evident under UV light), which was an indicator that the carbon source added to the medium is being used. *Curvularia lunata* and *Saprolegnia* sp. again did not show any degradation activity even over this changed substrate confirming the absence of cellulolytic ability in them [Fig-2] and [Table-3].

Fig-2 Carboxymethylcellulose Assay

Methylene Blue Assay

In the assay with 7 species, *Aspergillus flavus, A. fumigatus, Curvularia lunata* and *Penicillium notatum* showed lignin degradation capability as a development of decolourized periphery in the blue coloured media, around the growing colonies which can be visualised under UV illuminator. *Fusarium graminearum, Rhizoctonia solani* and *Saprolegnia* sp. were not able to form colourless zone, affirming the absence of ligninolytic ability in these species [Fig-3] and [Table-3].

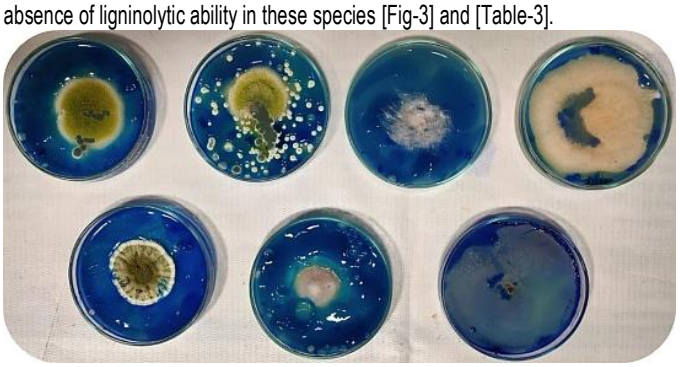

Fig-3 Methylene Blue Assay

In experiments of Thatheyus *et al.,* [12] and Namunch *et al.,* [13] with similar attempts to determine the cellulolytic activity, results were synonymous for *Aspergillus flavus*, where highest cellulolytic activity was observed in isolates identified as *A. flavus*. However, in the case of *A. fumigatus*, Ify *et al*., [14] produced results where the fungi showed acceptable levels of cellulolytic activity over filter paper powder which differed from the filter paper assay results in the current experiments but CMC degradation patterns were identical for *A. fumigatus* with narrow clearance zones. Both the *Aspergillus* species, have previously been proved to be associated with the ligninolytic activity but to considerably low levels. Li *et al*., [15] in his study over *A. flavus* showed that the fungi is capable of degrading lignin and its analogue to a maximum level of 34.6%, with initial sharp degradation followed by declined activity. Milstein *et al*., [16] performed degradation experiments on several *Aspergillus* species including *A. fumigatus* by observing deterioration and alteration of various lignocarbohydrate complexes possessing varying acids Ferulic, Veratric, vanillic and coumaric acid. Disappearance of the acidic components concluded the ligninolytic activity in the fungi, which showed positive results for *A. fumigatus*.

Curvularia lunata in an experiment conducted by Yadav and Vivekanand [17] was found to produce highest amount of ligninolytic activity over guaicol as substrate for a period of 6 days, among the isolates under observation, which has a striking resemblance to the results obtained here with wide discolouration zones around the colony periphery. Although, *Fusarium graminearum* did not show any ligninolytic activity but its cellulolytic activity was found on both the substrates (Filter Paper as well as CMC). Similar results were found in an investigation done by Sakai *et al*., [18] where in the analysis of enzymatic activities of several fungal isolates, only *Fusarium graminearum* was found to be capable of utilizing CMC as carbon source for growth showing its cellulolytic activity which clearly matches the results explained above. Most of the *Penicillium* species have been recorded as potent fungi with degradation capabilities for cellulose and lignin in the past. Penicillium notatum proved to be capable of degrading cellulose in both the substrates (Filter Paper as well as CMC) along with lignin degradation, proving to be the most potent species among all the tested isolates in terms of degradation capabilities. These results have been in line with the findings of Park *et al*., [19] where *P. notatum* was found to be able to degrade CMC and cellobiose both in cellulolytic assays among 53 species of *Penicillium* tested.

In a comparative study between *Pleurotus ostreatus* and *Penicillium chrysogenum* for the ligninolytic ability by Juárez- Cisneros *et al*., [20] veratryl alcohol oxidation method in cultures complemented with carbon nanotubes was used. Analysis of the results showed that both the fungi under study were proficient to grow on media containing lignin but in presence of carbon nanotubes.

Table-2 *Dominance of Mycoflora in different varieties*

SN	Mycoflora	Gopal Bhog	Kala Namak	Sambha	Malaysia
1	*Achlya americana*	-	-	-	+
2	*Achlya bisexualis*	-	+	-	+
3	*Alternaria padwickii*	-	++	-	-
4	*Aspergillus flavus*	+++	-	-	-
5	*A. fumigatus*	+++	-	-	-
6	*Bipolaris oryzae*	-	+	-	-
7	*Curvularia lunata*	+++	+++	+++	-
8	*Fusarium fujikuroi*	-	-	++	-
9	*F. graminearium*	-	+++	-	-
10	*Penicillium expansum*	++++	-	+	-
11	*P. notatum*	-	+++	++	+++
12	*Rhizoctonia solani*	+++	+++	+++	+++
13	*Saprolegnia* sp.	+++	-	-	-

Notes: +; Presence, -; Absence

Table-3 *Cellulolytic and Ligninolytic activity of dominant mycoflora*

SNo	Mycoflora	Cellulolysis		Ligninolysis
		Filter Paper	CMC	Methylene Blue
1	*Aspergillus flavus*	-	+	+
2	*A. fumigatus*	-	+	+
3	*Curvularia lunata*	-	-	+
4	*Fusarium graminearum*	+	+	-
5	*Penicillium notatum*	+	+	+
6	*Rhizoctonia solani*	+	+	-
7	*Saprolegnia* spp.	-	-	-

Notes: CMC- Carboxymethlcellulose, +; Presence of activity, -; Absence of activity

Clocchiatti *et al*., [21] observed the growth of *R. solani* on various cellulose containing substrates (crushed sawdust and paper pulp) as a parameter for its ability to degrade cellulose and saprophytic nature. In his observations, *R. solani's* growth was observed rendered the presence of natural inhabitants of the substrates. Though, the growth varied in respect to the different cellulose sources. Cellulolysis on various cellulose substrates comes in equivalent to the results of Filter Paper degradation and CMC degradation.

Conclusion

Four different rice straw samples showed association with 13 fungal species belonging to 9 different genera, 7 species being dominant out of all 13. Among these 7 dominant fungal species, only 5 species showed positive results for cellulolytic assays over two different substrates; *Aspergillus flavus, A. fumigatus, Fusarium graminearum, Penicillium notatum* and *Rhizoctonia solani*. 4 species showed positive response for the ligninolytic assay over one structural analogue of lignin; *Aspergillus flavus, A. fumigatus, Curvularia lunata, Penicillium notatum*.

Application of research: The degradation ability for the structural components observed in the fungal isolates is independent of the straw nature or the wood pulp and hence can be utilized for various other plant substrates.

Research Category: Mycology and Plant Pathology

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Study area / Sample Collection: Gorakhpur, Uttar Pradesh, India

Cultivar / Variety / Breed name: Rice

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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