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Cellulolytic Activity of *Bacillus* and *Pseudomonas* species isolated from Sugarcane Rhizoplane and its Correlation with Carbohydrate Utilization

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ABSTRACT: Cellulose is the most abundant and complex organic macromolecule on Earth. And Sugarcane is one of the important cash crop in India, leaves a residue of about 10-15 tons/hectare after harvest with abundant cellulose which necessitates finding simpler and most effective method for the decomposition of this vast abundant cellulose. In search for the best alternative for the degradation of the cellulose present investigation was aimed to isolate *Bacillus* and *Pseudomonas* species which have the best ability to degrade cellulose. Thus in the present study twenty four isolates of *Bacillus* and fourteen isolates of *Pseudomonas* from the rhizoplane of sugarcane by serial dilution method on *Bacillus* Agar Medium and Kings B Medium respectively and purified by following standard protocols. Cellulase activity of these isolates was determined by inoculating and incubating bacterial isolates on carboxy methyl cellulose agar medium at 37°C for 24 - 48 hours. Cellulase activity was assessed by measuring the clear zone formed around the point of inoculation after flooding the plates with Gram's iodine solution. The diameter of clear zone was proportional to cellulase production. Out of 24 isolates of *Bacillus* screened, RB6 (Rhizoplane *Bacillus* 6) showed highest cellulase activity (2.23cm) followed by RB10 (2.13cm), RB15 (2.1cm), RB22 (2.1cm) and RB4 (2.0 cm). Out of 14 isolates of *Pseudomonas,* RP9 (Rhizoplane *Pseudomonas* 9) showed highest cellulase activity (2.2cm), followed by RP5 (2.1cm), RP2 and RP12 (2.0 cm). The isolates, RB6 and RP9, were identified as *Bacillus amyloliquefaciens* and *Pseudomonas putida* based on 16S rRNA sequence homology. Those isolates showing promising cellulase activity were tested for carbohydrate utilization, where it was found that organisms lacking cellulase activity could breakdown other sugars but not cellulose since it is a complex polymer. RB6 isolate showing highest cellulase activity did not use most of the sugars tested whereas RP11 isolate showing no cellulase activity has hydrolysed most of the sugars. Therefore from the results *Bacillus amyloliquefaciens* (Strain RB6) and *Pseudomonas putida* (Strain RP9), can be effectively utilized for the biodegradation of Cellulose of Sugarcane. Nevertheless further research may be directed for decomposition of sugarcane trash at field level.

Keywords: Cellulase activity, *Bacillus, Psuedomonas,* cellulose, carbohydrate utilization.

INTRODUCTION

Sugarcane is the world's largest crop by production. India is the second largest sugarcane producer in the globe next to Brazil (Anon, 2020). Sugarcane, an important cash crop leaves a residue of 10-15 tons/ hectare after harvest. This huge agro waste rich in cellulose is subjected to combustion traditionally by our farming community leading to untoward environmental hazards resulting in the emission of particulate matter and smoke, resulting in poor air quality and a problem for public health (Tsao *et al*., 2011; Wood, 1991; Thorburn *et al*., 2012; de Oliveira *et al*., 2015).

Cellulose is a complex organic polysaccharide and the most abundant macromolecule on Earth. It is a linear chain of several 100's to 1000's of β-linked D-glucose units. Sugarcane waste is rich in cellulose and is one of the most complex molecules to degrade. Thus ecologically balanced alternative is highly needed to degrade this abundant complex molecule in an environmentally safe manner (Boopathy *et al*., 2001).

