
Study of fungi associated with decomposition of rice stubble and their role in degradation of lignin and holocellulose

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Twenty-seven species belonging to 15 genera (excluding one unidentified fungus) were isolated from decomposing rice stubble by dilution plate technique. The highest number of colonies (1166 cfu/g of rice stubble) was recorded in 45 days-old stubble and lowest (525) in 30 days-old stubble. Twelve dominant fungi capable of degradation of rice stubble, especially lignin and holocellulose, were tested for decomposition of rice stubble at three incubation periods (20, 40, 60 days). The study revealed a positive correlation between species inoculated and loss in dry weight of stubble with respect to different incubation periods. Highest loss in dry weight was recorded after 60 days of incubation. *Penicillium citrinum* showed the highest lignin degrading ability while *Aspergillus flavus* proved to be the most efficient degrader of holocellulose. All experiments were validated through ANOVA test.

Key words–incubation- stages of decomposition

Article

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Introduction

Rice is the principal crop and staple food of the people of Assam and it is grown extensively from low lying traditional paddy fields to upland hill sides. In rice-based cropping systems it is a common practice that after harvest the rice stubble is left in the field and becomes exposed to microbial degradation. Decomposition of rice straw depends on those microbes that can colonize and degrade its various constituents, especially cellulose, holocellulose and lignin (Abdel-Hafez et al. 1978, Coronel et al. 1991). Decomposition is a complex process made up of a number of sub-processes and involves a multitude of organisms, where fungi play an important role (Hudson 1971). Soluble components such as simple sugars and storage materials are utilized first and relatively quickly, followed by structural

polymers like cellulose and hemicelluloses and then lignin and lignocelluloses (Susan 1998). There are many reports on cellulose (Abdel-Hafez et al. 1978, Moubasher et al. 1985) lignin and holocellulose (Chatterjee & Nandi 1981b) decomposing fungi but little work has been done on decomposition of lignin and holocellulose in rice stubble.

The present investigation reports on the mycoflora associated with decomposition of rice stubble at different stages in the field and after rice harvest, and the role of fungi in degradation of lignin and holocellulose.

Methods

Site location and sampling technique

The study area was located near to Gauhati University campus, Jalukbari, Assam. The area is situated south of the river Brahma-

putra (26°12"N, 91°50"E). Stubble was collected one week after harvest from different localities of the experimental field; no fertilizer had been used. Rice stubble was mixed together and placed in 15 nylon bags (30 cm x 25 cm, mesh size 2 mm²). The bags were returned to the rice field and placed together on the soil surface. Five bags of decomposing stubble were removed for study at 30, 45 and 60 days after harvesting.

Estimation of rice stubble associated fungi

The rice stubble inhabiting microfungi were studied following the method of Sinha & Dayal (1983) and using a dilution plate technique (Warcup 1960). The stubble samples were ground aseptically into powder using a grinder and 1 g of the powder was suspended in 10 mL of sterile distilled water. The suspension was vigorously shaken and from this mixture a dilution series was prepared with a final dilution of 10⁻⁴. Five replicates of 1 mL from this final suspension (10⁻⁴) were added to Petri dishes containing 20 mL molten and cooled Czapeck's Dox agar medium with 100 ppm streptomycin to suppress bacterial growth. The plates were incubated at 27±1° C for 7 days. Fungal colonies were counted and the number of colonies/g of air dried stubble was calculated. Pure cultures were made for the identification of individual fungi (Barnett & Hunter 1972).

Decomposition of stubbles

In order to study those microfungi that are capable of degradation of rice stubble, especially lignin and holocellulose, 12 species of dominant fungi were selected. These fungi were then inoculated separately onto rice stubble in order to study their efficiency in biodegradation. Experiments were carried out under laboratory conditions for a period of 2 months.

Dried pieces (2.5 cm long) of rice stubble (10 g) of *Aijung* variety were added to 100 mL of sterile distilled water in 250 mL Erlenmeyer flasks. The flasks were plugged and sterilized for 1 hour at 15 lbs pressure. The flasks were then inoculated with spore suspension (2 mL) of each fungus separately, and thoroughly shaken. For each fungus nine replicates were prepared. Parallel sets of control (stubble + distilled water) were also maintain-

ed. The flasks were incubated at 30°C for 20, 40 and 60 days with periodic shaking at 2-day intervals to spread the fungus uniformly. Partially degraded stubble was taken out of the flasks in triplicate and dried to constant weight at 80°C and powdered. Lignin and holocellulose constituents of the rice stubble were estimated quantitatively.

