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Molecular characterization of cellulolytic nitrogen-fixing bacterial species isolated from Khyber Pakhtunkhwa

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Key words: Nitrogen free Carboxymethyl cellulose medium (NF-CMC), Nitrogenase gene, 16sRNA sequencing, Cellulase, Nitrogenase activities.

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Abstract

Bacteria inhabiting the soil rhizosphere had been recognized for their properties of promoting plant health. The current research was undertaken for the molecular characterization of cellulolytic nitrogen-fixing bacterial species from the rhizosphere. Two hundred and fifty soil samples were collected from Wheat and sugarcane rhizosphere in few districts of KPK and were grown on Nitrogen free Carboxymethyl cellulose medium for cellulose and nitrogenase enzyme production. Bacterial isolates from Wheat in Congo red assay showed clearance zone ratio on CMC substrate in the range of 1.6 - 3.0mm, while bacterial isolates from Sugarcane samples showed clearance zone ratio in the range of 1.3 - 2.71mm. Quantitative analysis revealed that wheat bacterial isolates were releasing cellulase in the range of 0.751– 4.784 U/mL on NF-CMC medium (with 5% CMC+pH 7) while they produced NH_4-N in the range of 0.104 – 1.115 mM in NF medium (pH 7). Bacteria from Sugarcane samples showed cellulase activities in the range of 0.418 – 4.155 U/mL and produced NH_4-N in the range of 0.102 – 1.070 mM under the same conditions. The presence of nitrogenase gene only in fourteen bacterial isolates was confirmed and identified by 16sRNA sequencing as bacterial strains *Achromobacter* sp. MFA1, *Paenibacillus pabuli* HSSC 492, *Streptomyces* sp. strain VITBVK2, *Stenotrophomonas* sp. Vi65, *Uncultured Actinomyces* sp. clone O-71, *Beijerinckia fluminensis, Alcaligenes faecalis* CCM5B, *Stenotrophomonas maltophilia* strain CM30, *Brevibacillus parabrevis* strain TJ2.3, *Ochrobactrum intermedium* strain BE1, *Brevibacillus parabrevis* strain TJ2.3, *Cupriavidus sp. christensen*.ICD.09 and *Bacillus cereus*.

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Introduction

Pakistan's economy depends largely on Agriculture because it not only provides income but also provide raw materials to the industrial sector. In the past few decades, fluctuations in growth in the agriculture sector however had a great impact on the economy (Hassan *et al*., 2016). The green revolution has now been started because of the decline in crop yields due to imbalanced nutrient management and decreased soil organic matter. Thus the development of sustainable agriculture and environment-friendly agricultural practices becomes a prerequisite in this regard (Aatif A *et al*., 2011). The utilization of beneficial microorganisms of soil is a safe way for sustainable crop production. Such types of microorganisms generally comprise rhizobacteria, mycorrhiza and nitrogen-fixing cyanobacteria (Boddey *et al*., 1995) Extensive and wide-scale research is going on plants associated with microorganisms for new strains of bacteria to meet the demands of the current day (Pereira *et al*., 2011).

Nitrogen fixers are known to be essential in plant fertility. Numerous bacterial genera had been reported so far. Although *Azotobacter* and *Rhizobium* are well-reported species there are many other unknown species of nitrogen fixers e.g. *Stenotrophomonas* strain. Other species include *Azotobacter, Azospirrilum, Rhizobium, stenotrophomonas, Paenibacillus, Achromobacter, Bradyrhizobium, cyanobacteria,* etc. (Reinhardt *et.al.* 2008). A functional nitrogenase enzyme is required for the fixation of elemental nitrogen. For this purpose, *nif* genes (Nitrogenase reductase) must be present in the DNA of a cell (Cocking EC., 2003). Researchers have been failed to design a universal primer for *nif*H gene because of the sequence variation among species of different phylogenetic groups (Franche *et al*., 2009).

Cellulose is the natural, abundant bio-resource and is an environmentally feasible renewable source of energy. It is degraded by cellulase enzymes converting cellulose into simple sugars. For this purpose isolation of cellulolytic microorganisms and production of such enzymes have been started for a long (Callow N *et al*., 2016). Nitrogen-fixing microorganisms have also been studied regarding cellulolytic potential. They included *Azotobacter, Azospirillum, Pseudomonas, Paenibacillus, Stenotrophomonas,* and *Achromobacter,* etc. *Paenibacillus* is the genus of nitrogen fixers which is also been reported widely in scientific research. *Paenibacillus terrae* ME27-1 isolated from the China region has been studied for the cellulolytic potential of 2.08 U/mL at 50 °C with an optimum pH of 5.5 (Yan-Ling L *et al*., 2014).

Municipal solid waste generation and accumulation on large scale are becoming a menace for all the developing countries (Al-Khatib IA *et al*., 2015). Municipal solid wastes containing cellulosic wastes and their inappropriate treatment procedures are also the failure for the developing countries to overcome the problems associated with it (Pin-Jing H., 2012). For the disposal of the huge amount of generated wastes, proper management is necessary as our natural habitats are continuously contaminating causing multiple environmental issues. Therefore, cellulosic wastes are required to recycle through effective techniques (Kazaragis A., 2005).

These wastes are also thought to be one of the renewable sources of energy. PGPR microorganisms can degrade cellulose thus this potential can be utilized for the development of biofertilizers in industries (Zhou H *et al*., 2015).

The study comprised of molecular characterization of Cellulolytic Nitrogen fixing bacteria isolated from soil. These bacterial isolates were qualitative and quantitative analyzed for cellulase and nitrogenase production.

Materials and methods

Sample size and location

Wheat and sugarcane rhizosphere were selected as samples for the study. A total of 250 rhizosphere soil samples equally from each plant were collected from different districts of Khyber Pakhtunkhwa.

