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(Research Article)

Isolation, characterization and identification of root nodule bacteria from *Macrotyloma uniflorum* (Lam.) Verdc

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Abstract

The present study was conducted to characterize the native plant growth promoting rhizobacteria (PGPRs) from the nodules of *Macrotyloma uniflorum* (Lam.) Verdc. The seeds of *Macrotyloma uniflorum* was grown in two different soils i.e., sterile and non-sterile soil and four bacterial isolates were isolated. The bacterial isolates were characterised on the basis of morphological and different biochemical analysis. Phylogenetic identification was done on the basis of 16S rRNA analysis. Four isolates obtained belongs to *Bacillus velezensis* and *Enterobacter cloacae*.

Keyword: PGPRs; Root nodules; Rhizobacteria; *Macrotyloma uniflorum*; 16S rRNA

1. Introduction

Plants require nitrogen in large quantities. It is the building block of proteins[1],. It greatly affects the growth and reproduction of organisms. Therefore, nitrogen plays the role of an important nutrient for plants and plants require it in the highest quantity. Plants cannot grow normally when there is a lack of nitrogen in the soil. The composition of nitrogen in the atmosphere is about 78%, but it cannot be used by plants directly. Biological nitrogen fixation is an important process and it is carried out by nitrogen fixing bacteria. It converts free nitrogen gas in the atmosphere into ammonium[2,3]. Nitrogen fertilizers are one of the most widely used chemical fertilizers. Excessive use of chemical fertilizers can damage fertile land and cause a decrease in the quality of agricultural land. One solution to this problem is the use of organic fertilizers[4, 5]. Biological nitrogen fixation via leguminous plants is used to improve agricultural sustainability by reducing the global use of synthetic nitrogen fertilizers[6, 7,8, 9].

Legumes are one of the most widely cultivated plants on earth[10, 11]. They can grow even in poor soils because they have the ability to fix nitrogen by forming a symbiotic relationship with nitrogen-fixing bacteria. This symbiotic relationship of legumes with nitrogen-fixing bacteria can improve soil quality and also increase crop production. Several plant growth-promoting rhizobacteria (PGPR) with nitrogen-fixing ability have been reported to be a very important source of nitrogen in maintaining soil fertility and sustainable crop production[12, 13]. Identification of potentially important PGPR is important for promoting crop growth and yield. Therefore, identification of efficient strains is very important from both ecological and economic perspectives.

Symbiotic nitrogen fixing bacteria in the root nodules and rhizospheric soils of leguminous plants are associated with many different bacterial species[14]. Identification of better bacterial strains for fixing nitrogen in leguminous plants can improve food security around the world. Discovery of bacterial species with plant promoting effects plays an important role in biological nitrogen fixation[15].

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Macrotyloma uniflorum Lam. (Vardc.), formerly known as *Dolichos biflorus*, belongs to the Fabaceae family, is a legume plant grown in Asia, Africa and Australia. The seeds are oval shaped, 4-6 mm long and 3-5 mm broad and usually reddish brown in colour. The seeds contain carbohydrates, proteins, amino acids, lipids, phenolic compounds, flavonoids, anthocyanidins, tannins, saponins, fatty acids, phytosterols and minerals such as iron, calcium and molybdenum in varying quantities. *Macrotyloma uniflorum* seeds are used in traditional medicine as anthelmintic, diaphoretic and are useful for tumours, bronchitis, liver diseases, kidney stones and asthma[16, 17,18, 19].

Very little work has been done on the isolation and characterization of *Macrotyloma uniflorum*. Therefore, through morphological, biochemical and molecular characterization of root nodule bacteria from *Macrotyloma uniflorum*, efficient bacterial isolates can be identified. These isolates can be used as inoculum to restore soil fertility to improve crop production.

2. Materials and methods

2.1. Collection of seeds

Seeds of *Macrotyloma uniflorum* (variety HG4) were obtained from Birsa Agricultural University, Kanke, Ranchi, Jharkhand and grown in sterile and non-sterile soil.