Estimation of lignin decomposition

Lignin content of rice stubble was estimated quantitatively following the procedure of Saeman et al. (1954). In this method the total carbohydrate of powdered stubble was hydrolysed in sulphuric acid. 400 mg of stubble powder was macerated first in 3 ml of ice cold 72.01 % (w/v) sulphuric acid for 2 minutes. The acid mixture was agitated for 1 hour for efficient penetration of the acid. The acid was then diluted to 4% by adding water (84 mL) and then autoclaved at 121°C for 1 hour. After cooling, it was filtered through G4 sintered glass filter and washed several times with distilled water to render the lignin free of acid. The lignin residue was then air dried to a constant weight.

Estimation of holocellulose decomposition

Holocellulose in the powdered stubble was estimated quantitatively following the TAPPI standard (1954) and Cowling (1961). Powdered stubble (100 mg) moistened with ice water was placed in a glass crucible and pre-extracted with ethanol: benzene, ethanol and hot water to remove extraneous substances. This was followed by a succession of chlorination and monoethanol-amine extraction treatments to remove lignin. This extraction process was repeated until the sample became completely white. The paper-white holocellulose residue thus obtained was dried to constant weight.

Statistical analysis

The data were analyzed through one-way ANOVA and differences between means were calculated at 5% probability level using SPSS software program (ver. 9).

Results

Different fungi were found associated with rice stubble in different stages of

decomposition and their number varied. Twenty-seven species belonging to 15 genera (excluding 1 unidentified fungus) were isolated from the decomposing rice stubble by dilution plate technique (Table 1). The most diverse fungal flora was obtained from 45-days-old stubble (15 genera, 26 species) with a total colony count of 1166/g dry stubble. The least number of fungi were found after 30 days (8 genera, 15 species) with 525 colonies/g dry stubble. After 60 days (12 genera, 18 species) 901 colonies/g dry stubble were found. Analysis of variance between decomposition stages and fungal colonies showed significant differences at .05 level of significance. The predominant genera were *Aspergillus*, *Penicillium*, *Mucor*, *Helminthosporium* and *Trichoderma*. In 30-day-old stubble the dominant fungi were *Aspergillus* (191 cfu), *Cladosporium* (51cfu), *Helminthosporium* (43 cfu), and *Fusarium* (42 cfu) followed by *Mucor* (39 cfu), *Alternaria* (34 cfu) and *Penicillium* (33 cfu). In 45-day-old stubble *Aspergillus* (293 cfu), *Penicillium* (152 cfu), and *Trichoderma* (120 cfu) were the highest followed by *Fusarium* (99 cfu), *Mucor* (88 cfu) and *Helminthosporium* (43 cfu). In 60 day-old stubble *Aspergillus* (296 cfu), *Penicillium* (208 cfu), and *Trichoderma* (90 cfu) were predominate. The isolation frequency of *Aspergillus* (29.9%) and *Penicillium* (15.1%) was highest followed by *Trichoderma* (8.5%) and *Fusarium* (8.4%). *Chaetomium* showed lowest (1.5%) isolation frequency. *Nigrospora* was found to be scantily distributed in the decomposed rice stubbles. The dominant species found throughout all stages of decomposition were *Aspergillus fumigatus* (8.6%), *A. flavus* (7.5), *A. niger* (6.8%) and *Alternaria alternata* (5.7%), followed by *Penicillium citrinum* (5.2%), *P. oxalicum* (5.1%), *Colletotrichum dematium* (5.1%) and *Trichoderma viride* (5.0 %).

All of the inoculated fungi had the capability to cause degradation of rice stubble (Table 2). After 20 days of incubation the maximum degradation ability was shown by *Aspergillus flavus* (9.94%) followed by *A. nidulans* (9.35%), *Penicillium citrinum* (8.75%), *Verticillium catenulatum* (8.60%) and *Aspergillus niger* (7.30%). The lowest degradation was shown by *Rhizopus stolonifer* (3.00%). After 40 days of incubation the

highest degradation was also shown by *Aspergillus flavus* (22.20%) and *A. nidulans* (19.22%), followed by *Penicillium citrinum* (18.66%), *A. niger* (18.60%) and *Trichoderma viride* (18.39%). A similar trend was seen after 60 days with *Aspergillus flavus* and *A. nidulans* showing 26.30% and 25.62% degradation, respectively, followed by *Penicillium citrinum* (23.27%), *Cladosporium herbarum* (20.69%) *Aspergillus niger* (20.60%) and *Trichoderma viride* (19.27%). The lowest degradation after both 40 and 60 days of incubation was shown by *Rhizopus stolonifer* (10.33 and 13.80%). Analysis of variance between loss in dry weight and incubation periods showed significant difference in mean at .05 level of significance.

