Indian Journal of Agricultural Sciences **94** (8): 908–911, August 2024/Short Communication
https://doi.org/10.56093/ijas.v94i8.124752

Microbial degradation of paddy (*Oryza sativa*) straw by submerged state fermentation

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Received: 13 June 2022; Accepted: 12 June 2024

Keywords: Consortium, FE-SEM, Lignocellulolytic, Paddy straw

Paddy (*Oryza sativa* L.) is considered as one of the three most important grain crops amongst the whole world. As per FAOSTAT (2021), paddy reached a total production of 787.3 million tonnes in 2021, occupying the second position amongst other crops. Paddy straw is a lignocellulosic residue which is composed of cellulose (32–37%), hemicellulose (29–37%) and lignin (5–15%). Burning of paddy straw for getting the field ready for next crop (usually wheat) is a serious issue because it causes loss of soil health and fertility by harming the rich and abundant beneficial microflora (Lohan *et al.* 2018). Various fungal and algal species have also been used to decompose the lignocellulose rich straw but they are not considered as efficient as the bacterial cultures because of bacteria's small generation time and very fast growth rate along with vast variety in species that can work under milder condition (Zhao *et al.* 2014). Bacterial enzymes play important role in lignin modification or straw decomposition (de Gonzalo *et al.* 2016). Present study was aimed to evaluate the enzyme production by different microbes that can play significant role in causing *in situ* degradation of paddy straw by degradation of its structural components like cellulose, hemicelluloses and lignin.

The study was carried out during 2021–22 at Punjab Agricultural University, Ludhiana, Punjab. Paddy straw (PR121) was obtained from the research field of Punjab Agricultural University, Ludhiana, Punjab. Mushroom compost collected from mushroom research complex was used for the isolation of effective lignocellulolytic microorganisms. Standard bacterial cultures, viz. *Delftia* spp. PP4_S3 (GenBank Accession number JF274923.1), *Pseudomonas* spp. and *Bacillus* spp. were also procured from the Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab.

Screening of lignocellulosic cultures: The isolated and purified bacterial, actinomycete and fungal cultures along with the standard cultures were analyzed qualitatively for their lignocellulose degrading potential by Agar Plate Assay method suggested by Okino *et al.* (2000). Point inoculation of the microbial cultures was done on the solidified plates of paddy straw agar media (PSA). The colonies grown on carboxy-methyl cellulose plates were flooded with 1% Congo red while the plates with xylan were flooded with 0.15% I_2 solution. The clear halos around the colonies indicated the presence of cellulolytic and hemicellulolytic enzymes. Potency index (PI) was calculated as:

$$\text{Potency index (PI)} = \frac{\text{Size of clearance zone (mm}^2)}{\text{Size of colony (mm}^2)}$$

For lignin degradation evaluation, paddy straw agar media was supplemented with Remazol brilliant blue (RBB) (0.05%). Guaiacol (0.075 ml/litre) containing media was used to confirm presence of lignin peroxidase and manganese peroxidase or laccase. PSA supplemented with Azure B (0.1%) was used to evaluate presence of lignin peroxidase.

Quantitative screening for lignocellulolytic enzyme production: For quantitative screening of production of lignocellulosic enzymes, chopped paddy straw (1.5 g) was mixed with 150 ml paddy straw based minimal broth i.e. 1:100 (w/v) ratio in a flask (250 ml) and sterilized by autoclaving. After cooling, flasks were inoculated with 5 ml inoculum of 24 h old culture each of *Delftia* spp. PP4_S3, *Pseudomonas* spp., *Bacillus* spp. and isolated lignocellulolytic bacterial, actinomycete and fungal (@10^7spores/ml) cultures as consortium. Flasks inoculated with the consortia were kept for incubation at 30±2°C, 100 rpm in a shaking BOD incubator. Crude extract of enzyme was collected by sieving the flask constituents through Whatsmann No. 1 filter paper at regular intervals. Obtained enzyme extract was centrifuged to collect crude enzyme at 10,000 rpm for 15 min at 4°C in a cooling centrifuge. Obtained supernatant was analyzed for Endoglucanase (CMCase) (Mandels *et al.*1976), Exoglucanase (FPase) (Mandels *et al.* 1976), β-glucosidase (cellobiase) (Toyama and Ogawa 1977) and Xylanase (Singh *et al.* 2000) along

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with Laccase (Turner 1974), Lignin peroxidase (LiP) (Tien and Kirk 1988) and Manganese peroxide (MnP) (Paszczynski *et al.* 1988) activity.