2.2. Collection of nodules

Plants grown in sterile and non sterile soil were used to collect root nodules. Nodules were harvested from freshly uprooted healthy green plants. The plant roots were thoroughly washed under running tap water to remove mud and soil particles from surface of the nodule. Fresh, healthy and large nodules were carefully selected from each plant for the study [20, 21,22,23] .

2.3. Surface sterilization of the nodules

The collected healthy nodules were thoroughly washed under tap water and then separated from the root using a sharp and clean forceps. Then the undamaged nodules were surface sterilised with the help of 70% alcohol for 3 minute and then with 0.1% mercuric chloride for another 3 minutes and then washed several times in sterile distilled water under aseptic conditions [24,25].

2.4. Isolation of root nodule bacteria or Isolation of strains from root nodules OR Bacterial isolation and purification

The primary step in the isolation process was to crush the sterile nodules with the help of a sterile rod to obtain a Milky suspension of bacteroides. The crushed suspension was taken and spread on Yeast Extract Mannitol Agar (YEMA) media plates. The inoculum was spread evenly on the plates using a glass spreader sterilised with alcohol and flame. The plates were incubated at room temperature[21,26]. After 24 hour incubation at 28° C, bacterial colony growth was observed and morphologically dissimilar colonies were picked from master plate and subcultured on freshly prepared YEMA plate by streaking method. Similar procedure was repeated until morphologically identical bacterial colonies were obtained in single plate[27].

2.5. Culture maintenance and preservation

Isolates were subcultured for long term preservation and pure cultures of all isolates were obtained. Pure cultures were preserved in 20% glycerol at 4 °C in a refrigerator until further use. All isolates were replated on YEMA and checked for contamination at least every three months[26].

2.6. Identification of isolates

Identification of the isolates was done by morphological, biochemical and molecular analysis. Microscopy was done by observing morphology and cultural characteristics of the isolates.

2.6.1. Morphological colony characteristics of the isolates

Morphological analysis is macroscopic with direct observation to observe bacterial isolates. Colony characteristics (i.e. size, shape, colour, opacity, elevation, edge, margin of the bacterial colony and their growth rate) were determined by observing the colonies on YEMA plates of microorganism grown overnight at 28±2 °C. Microscopic observation of the isolates was done using Gram staining technique. [28]

Gram's reaction: (Vincent, 1970)

This was used to differentiate bacteria into two main groups - gram positive and gram negative.

Using a clean slide and sterile wire loop, a loop of normal saline was inserted in the middle of the slide and the loop was sterilised again. The sample was collected with the wire loop. A smear was made in a circular manner. After making the smear, it was heated on the slide by passing it gently over the Bunsen flame. Crystal Violet was put on the smear for 30 seconds and poured off with water. Iodine solution was poured on and left for a minute. Then it was washed with water. Then, acetone or ethanol was added for the purpose of decolorization and later, acetone/ethanol was removed with water immediately. Counter staining was done using safranin with water within a minute. After drying, the slide surface was observe through a compound microscope. The Gram negative cell appears pink - red in colour and the Gram positive cells appear violet in colour.

2.6.2. Cultural and metabolic characteristics

The isolates were characterised by various biochemical methods : methyl red test, VP test, catalase test (cover slip method), starch hydrolysis test, citrate utilization test, indole test, nitrate reduction test, gelatin liquification test, methylene blue test, hydrogen sulfide production test, casein Hydrolysis and triple sugar iron (TSI) agar test.

Congo red test

The strain culture was plated on CRYEMA media and incubated for 24 hours at 28° C. After this time, the dye absorption capacity of the bacteria was observed.

2.6.3. Molecular identification

All the four bacterial isolates – MUHG4NSI, MUHG4NSII, MUHG4NSIII and MUHG4SS were selected for Molecular characterization. DNA of isolates was extracted and its quality was evaluated on 1.0% Agarose Gel. The RDNA gene amplificiation was performed using the primers LCO 1490 and HCO 2198. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3. 1 cycle sequencing kit on ABI 3730 xl Genetic Analyzer.

