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Isolation and Screening of Lignocellulolytic Microbes from Cow Dung for Rapid Composting of Rice Straw

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present work aimed to isolate and screen the lignocellulolytic microbes from cow dung, partially decomposed straw and forest soil. A total of 60 isolates from which 41 bacterial, 15 fungal and 4 actinomycetes distinct isolates obtained were further subjected to lignocellulolytic screening. The 60 isolates when screened for their ability to produce cellulase enzyme 21 bacterial isolates showed positive. The highest halo zone was shown with greater hydrolysis capacity ranging from 1.04 to 2.58. The 60 isolates were then subjected to screening for lignolytic activity by using methylene blue, azure B as indicator for bacteria and tannic acid as indicator for fungi. Out of them 11 bacterial isolates showed decolourisation of methylene blue and azure B and 4 fungal isolates were to exhibit positive for tannic acid assay which is indication for lignolytic activity. Among these 60 isolates 3 bacterial isolates CD-5, PDS-3, and PDS-6 showed both cellulolytic and lignolytic activity.

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Keywords: Cellulose; cow dung; forest soil; lignin; Lignocellulolytic microbes and partially decomposed straw.

1. INTRODUCTION

Rice straw is a by-product of rice cultivation, for every ton of harvested rice grain, about 1.35 tons of rice straw remains in the field, which generates huge amount of straw annually [1]. The disposal of rice straw is a problem due to the huge bulk material largely composed of lignocellulosic materials amenable to general biodegradation. In many countries including India, a huge amount of straw is disposed through open burning, which causes serious environmental problems as well as a threat to public health. Rice straw is a renewable carbon resource, which is rich in sources consisting of cellulose (36-37 %), hemicelluloses (23-24 %) and lignin (15-16 %) along with a small quantity of protein, by making it wider in the C: N ratio.

Microbes are very crucial in decomposition process as they contain many enzymes, which play an important role in degrading celluloses, lignin and hemicellulose in rice straw. This results in the declining of carbon (C) and increase of nitrogen (N) that is available in the rice straw and formation of soluble sugars from cellulose in rice straw relies on the sequential action of individual components of cellulose complex (exoglucanase, endoglucanase and beta-D glucosidase) derived from the cellulolytic microorganisms. In nature, mostly white rot fungi efficiently degrade lignin, including many basidiomycetes.

Rice straw is a potential food source for microorganisms like bacteria, fungi and actinobacteria. It could be converted into a valuable product in a short period through a microbial composting process. Rice straw compost is most commonly applied to fields in many countries to improve soil fertility and increase yield [2]. To make the rice straw composting process economically viable, lignocellulolytic microbes based biodegradation may be an effective alternative to in situ burning [3]. The compost can serve as an excellent source of nutrients in organic farming to mitigate the increasing fertilizer use. Bacteria and actinobacteria are well known for their ability to decompose complex molecules, particularly lignocellulosic components, which make them important agents in composting, process [4]. Ruminants mostly feed on lignocelluloses agricultural materials, their stomach contains crude fibre such as cellulose, starch and xylan [5] were not completely converted to animal product in intensive animal farming, these materials are fermented by rumen microbial community like bacteria, actinobacteria and fungi.

The present investigation was formulated for isolation of microbes from fresh cow dung, partially decomposed straw and forest soil and screening of the isolates for cellulolytic and ligninolytic activity.

2. MATERIALS AND METHODS

Collection of sample: Fresh cow dung (CD) sample was collected from the cowshed, Rajendranagar, Hyderabad; the partially decomposed straw (PDS) was collected from ICAR-IIRR research farm and a forest soil (FS) sample from Yellapur forest, Uttara Kannada District of Karnataka by using sterile spatula into the sterilized cover.

Isolation and purification of microorganisms: The microorganisms were isolated by using the serial dilution and spread plate technique on Nutrient agar, Martin Rose Bengal Agar, Kusters and Williams Agar. One gram of each sample was added to the test tube containing 9 ml of sterile distilled water to make 10^{-1} dilution and by drawing 1ml from this further dilutions upto 10^{-7} were prepared.

For isolation of bacteria 0.1 ml of the suspension was taken from 10^{-5} and 10^{-6} dilutions and spread on Nutrient Agar media plates, fungi was isolated by taking 0.1 ml of suspension from 10^{-3} and 10^{-4} dilutions and was spread on Martin Rose Bengal Agar media, actinomycetes was isolated by taking 0.1ml of suspension from 10^{-4} and 10^{-5} and was spread on Kuster and Williams and the plates were incubated at 30^{o}C. All the plates were examined, bacteria, fungi and actinomycetes were enumerated based on colony forming units (cfu) and the isolates were differentiated based on their morphological parameters *viz.,* shape, size, colour and texture. The distinct morphotypes were selected and purified on their respective media plates. Purified isolates were preserved for further analysis in their respective broth and preserved in glycerol stocks at -20^{0}C for future use.