One of the best methods for the bio- degradation is usage of potential soil microbes (Alam *et al*., 2013). In nature many bacterial and fungal species of both aerobic and anaerobic nature have been reported with cellulolytic activities. *Chaetomium, Fusarium, Myrothecium, Trichoderma, Penicillium, Aspergillus* and so forth, are some of the reported fungal species responsible for cellulosic biomass hydrolysation (Milala *et al*., 2005). On the other hand cellulolytic bacterial species includes *Trichonympha*, *Clostridium*, Actinomycetes, *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Methanobrevibacter ruminantium* (Schwarz, 2001). Kim *et al*., (2012) identified certain strains of *Bacillus subtilis* (SL9-9, C5-16, and S52-2) which were found effective in cellulolytic enzyme activities. They confirmed these strains by morphological, physiological, and biochemical and 16S rRNA gene analysis as *Bacillus subtilis*. Similarly Goel *et al.* (2019) were attempted to isolate the cellulolytic microbes isolated from a waste dumping site. Of the 28 microbial isolates, five isolates produced cellulase on LB agar plates containing 1% CMC. The PCS-22 isolate, representing *Pseudomonas* sp. has the ability to produce cellulase found effective in degrading the cellulolytic material. Liu *et al*. (2021) have studied extensively the sugarcane rhizosphere to examine the different bacterial communities. In their study they found that Pseudomonas and Bacillus species very predominant in the rhizoplane of the sugarcane. With this literature support the present investigation was carried out with the following objectives: i) To determine the cellulolytic activity of *Bacillus* and *Pseudomonas* isolated from the rhizoplane of sugarcane and ii) To correlate the carbohydrate utilization with cellulolytic activity of *Bacillus* and *Pseudomonas* species.

MATERIALS AND METHODS

The present research was carried out by collecting different soil samples from the rhizoplane of the sugarcane crop in Visakhapatnam district. The details of the soil sample collection were mentioned in the Table 1. A total of 9 soil samples from different mandals of Visakhapatnam district were collected. These soil samples were subjected to serial dilution. The soil completely adhered to the root zone was considered for the isolation of the bacterial isolates. From the sugarcane rhizoplane approximately one gram of the soil sample was taken and it was subjected to serial dilution. Initially one gram of the soil was mixed in 10 ml of sterile distilled water thoroughly mixed using vortex shaker. One milliliter of this dissolved soil suspension was transferred in to 9ml of sterile distilled water in a test tube and thoroughly mixed using vortex shaker. Likewise, this step was continued six times till sixth level of dilution was achieved. From the sixth level diluted sample, 100µ1 suspension was transferred onto Hichrome bacillus agar and Psuedomonas agar base media, respectively.

The Hichrome bacillus media was supplemented with cetrinix supplement to promote *Bacillus* sp. growth. Similarly to obtain *Pseudomonas,* the Psuedomonas agar base is supplemented with *Psuedomonas* supplement 1 and 2 (Himedia). This *Cetrimide Agar medium* which is a selective medium for the species of *Pseudomonas* consists of a key component cetrimide which inhibits the growth of many bacteria including gram-positive bacteria and normal flora allowing the growth of only *Pseudomonas* species. To obtain uniform colonies in the Petri plate the 100µl suspension was uniformly spread with elbow spreader. The inoculated Petri plates were incubated at 37°C for 48 hrs. The colonies thus obtained were purified on nutrient agar and maintained for further studies. The colonies obtained on Hi chrome Bacillus agar and Pseudomonas agar base were designated as RB and RP isolates, respectively. To determine the cellulose activity these isolates were grown on carboxy methyl cellulose medium (CMC) with the following composition (Kasana *et al.,* 2008).

Chemical composition of CMC media.

Chemical	Quantity in grams for 1 Liter of water
Cellulase	10.0
K_2HPO_4	1.0
KH_2PO_4	1.0
$MgSO_4\ 7H_2O$	1.0
$NH4NO_3$	1.0
$FeCl_3\ 6H_2O$	0.05
$CaCl_2$	0.02
Agar Agar	20

Confirmation of cellulose degrading ability of bacterial isolates was done by transferring a bacterial disc (5 mm) on the CMC medium and allowed to grow for 48 hours at 37°C. After 48 hours of inoculation these Petri plates were flooded with 5 ml of tincture iodine. Colonies showing clear zones were taken as positive cellulose-degrading bacterial colonies (Lu *et al*., 2004), and only these were taken for further study. Each isolate was tested for cellulolytic activity in three replicates. Cellulose-degrading potential of the positive isolates was also qualitatively estimated by determining the carbohydrate utilization pattern (Hendricks *et al*., 1995). The carbohydrate utilization kits are used for the estimation of carbohydrate utilization pattern by these isolates.