The rRNA gene sequence was used to carry out BLAST with the database of NCBI genbank database to identify the bacterial strains. The 16S rRNA gene sequences of the isolates were aligned using Clustal W software. Phylogenetic trees were constructed using Maximum Likelihood method based on the Kimura 2-parameter model with MEGA 11 software package. The percentage of replicate tree were evaluated using the bootstrap resampling method based on 1000 replicates[29,30,31].

3. Result

3.1. Isolation and morphological characterization

A total of 4 strains were isolated from the root nodules of *Macrotyloma uniflorum* (var. HG4) collected from both sterile and non-sterile soils. All the isolates were subjected to Gram staining and microscopic observation showed that three (MUHG4NSI, MUHG4NSIII and MUHG4SS) isolates studied were Gram positive and one isolate (MUHG4NSII) is gram negative. Isolation of nitrogen fixing bacteria was done on YEMA selective media and all the samples were grown well on it. Colony morphology of the isolates was observed. Most of the isolates were found to be creamy white colony with round shape features.

3.2. Cultural and metabolic characterization

The results showed that in the qualitative test of the isolates grown in YEMA + Congo Red Media all grew and were pink, this showed that the isolates did not absorb the red color from the Congo Red indication present on the media.

3.3. Biochemical characterization

Various biochemical test were performed on the isolates for characterisation as per the Burgys Manual. The purity of the test isolates was reconfirmed by performing various biochemical tests on different media. The biochemical behaviour of the test strains is listed in table.

Table 1 Morphological characterization of the bacteria isolated from *Macrotyloma uniflorum* (Var. HG4)

S. No.	Isolates	Morphological characterization of isolated bacteria from *Macrotyloma uniflorum* (variety HG4)						
		Size	**Form**	**Elevation**	**Margin**	**Pigmentation**	**Gram staining**	**CRYEMA Media**
1	MUHG4NSI	Small, Moderate	Circular	Raised	Entire	White	+ve	Pale Pink
2	MUHG4NSII	Moderate	Circular	Raised	Entire	Off white	-ve	Pale Pink
3	MUHG4NSIII	Pin point, Small, moderate	Circular	Raised	Entire	White	+ve	Pale Pink
4	MUHG4SS	Small	Circular	Raised	Entire	White	+ve	Pale Pink

(MUHG4NS = Bacteria isolated from root nodule of *Macrotyloma uniflorum* of variety HG4 grown in normal soil; MUHG4SS = Bacteria isolated from root nodule of *Macrotyloma uniflorum* of variety HG4 grown in sterile soil.

Table 2 Biochemical characterization of the bacteria isolated from *Macrotyloma uniflorum* (variety HG4)

S. No.		**1**	**2**	**3**	**4**
Isolates		**MUHG4NSI**	**MUHG4NSII**	**MUHG4NSIII**	**MUHG4SS**
Biochemical characterization of isolated bacteria	Starch Hydrolysis	-	+	+	-
	Casein Hydrolysis	-	+	+	-
	Gelatin Hydrolysis	-	+	+	-
	Triple Sugar Iron Agar Test	-	-	-	-
	H2S Production	-	-	-	-
	Catalase Test	+	+	+	+
	Nitrate Reduction Test	+	+	-	+
	IndoleTest	-	-	-	-
	Voges Praskauer Test	-	-	-	-
	Methyl Red Test	+	+	+	+
	Citrate Utilization Test	+	+	+	+

(- =negative result, + = positive result) (MUHG4NS = Bacteria isolated from root nodule of *Macrotyloma uniflorum* of variety HG4 grown in normal soil. MUHG4SS = Bacteria isolated from root nodule of *Macrotyloma uniflorum* of variety HG4 grown in sterile soil.)