Production of reducing sugars was estimated by dinitro-salicylic acid (DNS) method (Miller 1959). Enzyme activity was expressed in terms of International units:

$$\text{Enzyme units} = \frac{\mu\text{M of reducing sugar produced/ml}}{\text{Incubation period (min)}}$$

Proximate and chemical analysis of paddy straw: Method of Soest (1963) was used for evaluation of proximate as well as chemical constituents of paddy straw i.e. cellulose, hemi-cellulose, lignin, total solids (TS), volatile solids (VS), total organic carbon (TOC) and ash.

The data provided reflects mean of the values and standard error in triplicates. Data was subjected to critical difference at 5% level with completely randomized design (CRD) design using CPCS1 software.

Potency index of isolated and standard cultures was calculated and expressed in Table 1. With respect to isolated cultures, B_6 reported as the best cellulase producer with PI of 1.78, followed by B_4 with a PI of 1.53. Highest xylanase activity was shown by isolate B_4 (PI=2.36). Bacterial isolate B_6 (PI=1.55) exhibits maximum RBB potency index. Azure B activity exhibited by isolate A_1 depicted PI value of 1.61. Among the standard cultures, maximum cellulase activity was shown by *Pseudomonas* spp. with a PI of 1.38. *Delftia* spp. PP4_S3 exhibited highest xylanase activity with potency index of 2.35. Higher cellulolytic and hemicellulolytic activities as compared to lignolytic activity could be possible due to low *p*H of PSA media (Chukwuma *et al.* 2023).

With increase in incubation period, activity of endoglucanase, exoglucanase, β-glucosidase, xylanase and manganese peroxidase was found to increase till the 28th day (Table 2) whereas laccase and lignin peroxidase showed increase only upto 21st day of incubation and started declining further.

Maximum reduction of TS, VS, cellulose, hemicellulose, lignin and silica content was studied in straw sample upto an incubation period of 28 days (Table 3). TS decreased from 99.2% (control) to 83.7% and VS decreased from 85.4% (control) to 75% by showing maximum reduction of 13.34% within 28 days. Cellulose content kept on decreasing from 35.4% (control) to 22.9% after microbial treatment. Hemicellulose content also decreased from 27.8% (control) to 19.2% after an incubation period of 28 days. Lignin content decreased from 16.1% (control) to 8.9% by showing maximum reduction of 32.91%. It is important to mention here that an incubation period of 28 days is required for sufficient degradation of paddy straw, but window period between paddy harvesting and wheat sowing is shorter than this. Earlier also, variable trend in delignification during surface retained paddy straw trials, highlights that with microbial decomposer application, the surface retained paddy straw is not appreciably decomposed in the provided window period of 3 weeks between paddy harvesting and wheat sowing (Katyal *et al.* 2023).

Longitudinal section of paddy straw before and after biological pre-treatment was visualized under FE-SEM (Fig. 1). Samples were processed by dehydrating followed by sputter coating with gold particles. After processing, the samples were viewed at an accelerating voltage of 20 kV. Changes in surface structure were clearly visible in basic tissue straw and sloughing off of material was observed. Silica granules were exposed but did not undergo any degradation. Similar results have also been reported by Raj *et al.* (2023) where treatment of straw with bacterial culture of *Pseudomonas aeruginosa* strain AMB-CD-1 resulted in effective separation of microfibrils and holo-cellulose resulting in increase in porosity in paddy straw's outer surface.

Table 1 Qualitative estimation of lignocellulolytic activity of isolated and standard lignocellulosic cultures

Isolate no.	Potency index (PI)				
	Cellulolytic activity	Hemicellulolytic activity	Lignolytic activity		
	Cellulase	Xylanase	RBB activity	Guaiacol activity	Azure B activity
B_1	1.40 ± 0.15	1.31 ± 0.06	1.53 ± 0.08	-ve	-ve
B_2	1.33 ± 0.04	1.7 ± 0.05	1.18 ± 0.02	-ve	1.05 ± 0.11
B_3	1.16 ± 0.10	1.43 ± 0.07	1.47 ± 0.02	-ve	-ve
B_4	1.53 ± 0.16	2.36 ± 0.01	1.32 ± 0.09	-ve	1.13 ± 0.01
B_5	1.24 ± 0.01	1.49 ± 0.09	1.13 ± 0.05	-ve	1.09 ± 0.13
B_6	1.78 ± 0.17	1.67 ± 0.06	0.85 ± 0.10	-ve	1.31 ± 0.24
A_1	1.40 ± 0.05	1.54 ± 0.02	1.33 ± 0.05	-ve	1.61 ± 0.19
F_1	0.52 ± 0.07	1.02 ± 0.06	0.84 ± 0.16	-ve	-ve
Delftia spp. PP4_S3	0.67 ± 0.19	2.35 ± 0.03	1.55 ± 0.16	-ve	-ve
Bacillus spp.	1.16 ± 0.07	1.31 ± 0.02	1.83 ± 0.02	-ve	1.44 ± 0.02
Pseudomonas spp.	1.38 ± 0.04	0.66 ± 0.28	1.38 ± 0.05	-ve	-ve
CD (*P*=0.05)	0.226	0.286	0.182	-ve	0.208

RBB, Remazol brilliant blue (0.05%); Guaiacol i.e. O-methoxy phenol (0.075ml/l); Azure B (0.1%).