Screening of purified isolates for cellulolytic and lignolytic activity: The purified cultures were screened for cellulolytic bacteria on minimal media supplemented with 1% CMC plates (1g KH_2PO_4, 0.5g K_2SO_4, 0.5g NaCl, 0.01g $FeSO_4$, 0.01g $MnSO_4$, 1g $(NH_4)NO_3$, 10g CMC and 20g agar at neutral pH). Small wells were formed on the media using a sterile gel puncher, the purified cultures were inoculated on to the well and were incubated at ambient temperature for 3 to 5 days. After incubation, the plates were flooded with 0.1 % Congo red solution and after 15-20 minutes they were de-stained with 1M NaCl [6]. Formation of clear halo zone around the well after staining and de-staining is the indication for the presence of cellulose degrading ability. The Hydrolysis capacity (HC) of the isolates is calculated by using the formula:

$$\mathbf{HC} = \frac{\text{diameter of clear zone or intense zone (mm)}}{\text{diameter of colony (mm)}}$$

To examine the cellulolytic activity of fungi and actinomycetes the basal medium containing 0.5 g $(NH_4)_2SO_4$, 1.5g KH_2PO_4, 5g K_2HPO4, 0.1g $MgSO_4.7H_2O$, 0.1g Yeast extract, 0.2g NaCl, 1.1% CMC per 1 litre, Agar- 20g and pH-5.5 for fungi; pH- 7.2 for actinomycetes. The purified cultures were point inoculated on the plates and were incubated at 37^0C for actinomycetes and 30^0 C for fungi. After 5 days, the plates were flooded with congo-red (1mg/ml distilled water). The dye was drained off after 15min and plates washed thrice with 1M NaCl solution. The cultures producing CMCase showed yellow zones around the growth.

The purified bacterial isolates were screened for lignolytic activity on the minimal media containing 4.55g K_2HPO_4, 5g NH_4NO_3, 0.5g H_3BO_3, 0.01g $CaCl_2$, 0.53g KH_2PO_4,; Trace element solution 1 ml (2.2g $ZnSO_4.7H_2O$, 0.5g Mn acetate, 0.5g $FeCl_3$,0.16g $CuSO_4.6H_2O$), 0.11g Molybdic acid, 5g Na_2EDTA and distilled water 1000ml [7]. The pure bacterial cultures were streaked on the petriplates and incubated at 30^0C for 2-3 days. Methylene blue and azure B was used as an indicator; decolorization of the dye around the streaked colony indicates the presence of lignin degrading ability.

To examine the lignolytic activity of fungi, the minimal medium containing 0.5g $NH_4H_2PO_4$, 0.15 KH_2PO_4, 0.01g KH_2PO_4, 1g Malt extract, 20g Agar and pH- 5.5 substituted 240mg of tannic acid per litre [8] was prepared. The pure fungal cultures were spotted on the media plates and incubated at 30^0C for 2-3 days. Development of dark yellow to brown colorization around the fungal colony indicates the presence of lignin degrading ability.

3. RESULTS AND DISCUSSION

Isolation and purification of bacteria, actinomycetes and fungi: For isolation of lignocellulolytic bacteria, cow dung, partially decomposed straw and forest soil were collected. Bacteria, actinomycetes and fungi were isolated from the three different sources by using nutrient agar, Kusters and Williams Agar and MRBA plates respectively and the plates were incubated. After incubation, the plates were examined for colony formation were enumerated. It was observed that cow dung sample had $116x10^5$ cfu/g bacteria, $21x10^3$ cfu/g fungi and $35x10^4$ cfu/g actinomycetes, similar reports were given by (Obi et al*.,* 2022 *; Ram* et al*.,* 2020*),* Partially decomposed straw had $124x10^5$ cfu/g soil bacteria, $26x10^3$ cfu/g soil fungi, $31x10^4$ cfu/g soil actinomycetes [9,10] have also isolated microbes from partially decomposed straw and had similar findings. $105x10^5$ cfu/g soil bacteria-, $29x10^3$ cfu/g soil fungi and $42x10^4$ cfu/g soil actinomycetes were observed in forest soil which is similar to the studies of [11] (Table 1).