Identification of promising cellulase producing isolates. The bacterial isolates were multiplied in Luria Bertani broth overnight and the DNA was extracted according to the protocol given by Sambrook and Russell (2001). Amplification of 16 S rRNA genes of *Bacillus* isolates was carried out by PCR using universal primers, FGPS6-63-GGAGAGTTAGATCTTGGCTCAG and FGPL 132-38-CCCGGTTTCCCCATTCGG (Normand *et al*., 1992). The thermocyclic conditions included initial denaturation at 95°C for 3 min followed by 35 amplification cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by final extension at 72°C for 3 min.

PCR for amplification of *Pseudomonas* isolates was performed in a volume of 25 µl containing 1 µl of DNA template, 2.5 µl of 10X PCR buffer, 1 µl of each dNTP, 2mM of $MgCl_2$, 1 µl of each primer and 0.5 U of Taq Polymerase. The 16S rDNA amplification was performed using the primers Psmn 289 (5'-GGTCTGAGAGGATGATCAGT-3') and Psmn 1258 (5'-TTAGCTCCACCTCGCGGC-3'). PCR was performed on Master Cycler Nexus Gradient (Eppendorf, USA). PCR programme was 5 min at 95°C; 30 cycles of 30s at 94°C, 30s at 53°C, and 1 min at 72°C; and a final extension for 10 min at 72°C (Kim *et al*., 2013).

The PCR products were analyzed in 1.5 % agarose gel in 1X Tris-acetate EDTA, run for 90 min at 100 V, and the amplified products were excised and outsourced (Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad) for partial sequencing. Similarity of 16 S rRNA gene sequence was aligned using BLAST Programme of GenBank database (NCBI).

RESULTS AND DISCUSSION

Plating of serially diluted rhizoplane soil samples on *Bacillus* and *Pseudomonas* selective media resulted in distinct colonies, 48 hours after incubation. The colonies with rough, opaque, fuzzy white or slightly yellow with jagged edges were noticed on Hichrome Bacillus Agar medium and presumed as *Bacillus* species (Bai *et al*., 2013; Ming, 2008). Similarly colonies on Pseudomonas agar base were nearly colourless, but off-white, cream, and yellow colony pigmentation, with fluorescent colonies could be readily visualized under ultraviolet light with circular shape of 1-3 mm size, convex elevation, smooth to mucoid surface with greenish blue colour with opaque structure which were designated as *Pseudomonas* sp (Lowbury and Collins 1955). Based on the colony features a total of 24 *Bacillus* and 14 *Pseudomonas* isolates were isolated from rhizoplane of sugarcane genotypes. The details were furnished in the Table 2. The isolates obtained on Hichrome bacillus media were designated as RB series and on the Pseudomonas Agar base media as RP series. All these isolates were further sub-cultured on nutrient Agar media for cellulolytic studies.

Table 1: Collection of different isolates of *Bacillus* and *Pseudomonas* from the rhizosphere of different soils of Visakhapatnam District.

Sr. No.	Place of Collection	Rhizosphere Sugarcane Variety	Isolates obtained
1.	Choochikonda	87A 298	RB-1, RB-2,R RP-1 RP-2
2.	Jutthada	87A 298	RB-3, RP-3, RP-4
3.	Chodavaram	2009A 107,87A 298	RB-4, RB-5, RB-6, RB-7, RB-10, RP-5,
4.	Munagapaka	87A 298	RB-8, RP-9, RB-4, RP-6
5.	G Madugula	87A 298	RB-9, RB-10,RB-11, RB-12, RP-7,RP-8
6.	Narsipatnam	87A 298	RB-11, RB-12, RB-13, RB-14, RB-15, RP-10
7.	Cheedikada	2009A 107	RB-16, RB-17, RB-18, RB-19, RP-11
8.	Kasimkota	87A 298	RB-20, RB-21, RP-12, RP-13
9.	Atchuthapuram	87A 298	RB-22, RB-23, RB-24, RP-14