Lignin loss in decomposing stubble increased considerably with an increase in the incubation period (Table 3). Lignin content of fresh stubble (32.5%) decreased as a result of degradation by different test fungi. Maximum lignin degradation was achieved by genus *Aspergillus* followed by *Fusarium*, *Penicillium* and *Verticillium*. However, with respect to individual fungal species the highest lignin degradation was shown by *Penicillium citrinum* (9.95%, 13.60% and 16.54%) in all three incubation periods. After 20 days *Penicillium citrinum* (9.95%) showed highest lignin degrading ability followed by *Aspergillus flavus* (8.75%), *Verticillium catenulatum* (8.40%) and *Aspergillus niger* (8.30%). The lowest degradation ability was shown by *Fusarium oxysporum* (3.11%). At 40 days of incubation *Penicillium citrinum* (13.60%) was followed by *Verticillium catenulatum* (12.56%), *Aspergillus flavus* (12.35%) and *A. nidulans* (11.50%). The lowest degradation at 40 days of incubation was shown by *Rhizopus stolonifer* (6.63%). Similarly, after 60 days of incubation also *Penicillium citrinum* (16.54%) is followed by *Verticillium catenulatum* 15.75%) and *Aspergillus flavus* (15.22%). The lowest degradation was shown by *Trichoderma viride* (9.49%). Analysis of variance between percentage of lignin degradation (in dry weight) and (incubation period showed significant difference in mean at .05 level of significance.

There was a decrease in holocellulose content of the stubble with an increase in the

Table 1 Microfungi isolated from decomposing rice stubble.

Species	Decomposition (days)					Isolation frequency (%)
	30	45	60	Total	Sem	
	No. of colony forming units (g ⁻¹ stubble ×10 ⁴)					
<i>Alternaria alternata</i> (Fr.) Keissl.	34	50	65	149	8.95	5.7
<i>Aspergillus flavus</i> Link	52	64	80	196	8.11	7.5
<i>A. fumigatus</i> Fresen.	60	72	91	223	9.02	8.6
<i>A. niger</i> Tiegh.	45	58	75	178	8.68	6.8
<i>A. sydowii</i> (Bainier & Sartory) Thom & Church	34	45	-	79	13.54	3.0
<i>A. terreus</i> Thom	-	54	50	104	17.37	4.0
<i>Chaetomium globosum</i> Kunze	12	18	-	30	5.29	1.5
<i>Cladosporium cladosporioides</i> Fresen.	15	21	-	36	6.24	1.4
<i>C. herbarum</i> (Pers.) Link	36	-	-	36	12.00	1.4
<i>Colletotrichum dematium</i> (Pers.) Grove	-	56	76	132	22.74	5.1
<i>Curvularia lunata</i> (Wakker) Boedijn	26	30	-	56	9.40	2.1
<i>Fusarium moniliforme</i> J. Sheld.	21	33	55	109	9.95	4.2
<i>F. oxysporum</i> Schldtl.	-	27	23	50	8.41	1.9
<i>F. solani</i> (Mart.) Sacc.	21	39	-	60	11.26	2.3
<i>Helminthosporium oryzae</i> Breda de Haan	43	76	-	119	22.00	4.6
<i>Mucor racemosus</i> Fresen.	39	88	-	127	25.45	4.9
<i>Nigrospora sphaerica</i> (Sacc.) E.W. Mason	11	25	-	36	7.23	1.4
<i>Penicillium chrysogenum</i> Thom	-	35	40	75	12.58	2.9
<i>P. citrinum</i> Thom	10	50	76	136	19.19	5.2
<i>P. oxalicum</i> Currie & Thom	23	45	64	132	11.84	5.1
<i>P. rubrum</i> Stoll	-	22	28	50	8.51	1.9
<i>Phoma herbarum</i> Cooke	-	29	34	63	10.59	2.4
<i>Rhizopus stolonifer</i> (Ehrenb.) Vuill.	11	23	54	88	12.81	3.4
<i>Trichoderma harzianum</i> Rifai	12	36	-	48	10.58	1.8
<i>T. koningii</i> Oudem.	-	44	-	44	14.66	1.7
<i>T. viride</i> Pers.	-	40	90	130	26.03	5.0
<i>Verticillium catenulatum</i> (Kamyschko ex G.L. Barron & Onions) W. Gams	-	53	-	53	17.66	2.0
Sterile mycelia	25	33	-	53	9.93	2.0
Total	525	1166	901	2592	(2.8)	
No. of genera	12	15	8			
No. of species	18	26	15			