Sample collection

Soil rhizospheric samples were assembled over a few months in 2016 (March to May). For each sample, the soil was first ploughed and the soil associated with roots of the plants (≤15 cm deep) was taken in a clean polythene bag. The collected soil for each sample was mixed thoroughly and moved to sterile zip lock bags and sealed. They were labeled properly with sample number, date, location, districts and sample plant and were preserved at 4°C for further processing. The same procedure was followed for all the soil samples. Each sample was taken in triplicate.

Isolation of cellulolytic nitrogen-fixing bacterial species

Isolation of cellulolytic Nitrogen-fixing bacterial species was achieved by enrichment method on NF-CMC medium. It was made ($MgSO_4.7H_2O$, $MnSO_4.H_2O$, $FeSO_4.7H_2O$ and $CaCl_2.2H_2O$) and its pH was adjusted to 10.5 by 5 % NaOH. A hundred microliters of the medium were transferred to separate flasks and were sterilized in an autoclave at 121°C for 15 min. Each soil sample (1g) was added to the individual medium flask and was retained at 28°C for 7 days of incubation. An inoculum of 1ml was spread onto already prepared sterile NF-CMC agar plates (in triplicates) and was kept for 7 days at 28 °C for incubation.

Screening of cellulolytic nitrogen-fixing bacteria

NF-CMC plates after incubation were subjected to Congo red assay for screening of cellulolytic nitrogen-fixing bacteria. Plates were flooded with 0.2 % Congo red solution for 20 min and then poured off. A sterile solution of NaCl (1M) was poured over the plates and kept for 20 min and then discarded. Clear zones around the individual colonies were observed and marked for further purification (Ponnambalam AS *et al.*, 2011).

Purification and storage of bacterial isolates

Bacterial isolates were subcultures on NF-CMC plates several times to get purified colonies. Gram staining was carried out for the selection of single bacterial species in a selected colony. Storage of bacterial isolates was done in nutrient broth with 20 % glycerine at 4 °C.

Qualitative detection of cellulase and nitrogenase producing activity of bacterial isolates

Qualitative analysis for cellulase activity

Cellulase production was qualitatively determined by using Congo red assay. CMC plates were prepared and inoculated with the purified bacterial isolates. As compared to fungi, bacteria are not effective producers of the cellulase enzyme, therefore clear zones are difficult to observe. For efficient isolation, the inoculum was applied in the form of spots in concentric circles, labeled and incubated at 28°C for 7 days (Ponnambalam AS *et al.*, 2011). Plates were flooded with 0.2% Congo red solution for 20 min. The excess Congo red solution was poured off and the plates were further flooded with 1M NaCl solution for 20 min. Clear zones around the individual colonies were observed. Zones and colony diameter were measured in millimeters and the ratio was determined (Table 2). *Bacillus subtilus* was used as positive while *E.coli* as the negative control.

Qualitative analysis for nitrogenase activity

Nitrogen-fixing ability of bacterial isolates was checked by growing them on Glucose Nitrogen free Mineral Medium (GNFMM) supplemented with Bromothymol Blue (BTB) dye. Each bacterial isolate was inoculated on GNFMM plates in the center followed by incubation at 28 °C for 7 days. Colour change of medium due to bacterial colonies were observed (Table 2) (Zaw K L *et al.*, 2013). *Azotobacter Vinelandii* was used as positive and *E.coli* as the negative control.

Qualitative detection of Cellulase and Nitrogenase producing activity of bacterial isolates

Quantitative analysis of bacterial isolates for Cellulase activity

Carboxymethyl cellulase activity was determined by DNS method of Miller (1959) (Miller. G.L., 1959). The reaction mixture contained 0.5 mL of 2 % CMC substrate; 0.5 mL of the crude enzyme (supernatant). Citrate buffer (0.5mL) was added to it and incubated

for 30 min in the water bath at 50°C. DNS solution (3mL) was added and incubated for 5 min in a boiling water bath for colour development and cooled rapidly. Glucose standard solutions were also prepared (0.1, 0.2, 0.3, 0.5, 0.6, 1 mg/0.5mL). The activity of sample and glucose standard tubes were measured against a reagent blank at 540 nm in a UV spectrophotometer as described by Sasidharan Sreedevi *et al.*, 2013.

Quantitative analysis for Nitrogenase activity

Quantitative analysis of bacterial isolates has been done by using Nesslerization spectrophotometric method (Emtiazi G *et al.*, 2007). The supernatant was taken from freshly grown cultures in 100 mL NFGM broth, centrifuged at 8000 g. Ammonium standard solutions were formulated by using the stock solution of Ammonium (Table 1). The reaction mixture contained 10 mL of test samples, 0.2 mL KNa Tartarate and 0.2 mL of Nessler's reagent. Reading on spectrophotometer was noted at 425 nm for each sample, reagent blank and ammonium standards after 5 minutes. Test sample readings were matched with the standard samples for the measurement of protein concentration in a solution. Ammonium nitrogen concentration (mg/L) in supernatant for all of the bacterial cultures have been determined and millimolar concentrations of ammonium nitrogen were calculated according to the formula. *Azotobacter Vinelandii* was used as positive and *E.coli* as a negative control.

Marker genes detection on bacterial isolates

Fixation of nitrogen is a very complicated process carried out by a sophisticated nitrogenase enzyme system present in bacterial cells. Among bacteria and Archae, this system is widely distributed with highly conserved protein sequences across kingdoms. Just for confirmation of nitrogenase and cellulase system in bacterial isolates, commonly used primer set for *nif*H (F= 5'- TTCCATCAGCAGCTCTTCGA-3', R= GGCAAAGGTGGTATCGGTAA-3') and *Cel*12 (F=5'-TCCATTCTGCTATGGGGTGC-3', R=5'-CGGACCGTTACGTCCCAAT-3') were applied on all the fourteen (n=14) selected isolates under the optimized conditions. The reaction conditions were set accordingly as 95 °C for 3 min, 57 °C for 30 sec and 72°C for 45 sec. Finally, the extension was done at 72°C for 7 min. 30 cycles were set on PTC-06 ICCC Thermocycler. Amplified gene product obtained was visualised on Agarose Gel electrophoresis system.