The colonies were further purified based on their morphological parameters like colour, size, shape and texture. A total of 60 distinct morphotypes were isolated of which 41 were bacteria, 4 were actinomycetes and 15 were fungi (Table 2). Out of 60 isolates 19 (11 bacteria, 6 fungi, 1 actinomycetes) from cow dung, 26 (17 bacteria, 7 fungi, 2 actinomycetes) were from partially decomposed straw, and 15 (9 bacteria, 5 fungi, 1 actinomycetes) from forest soil. The purified isolates were named after the source they have been isolated.

Screening for the cellulose degrading organisms: All 60 purified microbial isolates were screened for cellulose production on CMC agar. Out of sixty isolates 21 bacterial isolates exhibited positive cellulase activity and the remaining isolates showed negative (Table 3). No fungal and actinomycetes showing positive activity were reported. The CMCase activity was determined based on the creation of a zone of hydrolysis or halo zone that results from the secretion of cellulase and subsequent hydrolysis of cellulose by the bacterial isolates described by many researchers [12]. Among 21, cellulose degrading bacterial isolates CD-2, PDS-1, PDS-9

Table 1. Summary of bacterial, fungal and actinomycetes isolates from different sources

Source of sample	CFU/ gram of sample		
	Bacteria (10^5)	Fungal (10^3)	Actinomycetes (10^4)
Cow dung	116	21	35
Partially decomposed straw	124	26	31
Forest soil	105	29	42
Grand total	345	76	108

Table 2. Morphotypes isolated from different sources based on morphology

Source of sample	No. of isolates	Bacterial	Fungal	Actinomycetes
Cow dung	19	13	5	1
Partially decomposed straw	26	17	7	2
Forest soil	15	11	3	1
Grand total	60	41	15	4

Table 3. Isolates having cellulase and lignase activity from different samples

Source of sample	Cellulolytic microbes			Lignolytic microbes		
	Bacteria	Fungi	Actinomycetes	Bacteria	Fungi	Actinomycetes
Cow dung	7	0	0	3	1	0
Partially decomposed Straw	9	0	0	5	2	0
Forest soil	5	0	0	3	1	0
Grand total	21	0	0	11	4	0

Table 4. Evaluation of cellulase activity of cellulose degrading bacteria in CMC agar plate through the maximum clearing zone and hydrolysis capacity (HC)

Isolates	Isolation source	Mean clear zone diameter(ZD) (mm)	Mean colony diameter (CD) mm	HC value (ZD/CD)
CD-2	CD	49	19	2.58
PDS-1	PDS	43	18	2.39
PDS-9	PDS	37	17	2.18
CD-4	CD	40	19	2.11
CD-7	CD	29	14	2.07
CD-3	CD	39	19	2.05
PDS-2	PDS	46	24	1.92
PDS- 3	PDS	32	18	1.78
FS-1	FS	28	17	1.65
PDS-7	FS	34	21	1.62
PDS-6	PDS	34	23	1.48
CD-5	CD	41	29	1.41
CD-1	CD	28	21	1.33
PDS-4	PDS	34	26	1.31
PDS-8	PDS	29	24	1.21
FS-4	FS	29	24	1.21
FS-3	FS	36	30	1.2
CD-6	CD	25	21	1.19
PDS-5	PDS	32	27	1.19
FS-2	FS	21	20	1.05
FS-5	FS	26	25	1.04