SEM-Standard Error of Means

ANOVA between decomposition stages and fungal colonies

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	7290.024	2	3645.012	5.813	.004
Within Groups	50786.393	81	626.993		
Total	58076.417	83			

- The mean difference is significant at the .05 level.

incubation period (Table 4). At 20 days of incubation maximum degradation of Holocellulose was shown by *Aspergillus flavus* (13.35%) and least by *Helminthosporium oryzae* (4.29%). After 40 days of incubation highest degradation was achieved by *Aspergillus niger* (22.65%) and lowest by *Fusarium oxysporum* (9.42%). After 60 days of incubation *Aspergillus flavus* (35.36%) was the

highest holocellulose degrader and the lowest degrading ability was shown by *Fusarium oxysporum* (12.17%). Thus, in all the three incubation period *Aspergillus* was found to be the dominant holocellulose degrader. Analysis of variance between percentage of holocellulose degradation (in dry weight) and incubation period shows significant difference in mean at .05 level of significance.

Table 2 Degradation of rice stubble by some dominant fungi.

Organisms//Incubation Days	20	40	60
	Loss in dry weight (%)		
<i>Aspergillus niger</i>	7.30	18.60	20.60
<i>Aspergillus flavus</i>	9.94	22.20	26.30
<i>Aspergillus nidulans</i>	9.35	19.22	25.62
<i>Penicillium citrinum</i>	8.75	18.66	23.27
<i>Cladosporium herbarum</i>	6.30	17.21	20.69
<i>Trichoderma viride</i>	6.54	18.39	19.27
<i>Fusarium solani</i>	5.30	15.74	17.21
<i>Fusarium oxysporum</i>	4.40	13.82	16.90
<i>Verticillium catenulatum</i>	8.60	17.74	21.17
<i>Helminthosporium oryzae</i>	4.50	13.16	15.20
<i>Mucor racemosus</i>	3.80	11.36	14.50
<i>Rhizopus stolonifer</i>	3.00	10.33	13.80
Control	0.20	0.20	0.20

ANOVA between loss in dry weight and incubation periods

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1028.20	2	514.10	18.29	.000*
Within Groups	1011.77	36	28.10		
Total	2039.98	38			

*The mean difference is significant at the .05 level.

Discussion

Fungi are a heterogeneous group of microorganism and their ability to inhabit rice stubble different stages of decomposition may be based on their decomposing activity mediated through the production of different enzymes. Cellulose decomposing fungi like *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* occur frequently and act upon the cell wall material of the rice stubbles. High occurrence of *Fusarium* on cellulose medium from wheat and rice straw was reported by others (El-Kady et al. 1981, Helal 2005, Rai et al. 2001). *Aspergillus*, *Penicillium* and *Trichoderma*, which were known to be most prevalent fungi from rice straw as xylan decomposer, were also isolated in the present investigation (Abdel-Sater & El-Said 2001). *Trichoderma* species are often cited as high cellulose decomposers (Zayed & Abdel-Motaal 2005). Many wood rotting basidiomycetes are also known to be very efficient in utilizing cellulose, but none were detected in the present study. The degradation process is mainly influenced by the chemical composition of degrading materials as well as the population of the degraders. Different types of

fungi with variable colony forming units were found in each stage of decomposition of rice stubble. This variability may be due to abiotic variables, nutrients within the stubble and due to secretion of metabolites by the inhabiting fungi resulting in toxic effect to the sensitive group of fungi or may be due to competition (Pandey & Sinha 2008, William & Gray 1974, Rai & Srivastava 1982, Khanna 1964, Thormann et al. 2003). The study revealed positive relationships between species inoculated and loss in dry weight of stubbles with respect to different incubation period (Table 2). Due to the degrading ability of the inoculated fungi the dry weight of the initial material reduced with the increase in the incubation period. Highest loss in dry weight of stubble was recorded after 60 days of incubation. This may be due to increased activity of microbes owing to the favourable edaphic and climatic conditions (Kumar et al. 2010, Cookson et al. 1998, Beare et al. 2002). Weight loss as a percentage from initial dry mass within a time period is an indirect measurement of the decomposition of stubble. All the treatments showed a higher weight loss than the control throughout the experiment

Table 3. Degradation of lignin in rice stubble by some dominant fungi.