The amplified gene product appeared as bright bands under the Gel documentation system confirmed the presence of the gene in the sample strain (Mohammad D *et al.*, 2013).

Identification of bacterial isolates

Biochemical identification of bacterial isolates

Bacterial identification was carried out by Bergey's Manual of Determinative Bacteriology (Krieg NR *et al.*, 1984). These tests include Gram staining, oxidase, catalase, gelatin hydrolysis, starch hydrolysis, citrate, motility, indole tests.

Molecular identification of bacterial isolates

Among biochemically identified bacterial isolates only a few species were selected for molecular identification. For this purpose DNA was isolated by using the proper kit protocol for DNA isolation (Thermo fisher scientific). They were quantified and then sent to Macrogen Korea for 16sRNA amplification. The amplified product of sequencing was compared with the sequence obtained from the Nucleotide database of the National Centre for Biotechnology Information (NCBI) (Lubanza N *et al.*, 2013).

Results and discussion

Cellulolytic Nitrogen-fixing bacterial isolates obtained from soil samples

Two hundred and seventy-four bacterial colonies showed growth upon repeated subculturing. Among them, only one hundred and twelve bacterial isolates had shown growth on Carboxymethyl cellulose medium and GNFM medium. An equal number of bacterial isolates (n=15) from each plant sample was selected for enzyme production. These isolates were phenotypically CMC degraders on CMC medium and nitrogen fixers on nitrogen-free medium.

Table 1. Concentration of Ammonium standard solutions.

Tube	Molarity of NH_4Cl solution mol/L	Amount of stock solution (mL)	Amount of DDI water (mL)	Final volume of the solution (mL)
S1	0.012	10	60	70
S2	0.022	10	22.72	32.75
S3	0.032	10	12.5	22.50
S4	0.042	10	7.14	17.14
S5	0.052	10	3.84	13.84
S6	0.062	10	1.61	11.61
S7	0.072	10	0	10

Cellulase producing activity of bacterial isolates (Qualitative assay)

Zones of clearance of fifteen bacterial isolates (having dual properties) have been estimated by finding the ratio of colony diameter and zone size as given by Lester Hankin *et al* in 1977. Clearance zone ratio (ZR) of 1.3 and above were considered as positive isolates for activity as was done by Lester. Bacterial isolates from Wheat samples showed ZR on CMC substrate in the range of 1.6-3.0 (Fig. 1 a) while bacterial isolates from Sugarcane samples showed ZR in the range of 1.3-2.71. Among Wheat isolates, Minimum ZR was shown by WS18 and maximum by WS11. In Sugarcane isolates, minimum ZR was shown by SCC6 and maximum by SCS12 (Fig. 1 b). CMC was considered as the best substrate for the effective and quick isolation of cellulose-degrading microorganisms as was compared with the less hydrolyzing substrates such as cotton fiber or paper (Lester H *et al.*, 1997).

Table 2. Qualitative analysis of cellulolytic and Nitrogen Fixing activity of selected bacterial isolates (n=15).

S. No	Wheat samples			Sugarcane samples		
	Isolate no	CMC Zone ratio (mm)	Nitrogen fixation	Isolate no	CMC Zone ratio (mm)	Nitrogen fixation
1	WP2	1.71	+	SCP3	1.75	+
2	WP8	2.4	+	SCP8	1.71	+
3	WP13	1.85	+	SCS1	1.4	+
4	WS3	2.0	+	SCS5	1.3	+
5	WS11	3.0	+	SCS6	2.25	+
6	WS14	2.4	+	SCS12	2.71	+
7	WS18	1.6	+	SCS19	2.5	+
8	WS20	2.5	+	SCS20	2.0	+
9	WM9	2.6	+	SCM2	1.4	+
10	WM13	2.0	+	SCM4	1.6	+
11	WM21	2.0	+	SCM7	2.5	+
12	WM24	2.2	+	SCC6	1.3	+
13	WC4	1.8	+	SCC16	2.6	+
14	WN10	1.85	+	SCN7	1.85	+
15	WN15	2.71	+	SCN15	1.75	+
Controls	*B. subtilis*	2.71	+	*E.coli*	-	-

Scale: W=Wheat, SC=Sugarcane, P=Peshawar, S=Swabi, M=Mardan, C=Charsada, N=Nowshera, Blue color = +ive, Green color= -ive.

Nitrogenase producing activity of bacterial isolates (Qualitative analysis)

All the thirty bacterial isolates changed the color of the medium from greenish to blue (Fig. 2 a, b). This indicated the production of ammonium ions, an indication of nitrogen-fixing ability because the medium lacks nitrogen as described by Zaw K L, 2013.

Cellulase producing activity of bacterial isolates (Quantitative analysis)

Bacteria from Wheat samples showed activities of

cellulase of 0.751 – 4.784 U/mL on 0.5 % CMC at pH 7 on NF-CMC medium (Fig. 3). Minimum cellulase activity of 0.751 U/mL in sample WS18 while the maximum of 4.784 U/mL cellulase activity was seen in WS11. Bacteria from Sugarcane samples showed cellulase activities of 0.418 – 4.155 U/mL on 0.5 % CMC at pH 7 on NF-CMC medium (Fig. 4). Minimum cellulase activities of 0.418 U/mL in sample SCS5 while maximum cellulase activities of 4.155 U/mL were noted in SCS12.

Table 3. Biochemical properties of selected bacterial isolates.