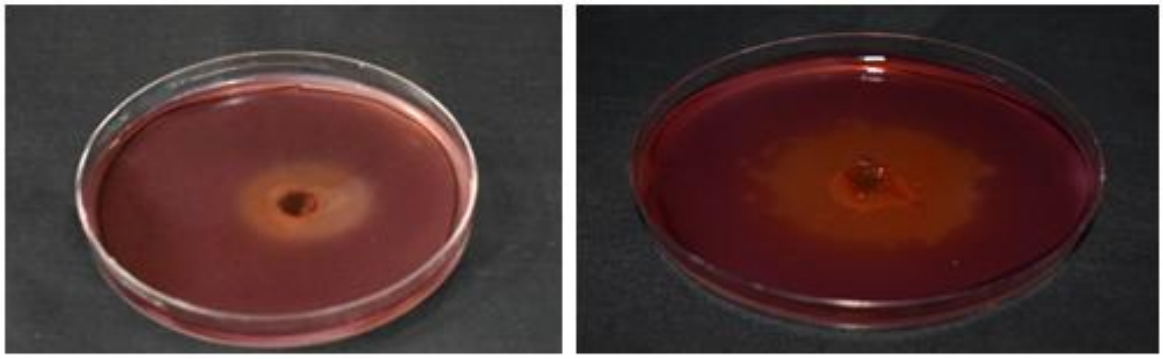

Plate 1. Cellulose Degrading Bacteria

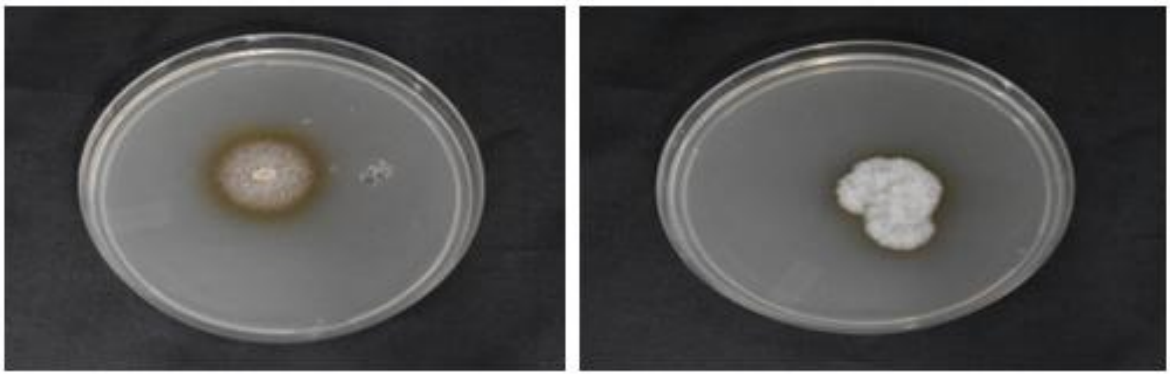

Plate 2. Lignin Degrading Fungi

showed greater halo zone in comparison to other isolates. The clearing zone diameter ranged from (21 to 49mm) and the HC (hydrolysis capacity) value ranged from (1.04 to 2.58) (Table 4). The findings of this study are closely related with the results reported by Rawway et al., [12]. Gaur et al., [13] had reported that hydrolysis capacity value greater than 1.0 indicated high cellulolytic activity. The partially decomposed straw was the richest source of decomposing microbes with 26 isolates, followed by the cow dung having 19 isolates and forest soil with 15 isolates.

Similarly cellulolytic microbes were isolated from cow dung by [14], partially decomposed straw by [9] and forest soil by [15,16].

Screening for the lignin degrading organisms: In order to break down lignin, microorganisms usually secrete extracellular enzymes. The breakdown of lignin by bacteria or fungi is dependent on the actions of certain enzymes, including LiP, Lac, and MnP [17,18]. All 60 purified microbial isolates were screened for lignase activity on the minimal media. Out of sixty isolates 15 isolates exhibited positive lignase activity and the remaining isolates were negative Table 3, and among these 11 bacterial isolates confirmed through the lignin peroxidase enzyme assay, decolourisation of methylene blue used as an indicator which confirms the isolates having the lignolytic ability [7].

Four fungal isolates CDF3, CDF4, CDF6, PDSF3 showed a dark yellow to brown coloration around the fungal colony, indicating that they were lignin degrading fungi in accordance to a tannic acid plate assay where tannic acid is used as sole carbon source. The results were similar with the findings of Sharma et al., [19]. Silva et al., [20] had reported that filamentous fungi particularly *Aspergillus niger* and *Penicillium* sp., which had lignolytic activity were capable of growing in tannic acid as the sole carbon source.

Similarly lignolytic microbes from cow dung samples were isolated by Sasikumar et al. [21], Umashankar et al. [7], from partially decomposed straw by Jagadeesh et al., 2022 and from forest soil by Banakar et al., [22][23-27].

4. CONCLUSION

The current study was carried out with the goal of isolation and screening of lignocellulolytic microbes from the three different sources, 60 distinct cultures were isolated, of which 21 isolates had the ability to degrade the cellulose and 15 isolates had ability to degrade the lignin. Among them 3 bacterial isolates CD-5, PDS-3 and PDS-6 had the ability of degrading both lignin and cellulose. It can be concluded that out of three sources, cow dung and partially decomposed straw are good sources for the isolation of diverse microbes having the ability to degrade cellulose and lignin. The isolates CD-5, PDS-3 and PDS-6, showed the cellulose and lignin degrading activities and further these can be potentiality used for the decomposition of rice straw.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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