Organisms//Incubation Days	20	40	60
	Loss in dry weight (%)		
<i>Aspergillus niger</i>	8.30	10.44	13.63
<i>Aspergillus flavus</i>	8.75	12.35	15.22
<i>Aspergillus nidulans</i>	7.81	11.50	12.15
<i>Penicillium citrinum</i>	9.95	13.60	16.54
<i>Cladosporium herbarum</i>	6.65	9.43	11.22
<i>Trichoderma viride</i>	4.44	7.83	9.49
<i>Fusarium solani</i>	5.54	9.75	11.92
<i>Fusarium oxysporum</i>	3.11	8.74	12.90
<i>Verticillium catenulatum</i>	8.40	12.56	15.75
<i>Helminthosporium oryzae</i>	5.35	8.78	12.16
<i>Mucor racemosus</i>	4.45	7.41	12.57
<i>Rhizopus stolonifer</i>	3.21	6.63	9.50
Control	0.32	0.32	0.32

Lignin content in fresh stubble-32.5%.

ANOVA between loss in dry weight and incubation periods

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	258.02	2	129.01	10.46	.000*
Within Groups	443.73	36	12.32		
Total	701.76	38			

* The mean difference is significant at the .05 level.

Table 4 Degradation of holocellulose in rice stubble by some dominant fungi.

Organisms//Incubation Days	20	40	60
	Loss in dry weight (%)		
<i>Aspergillus niger</i>	12.67	22.65	33.23
<i>Aspergillus flavus</i>	13.35	20.32	35.36
<i>Aspergillus nidulans</i>	9.45	18.53	28.65
<i>Penicillium citrinum</i>	10.98	12.67	14.45
<i>Cladosporium herbarum</i>	8.87	16.44	19.70
<i>Trichoderma viride</i>	6.60	11.40	12.80
<i>Fusarium solani</i>	9.81	10.54	14.76
<i>Fusarium oxysporum</i>	7.91	9.42	12.17
<i>Verticillium catenulatum</i>	8.49	11.34	16.92
<i>Helminthosporium oryzae</i>	4.29	10.19	15.45
<i>Mucor racemosus</i>	7.01	9.76	14.15
<i>Rhizopus stolonifer</i>	9.81	13.50	16.60
Control	0.25	0.25	0.25

Holocellulose content in fresh stubble-61.4%

ANOVA between loss in dry weight and incubation periods

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	602.23	2	301.11	6.72	.003*
Within Groups	1610.99	36	44.75		
Total	2213.22	38			

* The mean difference is significant at the .05 level.

period. This is consistent with the findings of Herath et al. (2004) who used effective microorganisms to accelerate the decomposition of rice straw. The rate of loss in

dry weight of the stubble was also related to the growth and proliferation of stubble mycoflora population, as during period of their active growth the weight loss was maximum.

Penicillium citrinum showed the highest lignin degrading ability among the test fungi. Other fungi which showed high lignin degrading ability are the species of *Aspergillus*, *Fusarium*, *Penicillium* and *Verticillium*. The role of these fungi as efficient lignin degraders was reported earlier (Alexander 1977, Chatterjee & Nandi 1981b, Gulyas 1967, Coronel et al. 1991). Lignin, which is the most important component of cell wall of rice stubble, is known to be relatively resistant to microbial decay (Kirk et al. 1977). Lignin is a complex substance covalently bound to side chains of xylans of cell wall. It represents an obstacle to microbial digestion of structural carbohydrates both because it is a physical barrier and because of the depressing effect on microbial activity, due to the phenolic compounds it contains. The most important phenolics are p-coumaric acid, ferulic acid, p-hydroxybenzoic acid and vanillic acid (Antongiovanni & Sergentini 1991). Lignin degrading fungi produce extracellular phenol-oxidizing enzymes, which act on the lignin through the oxidative process, with the formation of the primary structural groups like p-coumaric acid, vanillic acid, etc. (Nobles 1958). The role of fungi capable of oxidizing these primary structural groups is well established. The structural groups lose their methoxyl groups and are oxidized to quinines, which are polymerized and condensed with amino acids to form humic acids. It is unlikely that these structural units always participate in the formation of humus, since soil harbours a large population of microorganisms capable of assimilating these aromatic compounds. Garrett (1963) reported that fungi that are responsible for lignin decomposition appear in the last stage of decomposition and most of them are basidiomycetes.

It is clear from the present study that holocellulose, one of the major constituents of rice stubble, is degraded by all the inoculated fungi (Table 4). It is evident that the fungi which degrade lignin also degrade holocellulose at a higher rate. Among all the inoculated fungi *Aspergillus flavus* proved to be the most efficient degrader of holocellulose. Many research findings have shown that *A. flavus* is an efficient degrader of holocellulose

and lignin (Chatterjee & Nandi 1981a).

The present investigation has shown that diverse types of fungi are involved in the decomposition of rice stubble and it gives a clear insight into the diversity of mycoflora involved during decomposition in a sub-tropical rice based ecosystem.

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