S.No	Isolate	GS	Cat	Oxi	Cit	NO_3	Gelatin	Starch	Indole	MR	VP	Mot	Suggested Bacterial Genus
1	WP8	G-ive	+	+	+	+	+	-	-	+	-	+	*Achromobacter*
2	WS11	G+ive	+	+	+	+	+	+	-	-	+	+	*Bacillus*
3	WS14	G+ive	+	+	+	+	-	-	-	+	-	-	*Streptomyces*
4	WS20	G-ive	+	+	+	+	+	-	-	-	-	+	*Stenotrophomonas*
5	WM9	G+ive	+	+	+	+	+	+	-	-	+	+	*Bacillus*
6	WM13	G+ive	+	+	+	+	+	+	+	+	+	+	Actinomycetes
7	WM21	G+ive	+	+	+	+	+	+	+	+	+	+	*Rhizobium*
8	WM24	G-ive	+	+	+	-	-	+	+	+	-	+	*Alcaligenes*
9	WN15	G-ive	+	+	+	+	+	-	-	-	-	-	*Stenotrophomonas*
10	SCS6	G+ive	+	-	+	+	+	+	-	-	+	-	*Bacillus*
11	SCS12	G-ive	+	+	+	+	+	+	-	-	-	+	*Brucella*
12	SCS19	G+ive	+	-	+	+	+	+	-	-	+	-	*Bacillus*
13	SCM7	G-ive	+	+	+	+	+	+	-	+	-	+	*Bacillus*
14	SC16	G+ive	+	+	+	+	+	+	-	-	+	+	*Bacillus*

Scale: W=Wheat, SC=Sugarcane, P=Peshawar, S= Swabi, M= Mardan, C= Charsada, N= Nowshera, GS= Gram staining, Cat= catalase, Oxi= oxidase, NO_2=Nitrate reduction, MR= Methyl red, VP= Vogues prosker, Mot= Motility.

Our isolated bacteria were phenotypically nitrogen fixers and cellulose degraders. Studies have shown that nitrogen-fixing bacteria can be grown on cellulose-containing medium as demonstrated by G. Emtiazi in 2007 who have isolated Nitrogen-fixing *Paenibacillus* strain E from the soil by using NF-CMC medium. The strain in this study showed 4 IU/ml of cellulase activity. It can be demonstrated that many plant-associated bacterial strains might have cellulase-producing activities for the better establishment of plant-microbe interaction (Emtiazi G *et al.*, 2007).

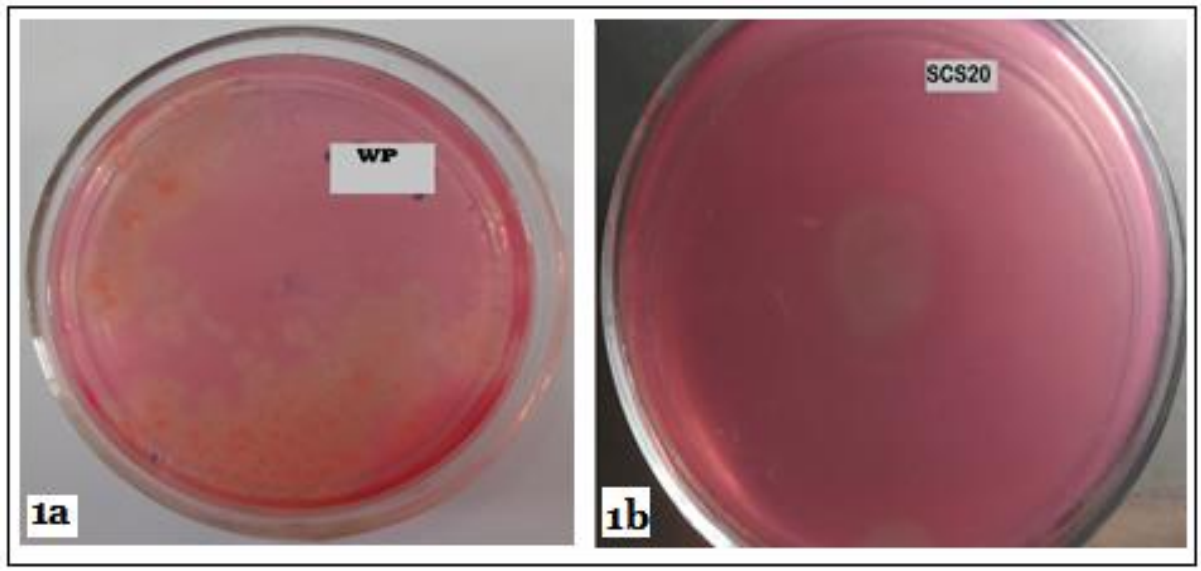

Fig. 1. a. Clear zone formation in Cellulase production by bacterial isolates from Wheat, b. Clear zone formation by bacterial isolate number SCS20.

Cellulase activities of bacterial isolates in our study are comparatively lower than the bacteria studied by Farjana *et al.* in 2018. They have isolated novel *Paenibacillus* sp isolated from molasses which showed 0.9 IU/mL (90 U/mL) of cellulase activities at pH 7 at 40 °C after 24 hrs of incubation on 1 % CMC as carbon source. When compared with Sasidharan Sreedevi *et al* 2013 who had reported maximum cellulase activities of *Achromobacter Xylosoxidans* BSS4 (from wood yards) as 68.37 U/mL at 6 hrs of incubation at pH 7 at 40 °C on 0.5 % CMC medium, our results of cellulase activities are low. Lower activities may be because the medium used in our study is a complex medium having CMC as a carbon source while lacking nitrogen source.

On the other hand, alkaline pH might be the reason behind the low activities of our isolates. Incubation temperature, Substrate concentration also may be the reason behind reduced activities however we can demonstrate that CMC is the effective medium for the quantification of cellulases. Optimization of bacterial cultural conditions however is suggested to be used (Farjana I *et al.*, 2018).