After 36 hours of inoculation of these isolates on the CMC medium, the cultures were flooded with Iodine solution and incubated for half an hour. The iodine specifically reacts with the degraded cellulose and forms a clear circular zone around the bacterial disc in Petri plate for cellulase producing isolates which forms the basis for the identification of the effective strains in degradation of the cellulose. The clear zone was measured using zone scale. The cellulolytic activity of the isolated strains was depicted in Table 1.

Based on the results the bacterial isolates can be categorized in to three distinct groups based on their cellulolytic activity which were depicted in Table 3. The group having zone range between 1.00 to 1.50 which is an indication of lowest cellulolytic activity, the second group having zone range between 1.51 to 2.00 with medium range cellulolytic activity and the third group zone was having range above 2.01 and above with highest cellulolytic activity. Accordingly the isolates RB5, RB8, RB9, RB18, RB19, RB20 and RB23 of *Bacillus* sp and RP7, RP10, RP13 and RP14 of *Pseudomonas sp*. have the least cellulolytic activity, whereas RP1, RP8 and RP 11 doesn't have any cellulolytic activity as they were within the range of 1.00 to 1.50.

From the results it is evident that maximum number of isolates have produced a clear zone of 1.51 to 2.00 cm with moderate level of cellulase activity. RB1, RB2,

RB3, RB4, RB11, RB12, RB13, RB14, RB16, RB17, RB21 and RB24 of *Bacillus* sp and RP2, RP3, RP4, RP6 and RP12 of *Pseudomonas* sp were within in this category. RB6, RB7, RB10, RB15 and RB22 of *Bacillus* sp and RP5 and RP9 of *Pseudomonas* sp have shown higher cellulolytic activity with clear zone of above 2.01. Among *Bacillus* sp RB6 have shown highest cellulolytic activity with a value of 2.23 followed by RB10 (2.13). Similarly, within the *Pseudomonas* sp RP9 has shown highest cellulolytic activity. Similar results were obtained by Sonia *et al*. (2013), who isolated cellulase producing bacteria from soil and identified potent cellulase producers as *Pseudomonas fluorescens*, *Bacillus subtilis*, *E. coli* and *Serratia marcescens*. In their study they found that among bacteria, *Pseudomonas fluorescens* as the best cellulase producer among the four, followed by *Bacillus subtilis*, *E. coli* and *Serratia marscens*.

Though in the present investigation some of good cellulolytic activity bacterial isolates were identified, the identification was done purely based on the qualitative parameter. Eida *et al*. (2013); Maki *et al*. (2009) opined that qualitative methods are not sufficiently sensitive compared to quantitative tests due to the poor correlation between enzyme activity and the resulting hydrolysis zone diameter. Thus, to more accurately evaluate cellulolytic and hemicellulolytic microorganisms, an efficient plate-screening method is required.

Bacillus and *Pseudomonas* isolates showing promising cellulase activity *viz*., RB6, RB7, RB10, RB15, RB22 and RP5, RP9, were further tested for carbohydrate utilization in comparison to isolate with non-cellulolytic activity (RP11), and the results were depicted in the Table 4. From the Table 4 it is evident that isolate lacking cellulase activity, *i.e*., RP11 was able to breakdown almost all the sugars. On the other hand RB6 isolate showing highest cellulase activity did not use most of the sugars tested. Interestingly all the isolates have utilized Citrate and Esculin. The isolates RB7, RB15 and RP9 utilized maltose, fructose, dextrose, trehalose and sucrose. Though some of the isolates have good cellulolytic ability, some have utilized other carbohydrates as well. This perhaps needs further investigation into the factors that determine the preference of various isolates towards utilization of diverse sugars. Similar examination was conducted on *Arthrobacter* strains from industrial polluted soil by Lokesha *et al*. (2019). They found that the sugar utilization pattern revealed that the isolated strains were able to grow in a vast variety of sugar/carbohydrate source and this ability of *Arthrobacter* genus make them widely distributed and abundant in soils of harsh environmental conditions.