Table 2 Quantitative estimation of cellulolytic, xylanolytic and lignolytic activity of consortium from paddy straw in submerged state fermentation

Incubation time (in days)	Enzyme activities (U/ml)						
	Endoglucanase	Exoglucanase	β-glucosidase	Xylanase	Laccase	Lignin Peroxidase	Manganese Peroxidase
0	0.12 ± 0.01	0.36 ± 0.09	0.40 ± 0.06	1.88 ± 0.33	0.13 ± 0.02	0.93 ± 0.05	0.29 ± 0.06
7	0.38 ± 0.05	0.50 ± 0.04	0.72 ± 0.07	3.83 ± 0.28	0.85 ± 0.63	2.21 ± 0.12	1.16 ± 0.71
14	0.51 ± 0.04	0.69 ± 0.07	1.53 ± 0.10	7.75 ± 0.06	1.42 ± 0.19	4.93 ± 0.14	2.15 ± 0.13
21	0.77 ± 0.04	1.03 ± 0.02	1.79 ± 0.04	11.93 ± 0.21	4.36 ± 0.07	6.71 ± 0.32	3.83 ± 0.07
28	1.00 ± 0.02	1.31 ± 0.02	2.42 ± 0.03	12.63 ± 0.39	3.71 ± 0.05	6.02 ± 0.02	5.43 ± 0.14
35	0.46 ± 0.13	0.63 ± 1.04	0.81 ± 0.28	4.15 ± 0.08	1.03 ± 0.12	3.65 ± 1.19	1.51 ± 0.01
CD (*P*=0.05)	0.21	0.154	0.192	0.887	0.243	0.192	0.451

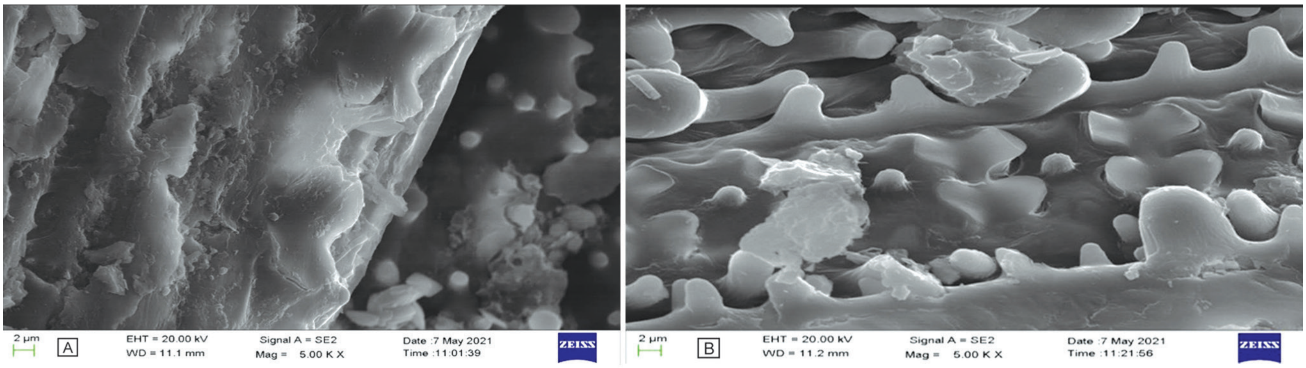

Fig. 1 Field emission scanning electron micrographs (FE-SEM) of paddy straw before and after biological treatment. (A) Longitudinal section of untreated paddy straw (control) at a scale of 2 µm; (B) Longitudinal section of paddy straw treated with microbial consortium for 28 days at a scale of 2 µm.