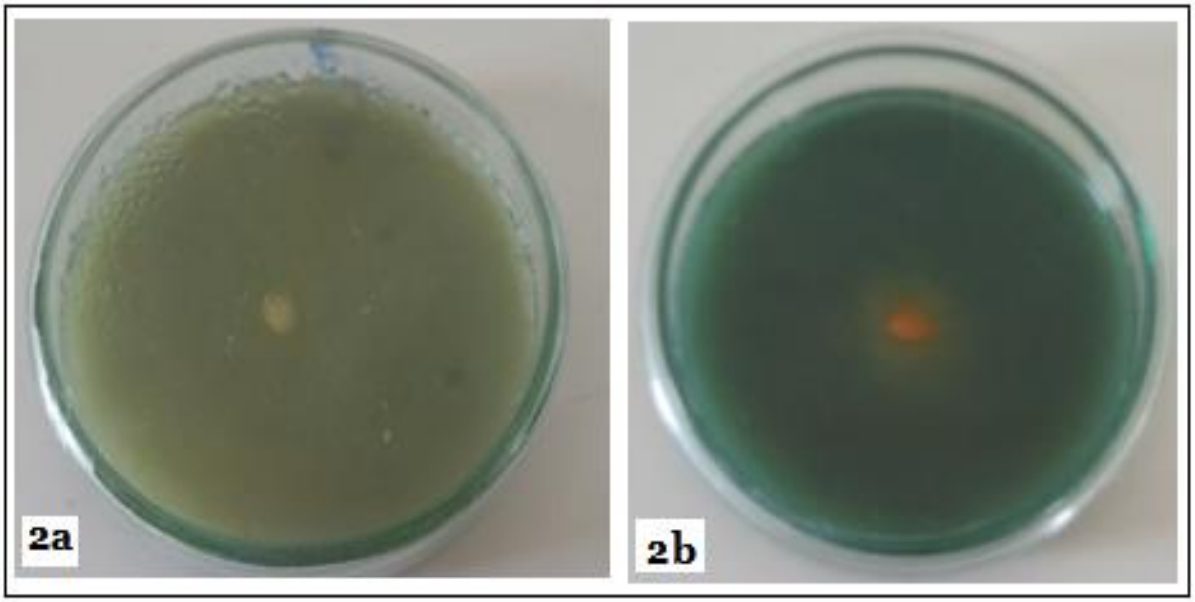

Fig. 2. a. Green media coloration before incubation, b. Bluish-green media coloration after incubation.

MBala Kumaran *et al.* in 2015 studied the cellulase activities of *Bacillus licheniformis* MTCC 429 which revealed maximum cellulase activities of 0.34 U/mL at pH 7.0 at 35°C after 48 hours on CMC medium. Upon comparison, all of our bacterial isolates have shown higher and different cellulase activities on CMC medium. This indicated as the diversity among cellulases is present among bacterial species, proper condition optimization is however required (Bala Kumaran MD *et al.*, 2015).

In different *Bacillus* sp, a variation on cellulase activities had been found out at optimum growth conditions. In another study by Sreeja *et al.* 2013, *Bacillus licheniformis* APS2 MSU isolated from estuarine fish *E.suratensis* gut showed maximum cellulase activity of 2.71 U/mL on CMC medium than other carbon sources used. While Shanmugapriya K, Saravana PS 2012 demonstrated the highest cellulase activity of *Bacillus* spp. at 1 % CMC at pH 6 at 40 °C as compared to sawdust and coir wastes. Our results are in agreement with them as CMC substrate gave us different activities. This may be because cellulose is considered as an inducer for cellulase production as reported by Paul and Verma in 1993 (Shanmugapriya K *et al.*, 2012).

It has also be seen that all the isolates were showing better zone diameter on solid CMC medium but their CMCase activities in liquid media were comparatively low, suggesting that the ability of CMCase secretion on the liquid medium is weak. It was described by

Yan Ling Liang at al 2014 that CMCase activities of *Paenibacillus terrae* in liquid medium were low as compared to zone diameter on solid medium, thus our results are analogous to the study (Yan-Ling L *et al.*, 2014).

Nitrogenase producing activity of bacteria isolates (Quantitative analysis)

Bacteria from Wheat samples produced 0.104 – 1.115 mM NH_4-N in NF medium at pH 7 and 28°C. Minimum NH_4-N of 0.104 mM was observed in sample WC4 while maximum NH_4-N of 1.115 mM was observed in WS11.

Bacteria from Sugarcane samples produced 0.102 – 1.070 mM NH_4-N in GNFM medium at pH 7 and 28 °C for 7 days. Minimum NH_4-N of 0.102 mM was observed in sample SCP3 while maximum NH_4-N of 1.070 mM was observed in sample SC16. Upon comparison, our results are analogous with a study where a novel cellulolytic nitrogen-fixing *Paenibacillus* strains E, H and SH were screened and also observed the ammonium production of 0.1 mM in GNFM medium at pH 7 after 7 days incubation. However, they also have found that *Paenibacillus* strains E, H and SH produced 1.3 mM ammonium on mannitol containing nitrogen-free medium (MNFM) while had produced 3.16 mM ammonium on MNFM medium. This indicates clearly that ammonium production by nitrogen-fixing bacteria varies upon using various carbon sources in the medium at the same optimum conditions (Emtiazi G., 2007).