Table 2: *In vitro* cellulolytic activity of different isolates of *Bacillus* and *Pseudomonas* obtained from sugarcane rhizoplane.

***Bacillus* isolates**				***Pseudomonas* isolates**	
Isolate	**Zone diameter (cm)**	**Isolate**	**Zone diameter (cm)**	**Isolate**	**Zone diameter (cm)**
RB1	1.93	RB15	2.10	RP1	0.00
RB2	1.97	RB16	1.91	RP2	2.00
RB3	1.67	RB17	1.63	RP3	1.91
RB4	2.00	RB18	1.13	RP4	1.93
RB5	1.13	RB19	1.37	RP5	2.12
RB6	2.23	RB20	1.25	RP6	1.97
RB7	2.10	RB21	1.80	RP7	1.00
RB8	1.40	RB22	2.10	RP8	0.00
RB9	1.07	RB23	1.07	RP9	2.21
RB10	2.13	RB24	1.63	RP10	1.15
RB11	1.92			RP11	0.00
RB12	1.92			RP12	2.00
RB13	1.50			RP13	1.25
RB14	1.50			RP14	1.43

Table 3: Cellulolytic activity range of different bacterial isolates.

Bacterial isolates	**1.00-1.50**	**1.51-2.00**	**2.01 and above**
RB Series	RB5, RB8 , RB9, RB18, RB19, RB20 and RB23	RB1, RB2, RB3, RB4, RB11, RB12, RB13, RB14, RB16, RB17, RB21 and RB24	RB6, RB7, RB10, RB15 and RB22
RP Series	RP1, RP7, RP8, RP10, RP11, RP13 and RP14,	RP2, RP3, RP4, RP6, and RP12	RP5 and RP9

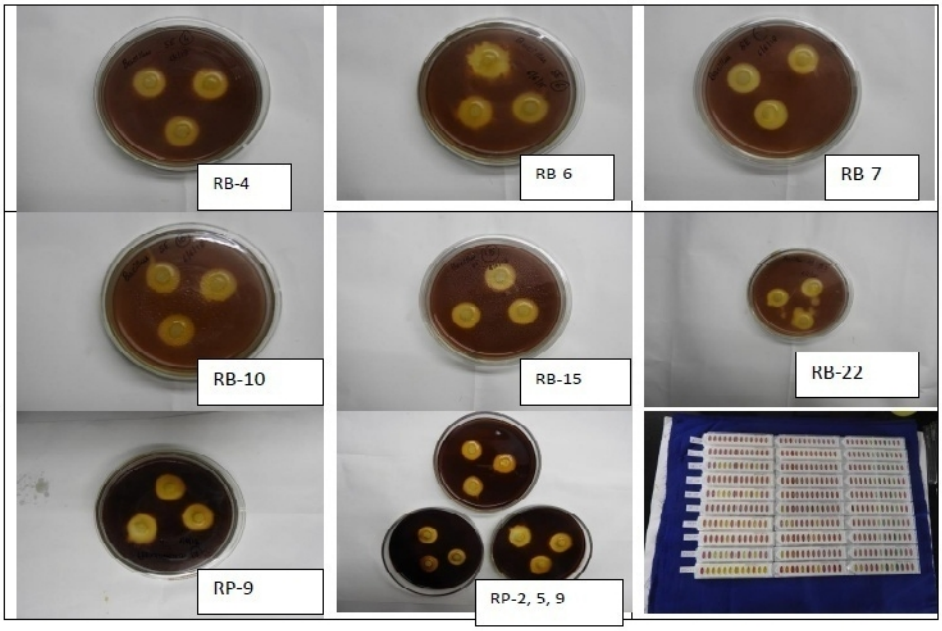

Fig. 1. Cellulolytic activity and carbohydrate utilization pattern of different isolates of sugarcane rhizoplane bacteria.