From the above study, it can be presumed that different isolates namely B_4, B_6, A_1 and F_1 and standard bacterial cultures *Delftia* spp. PP4_S3, *Pseudomonas* spp. and *Bacillus* spp. are efficient lignocellulose degraders. These findings are very much in line with earlier reports where higher yield of lignocellulosic enzymes has been observed by action of consortia than individual cultures (Sahil *et al.* 2023). In the present study, use of enzyme cocktail has proven to be more efficient in hydrolysis of straw resonating with results of Gao *et al.* (2023). Some fungal strains like *Phanerodontia chrysosporium* have shown exemplary delignifying abilities resulting in production of reducing sugars (Guo *et al.* 2022), which can be correlated with elevated levels of reducing sugars in present study. FE-SEM studies also revealed structural and morphological changes caused by consortium over straw's surface. This proves that

Table 3 Periodic change in proximate and chemical composition of paddy straw

Treatment (Days)	Composition of paddy straw (%)							Dry matter loss (%)
	Total solids	Volatile solids	Total organic carbon	Ash	Cellulose	Hemicellulose	Lignin	
Control#	99.2 ± 0.43	85.4 ± 0.18	47.44 ± 0.18	14.6 ± 0.31	35.4 ± 0.21	27.8 ± 0.17	16.1 ± 0.53	0.16 ± 0.05
7	94.5 ± 0.19	81.5 ± 0.44	45.27 ± 0.21	18.5 ± 1.11	31.4 ± 0.15	25.3 ± 0.89	14.7 ± 0.23	7.32 ± 1.67
	(4.73↓)*	(4.51↓)	(4.59↓)	(26.71↑)	(11.29↓)	(9.27↓)	(8.69↓)	
14	88.7 ± 0.75	78.6 ± 0.26	43.66 ± 0.27	21.4 ± 0.97	28 ± 0.33	23.7 ± 0.02	12 ± 1.13	15.6 ± 1.41
	(10.58↓)	(7.91↓)	(7.98↓)	(46.57↑)	(20.9↓)	(14.1↓)	(25.46 ↓)	
21	85.1 ± 0.53	75.1 ± 0.13	41.72 ± 0.44	24.9 ± 0.64	24.7 ± 0.46	23.1 ± 1.35	10.8 ± 0.78	21.81 ± 0.36
	(14.21↓)	(12.03↓)	(12.05↓)	(70.54↑)	(30.22↓)	(15.3↓)	(32.91↓)	
28	83.7 ± 0.28	74 ± 1.02	41.11 ± 0.58	26 ± 0.12	22.9 ± 0.20	19.2 ± 1.21	8.9 ± 0.22	25.51 ± 0.71
	(15.6↓)	(13.34↓)	(13.24↓)	(78.06↑)	(35.31↓)	(22.5↓)	(44.72↓)	
CD (*P*=0.05)	1.764	2.302	1.237	0.966	1.386	0.999	0.727	0.567

Control#, Soaked paddy straw; (↓), Decrease; (↑), Increase. *Figures in parenthesis indicate percent decrease with respect to control.

straw, apart from being cheap and easily available, can be used efficiently as an agro-residue for enzyme production by using various lignocellulosic cultures.

Microbial consortium can be exploited for *in situ* management of paddy straw but only when a sufficient window period is given for the degradation of straw. Alternatively, mechanical interventions like use of Happy Seeder, Super seeder or Surface seeder that have the ability to sow the wheat crop immediately after rice harvesting can be exploited instead of burning the rice straw.

SUMMARY

This study elucidates the effective lignocellulose degrading potential of microbial consortium by using paddy straw as the sole source under submerged fermentation conditions. Straw degradation was enhanced by bioaugmentation procedures where consortia of isolated and standard cultures resulted in maximum enzyme activities of endoglucanase (1 U/ml), exoglucanase (1.31 U/ml), β-glucosidase (2.42 U/ml), xylanase (12.63 U/ml) and manganese peroxidase (5.43 U/ml) after 28 days of incubation along with laccase (4.36 U/ml) and lignin peroxidase (6.71 U/ml) after 21 days of incubation. By the action of consortium, maximum reduction of cellulose, hemicellulose and lignin was found to be 35.31, 22.50 and 44.72%, respectively after 28 days of incubation. A significant increase in percentage loss in dry matter of paddy straw was observed till 4 weeks of fermentation. Field emission scanning electron microscope (FE-SEM) micrographs revealed sufficient loosening of cellulose and hemicellulose showing successful degradation of straw tissue. Overall, the above stated findings emphasize on potential of lignocellulosic microorganisms to be used as an alternative to traditional stubble burning practice. Although, these extracellular enzymes are well equipped in breaking down lignocelluloses to their monomeric or oligomeric subunits, but they may not be exploited at commercial level due to very slow rate of reaction. The efficacy of these cultures for straw degradation in field condition further needs elaborative experimental trials as environmental parameters are quite variable in the field.

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