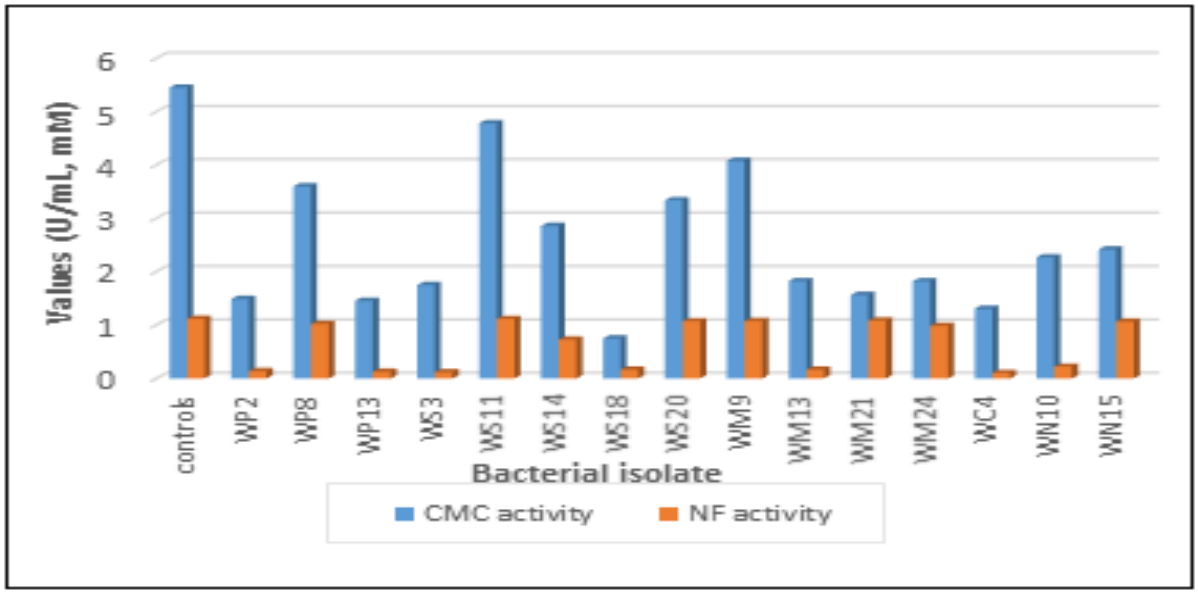

Fig. 3. Cellulase (Units/ml) and Ammonium nitrogen production (mM) of bacterial isolates from Wheat samples.

The range of our results is also analogous to another study by San San Yu *et al.* 2017 who isolated nitrogen-fixing bacterial strains *Azotobacter Beijirinkia*, *Azotobacter vinellandii* and *Stenotrophomonas maltophila* form agricultural soil samples. They had investigated the influence of different carbon sources on ammonium production. They have found out that *Azotobacter Beijirinkia* accumulated 0.48 mM ammonia after 96 hrs incubation in GNFM medium while *Stenotrophomonas maltophila* produced 0.52 mM ammonia on the same medium. *Azotobacter vinellandii* however produced 0.36 mM ammonia after 96 hrs incubation in fructose nitrogen-free medium. Variation in the values among samples is the indication that all the isolates might be different bacterial species with different nitrogen-fixing potential (San S Y *et al.*, 2017).

Enzyme gene detection in bacterial isolates

As all the bacterial isolates were phenotypically showing Nitrogen-fixing and CMC degradation

activity, only fourteen out of thirty isolates showed positive results when *nif*H primer was applied. DNA ladder of 1 Kb was employed in this study. The desired band length (786 bp) confirmed the presence of a nitrogenase system in the isolated bacteria (Fig. 5). *Azotobacter* was used as a positive control.

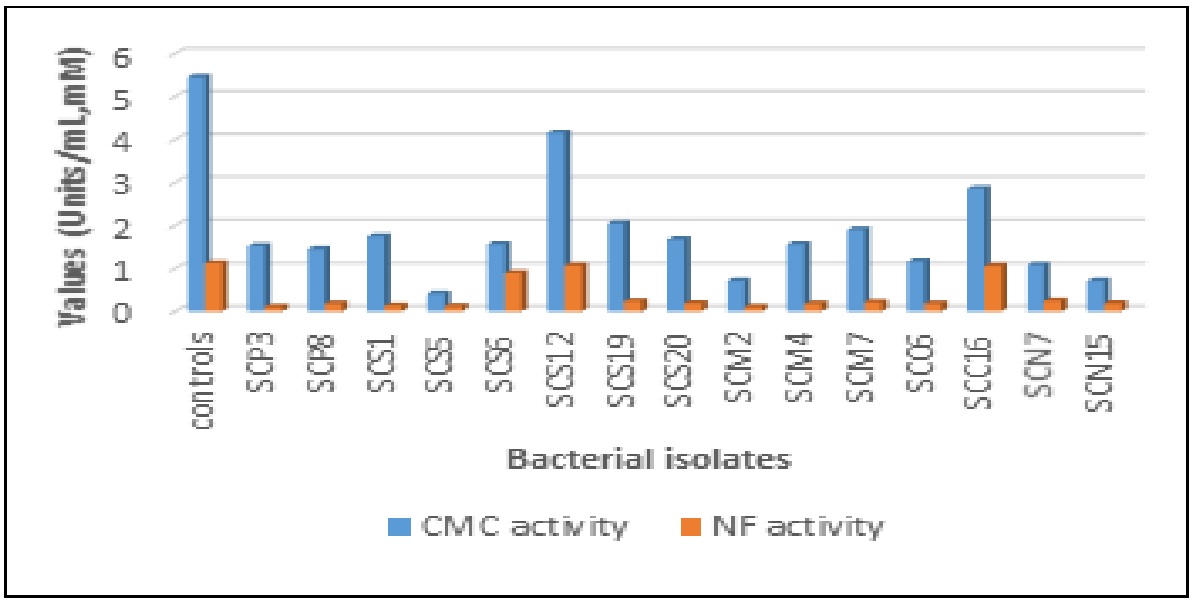

Fig. 4. Cellulase (Units/ml) and ammonium nitrogen production (mM) of bacterial isolates from sugarcane samples.

As *nif*H is a common primer used for detection of dinitrogenase reductase in bacterial DNA, however variation still exists among bacterial species due to which multiple primer pairs can be designed and used for the detection and amplification of nitrogen-fixing genes in them (Jonathan P Z *et al.*, 2013). Based on positive results, isolates were processed for biochemical and molecular identification. For the detection of endoglucanases in the bacterial isolates, a commonly used primer set of *cel*12 was applied.