Table 4: Carbohydrate Utilization Pattern of efficient cellulose degrading isolates.

Sr. No.	Sugars	RB-4	RB-6	RB-7	RB -10	RB -15	RB-22	RP-2	RP-5	RP -9	RP-11
1.	Lactose										
2.	Xylose										+
3.	Maltose			+		+				+	+
4.	Fructose			+		+				+/-	+
5.	Dextrose			+		+		+		+	+
6.	Galactose										+
7.	Raffinose										+
8.	Trehalose			+		+				+	+
9.	Mellibiose										+
10.	Sucrose			+		+				+	+/-
11.	L-Arabinose										+
12.	Mannaose										+
13.	Inulin						+			+/-	
14.	Sodium Gluconate										+/-
15.	Glycerol										+/-
16.	Salicin										+/-
17.	Dulcitol										+/-
18.	Inositol										+/-
19.	Sorbitol										+
20.	Mannitol										+
21.	Adonitol										
22.	Arabitol										
23.	Erythritol										
24.	α-methyl D- Glucoside										
25.	Rhamnose										+/-
26.	Cellobiose										+
27.	Melezitose										
28.	α-methyl D- Mannoside										
29.	Xylitol										
30.	ONPG	+					+				+
31.	Esculin	+	+	+	+	+	+	+	+	+	+
32.	D-Arabinose										+
33.	Citrate	+	+	+	+	+	+	+	+	+	+
34.	Malonate										+

+ Positive; -Negative; +/- variable reaction

Characterization of promising cellulase producing isolates. The bacterial isolate obtained on Hichrome Bacillus agar was *viz*., RB6 identified as bacteria of the genera, *Bacillus* by amplification of 16S rDNA genes using universal primers, FGPS6-63 and FGPL 132-38 (Normand *et al*., 1992). Comparison of 16S rDNA amplified genes to sequences of Genbank has shown identity to *B. amyloliquefaciens.* A similar study was conducted by the Singh *et al*., (2013). During isolation and characterization of different bacteria from the rhinoceros dung, Out of 36 isolates, isolate no. 35 exhibited maximum enzyme activity of 0.079 U/mL and was selected for further identification by using conventional biochemical tests and phylogenetic analyses. This was a Gram-positive, spore forming bacterium with rod-shaped cells. The isolate was identified as *Bacillus amyloliquefaciens* SS35 based on nucleotide homology.

The bacterial isolates from Pseudomonas agar was identified as bacteria of the genus, *Pseudomonas* by amplifying the DNA with *Pseudomonas* specific primers, Psmn 289 and Psmn 1258. The electrophoresis of PCR amplified products had produced amplification confirming the bacteria as *Pseudomonas* species. The elite *Pseudomonas* species *viz*., RP9 was further identified to species level by partial sequencing of amplified products. The NCBI blast analysis revealed the identity of the elite *Pseudomonas* species as *P. putida* (KX758437). Tozakidis *et al.*, (2016) while studying the effectiveness of cellulose activity of different *Pseudomonas sp* along with other bacteria they could establish *Pseudomonas putida* as host for the surface display of cellulases, and provided proof-of-concept for a fast and simple cellulose breakdown process at elevated temperatures.

CONCLUSION

RB6 isolate among *Bacillus* and RP9 among *Pseudomonas* were found to be elite cellulose degraders and their efficacy needs to be tested under field conditions for degradation of sugarcane trash and other agricultural wastes. During the study, it was observed that RP11 was utilizing most of the sugars but lacks cellulolytic activity and some of the isolates with cellulolytic activity could also utilize other carbon sources. Hence, a perfect correlation couldn't be established between carbohydrate utilization pattern and cellulolytic activity.

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Conflict of Interest. None.

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