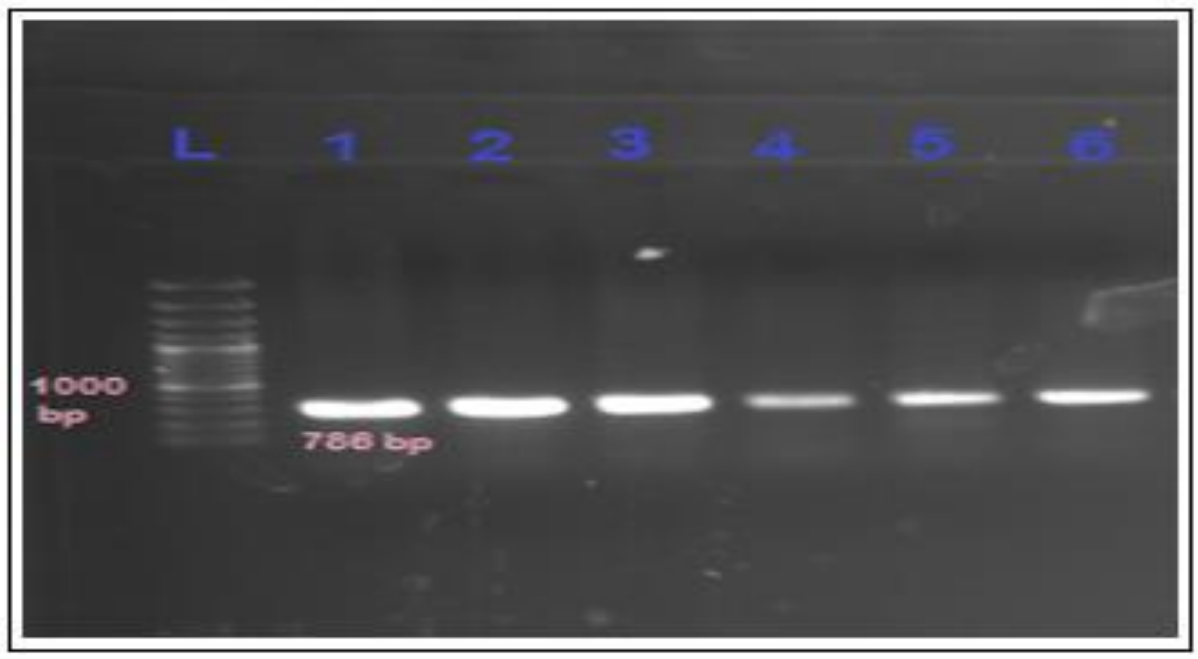

Fig. 5. *nif*H primer band of 786 bp on gel (Scale: L= Ladder, 1= Positive control, 2= WP8, 3= WS14, 4= SCS6, 5= SCS12, 6= SCS19).

All the bacterial isolates were found negative for this primer. Cellulolytic degradation is a complicated process performed by different enzymes. This may be due to the diversity in the cellulase system among the bacterial kingdom (David B W., 2011).

Biochemical properties of bacterial isolates

The biochemical test result of each bacterial isolate with their colony characteristics was compared and they showed that the bacterial isolates may belong to the bacterial genus *Stenotrohomonas, Alcaligenes, Achromobacter, Bacillus, Brucella, Actinomycetes* and *Rhizobium* (Table 3).

Bacterial strains identified by 16SrRNA sequencing

The sequence results of each isolate were checked in NCBI nucleotide Blast and they were identified as bacterial strains based on the maximum identity and match with the already known bacterial strains. They were identified as *Achromobacter* sp. MFA1 (WP8), *Paenibacillus pabuli* HSSC 492 (WS11), *Streptomyces* sp. strain VITBVK2 (WS14), *Stenotrophomonas* sp. Vi65 (WS20), *Paenibacillus pabuli* HSCC 492 (WM9), *Uncultured Actinomyces* sp. clone O-71 (WM13), *Beijerinckia fluminensis* (WM21), *Alcaligenes faecalis* CCM5B (WM24), *Stenotrophomonas maltophilia* strain CM30 (WN15), *Brevibacillus parabrevis* strain TJ2.3 (SCS6), *Ochrobactrum intermedium* strain BE1 (SCS12), *Brevibacillus parabrevis* strain TJ2.3 (SCS19), *Cupriavidus sp. christensen*.ICD.09 (SCM7) and *Bacillus cereus* (SC16).

The identified bacterial strains are now phenotypically and genotypically characterized as Nitrogen fixers while they are phenotypically characterized as CMC degraders.

Conclusion

Bacterial species that showed nitrogen-fixing and cellulose degradation ability were isolated from two hundred and fifty rhizosphere soil samples of Wheat and Sugarcane. Fifteen bacterial isolates from each sample plant were selected for qualitative and quantitative assay for cellulase and nitrogenase activities. Bacterial isolates from Wheat samples showed clearance zone ratio on CMC substrate in the range of 1.6 (WS18) - 3.0 (WS11) while bacterial isolates from Sugarcane samples showed clearance zone ratio in the range of 1.3 (SCC6) - 2.71 (SCS12). Upon quantitative analysis on DNS method, bacterial isolates from Wheat samples released cellulase in the range of 0.751 (WS18) – 4.784 (WS11) U/mL on 0.5 % CMC at pH 7 on NF-CMC medium. Bacteria from Sugarcane samples showed cellulase activities in the range of 0.418 (SCS15) – 4.155 (SCS12) U/mL on 0.5 % CMC at pH 7 on NF-CMC medium. Bacterial isolates from Wheat samples produced NH_4-N in the range of 0.104 (WC4) – 1.115 (WS11) mM in Nitrogen free medium at pH 7 and 28°C. While bacterial isolates from Sugarcane samples produced NH_4-N in the range of 0.102 (SCP3) – 1.070 (SC16) mM in glucose-containing Nitrogen free medium at pH 7 and 28°C for 7 days. Fourteen bacterial isolates out of thirty confirmed the presence of nitrogenase gene and were further identified as bacterial strains *Achromobacter* sp. MFA1, *Paenibacillus pabuli* HSSC 492, *Streptomyces* sp. strain VITBVK2, *Stenotrophomonas* sp. Vi65, *Uncultured Actinomyces* sp. clone O-71, *Beijerinckia fluminensis, Alcaligenes faecalis* CCM5B, *Stenotrophomonas maltophilia* strain CM30, *Brevibacillus parabrevis* strain TJ2.3, *Ochrobactrum intermedium* strain BE1, *Brevibacillus parabrevis* strain TJ2.3, *Cupriavidus sp. christensen*.ICD.09 and *Bacillus cereus*.

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References

Aatif A, Zakia L. 2011. Isolation and characterization of H_2S producing yeast to detoxify mercury containing compounds. International Research Journal of Microbiology **2(12),** 517-525.

Al-Khatib IA, Kontogianni S, Nabaa HA, Alshami N, Al-Sari MI. 2015. Public perception of

hazardousness caused by current trends of municipal solid waste management. Waste Management **36,** 323-330.

Bala Kumaran MD, Kalaichelvan PK, Santhi R. 2015. Exploration of Agro Industrial Wastes as Substrates for cellulose production by *Bacillus licheniformis* MTCC 429. Microbiology Journal **5(2),** 36-42.

Boddey RM, Dobereiner J. 1995. Nitrogen fixation associated with grasses and cereals: recent progress and perspectives for the future. Fertility Research Journal **42,** 241–250.

Callow N, Ray CS, Kelby MA, Ju LK. 2016. Nutrient control for stationary phase cellulase production in Trichoderma reesei Rut C-30. Enzyme and Microbial Technology **82,** 8-14.

Cocking EC. 2003. Endophytic colonization of plant roots by nitrogen-fixing bacteria, Plant and Soil 252 **(1),** 169-175.

David BW. 2011. Microbial diversity of cellulose hydrolysis. Current Opinion in Microbiology **14(3),** 259-263.

Emtiazi G, Pooyan M, Shamalnasab M. 2007. Cellulase Activities in Nitrogen Fixing Paenibacillus Isolated from Soil in N-free Media, World Journal of Agricultural Sciences **3(5),** 602-608.

Farjana I, Narayan R. 2018. Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses, Islam and Roy. BMC Research Note. **11,** 445.

Franche C, Lindström K, Elmerich C. 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant and Soil **321,** 35-59.

Hassan M, Ali KW, Amin FR, Ahmad I, Khan MI, Abbas M. 2016. Economical perspective of sustainability in agriculture and environment to achieve Pakistan vision 2025. International. Journal of Agricultural and Environmental Research **2(2),** 129-142.

Jonathan PZ, Bethany DJ, Steven MS, Greg FS. 2003. Nitrogenase gene diversity and microbial community structure: a cross system comparison. Environmental Microbiology **5(7),** 539-554.

Kazaragis A. 2005. Minimization of atmosphere pollution by utilization of cellulose waste. Journal of Environmental Engineering and Landsciences **2,** 81-90.

Krieg NR, Holt JG. 1984. Berjeys manual of systematic bacteriology, Ist edition.

Lester H, Sandra L. 1977. Solid Media Containing Carboxymethyl cellulose to Detect C_x Cellulase Activity of Micro-organisms. Journal of General Microbioloy **98,** 109-1 I 5.

Lubanza N, Boipelo E, Olubukola OB. 2013. Isolation and characterization of beneficial indigenous endophytic bacteria for plant growth promoting activity in Molelwane Farm, Mafikeng, South Africa. African Journal of Biotech **12(26),** 4105-4114.

Miller GL. 1959. Use of Dinitrosalisyclic acid reagent for determination of reducing sugar. Analytical Chemistry **31(3),** 426-428.

Mohammad D, Sedigheh M, Saeed I. 2013. Identification of ten N2 fixing bacteria using 16sRNA and their response to various zinc concentration, International Journal of Cellular and Molecular Biotechnology, 1-8.

Pereira P, Ibanez F, Rosenblueth M, Etcheverry M, Martinez-Romero E. 2011. Analysis of the bacterial diversity associated with the roots of maize (Zea mays L.) through culture-dependent and culture-independent Methods.

International Scholarly Research Notices. 1-10.

Pin-Jing H. 2012. Municipal solid waste in rural areas of developing country: do we need special treatment mode? Waste Management **32,** 1289-1290.

Ponnambalam AS, Deepthi RS, Ghosh AR. 2011. Qualitative Display and Measurement of Enzyme Activity of Isolated Cellulolytic Bacteria, Journal of Biotechnology. Bioinformatics and Bioengineering **1(1),** 33-37.

Reinhardt. 2008. Molecular characterization of nitrogen fixing bacteria isolated from Brazilian agricultural plants at Sao Paulo state. Brazilian Journal of Microbiology **39(3),** 414-422.

San SY, Ei PK, Tin ML, Zaw KL, Amy Aung TM S, May Thet N, Wai WM. 2017. The correlation of carbon source and ammonium accumulation in culture broth by nitrogen fixing bacterial isolates. Journal of Scientific an Innovative Research **6(2),** 63-67.

Sasidharan S, Sreedharan S, Sailas B. 2013. Cellulase Producing Bacteria from the Wood-Yards on Kallai River Bank. Advances in Microbiology 3, 326-332.

Shanmugapriya K, Saravana PS, Manoharan M, Mythili A, Joseph S. 2012. Isolation, screening and partial purification of cellulase from cellulase producing bacteria. International Journal of Advance Biotechnology Research **3,** 509-514.

Yan-Ling L, Zheng Z, Min WU, Yuan W, Jia-Xun F. 2014. Isolation, Screening, and Identification of Cellulolytic Bacteria from Natural Reserves in the Subtropical Region of China and Optimization of Cellulase Production by *Paenibacillus terrae* ME27-1, Biomedical research international **1,** 1-13.

Zaw K L, San san Y, Tin ML. 2013. Enhancement of Cellulolytic nitrogen fixing activity of Alcaligenes sp. By MNNG Mutagenesis. International Journal of Innovation and Applied Sciences **3(4),** 979-986.

Zhou H, Long YQ, Meng AH, Li QH, Zhang YG. 2015. Classification of municipal solid waste components for thermal conversion in waste-to-energy research. Fuel **145,** 151-157.