Figure 1 A. Plant of *Macrotyloma uniflorum* (variety HG4)

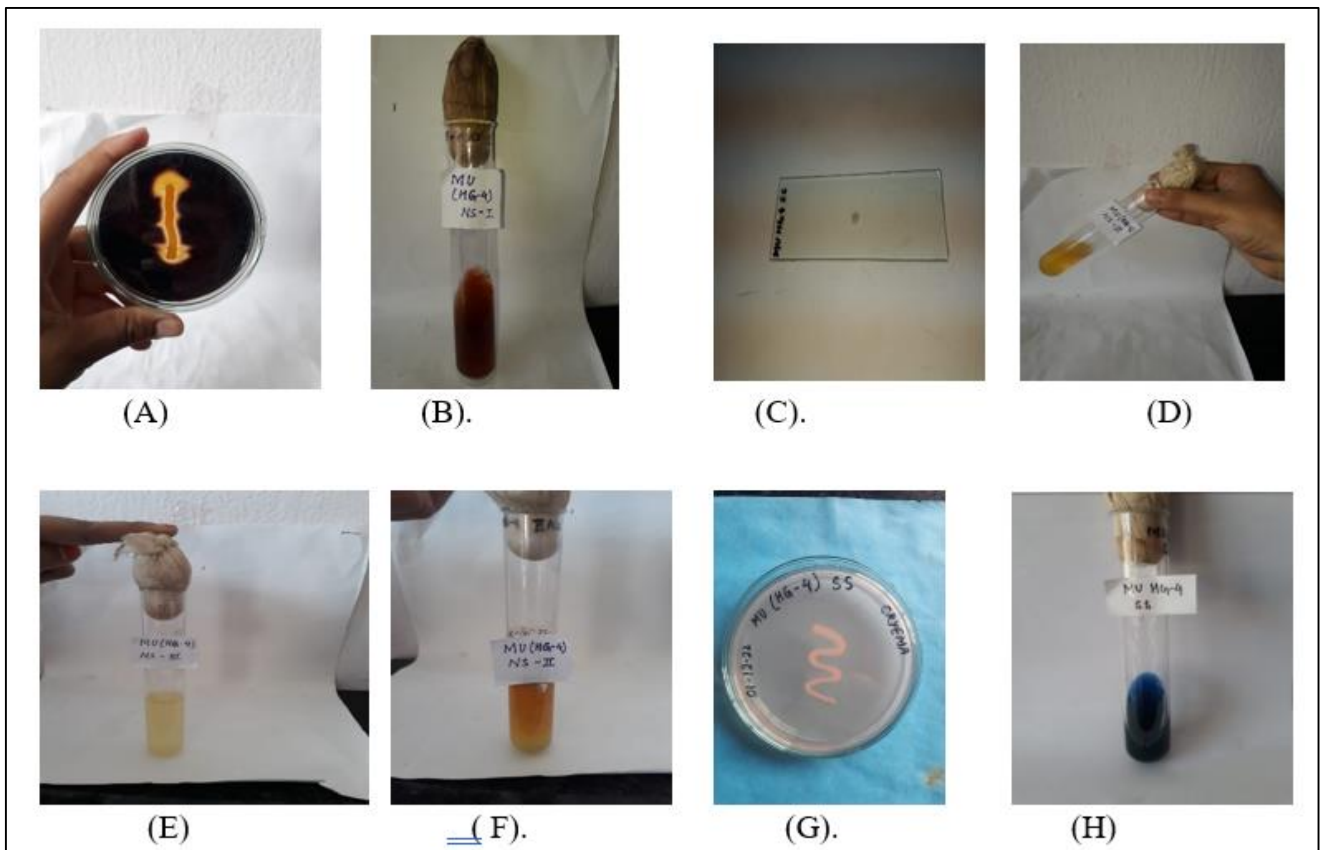

Figure 2 (A) Starch Hydrolysis test; (B) TSI test; (C) Catalase test; (D) Gelatin Hydrolysis test (E)Nitrate Reduction test; (F) Methyl Red Test; (G) CRYEMA; (H) Citrate Utilization test

3.4. Identification and Phylogenetic Analysis of Isolates

According to 16S rRNA sequencing, four rhizobacteria were identified. BLAST and Phylogenetic tree analysis revealed that 3 strains such as MUHG4NSI, MUHG4NSIII and MUHG4SS showed similarities of 91.29 to 99.02% with *Bacillus velezensis* and one strain MUHG4NSII showed similarity of 99.43% with *Enterobacter cloacae*.

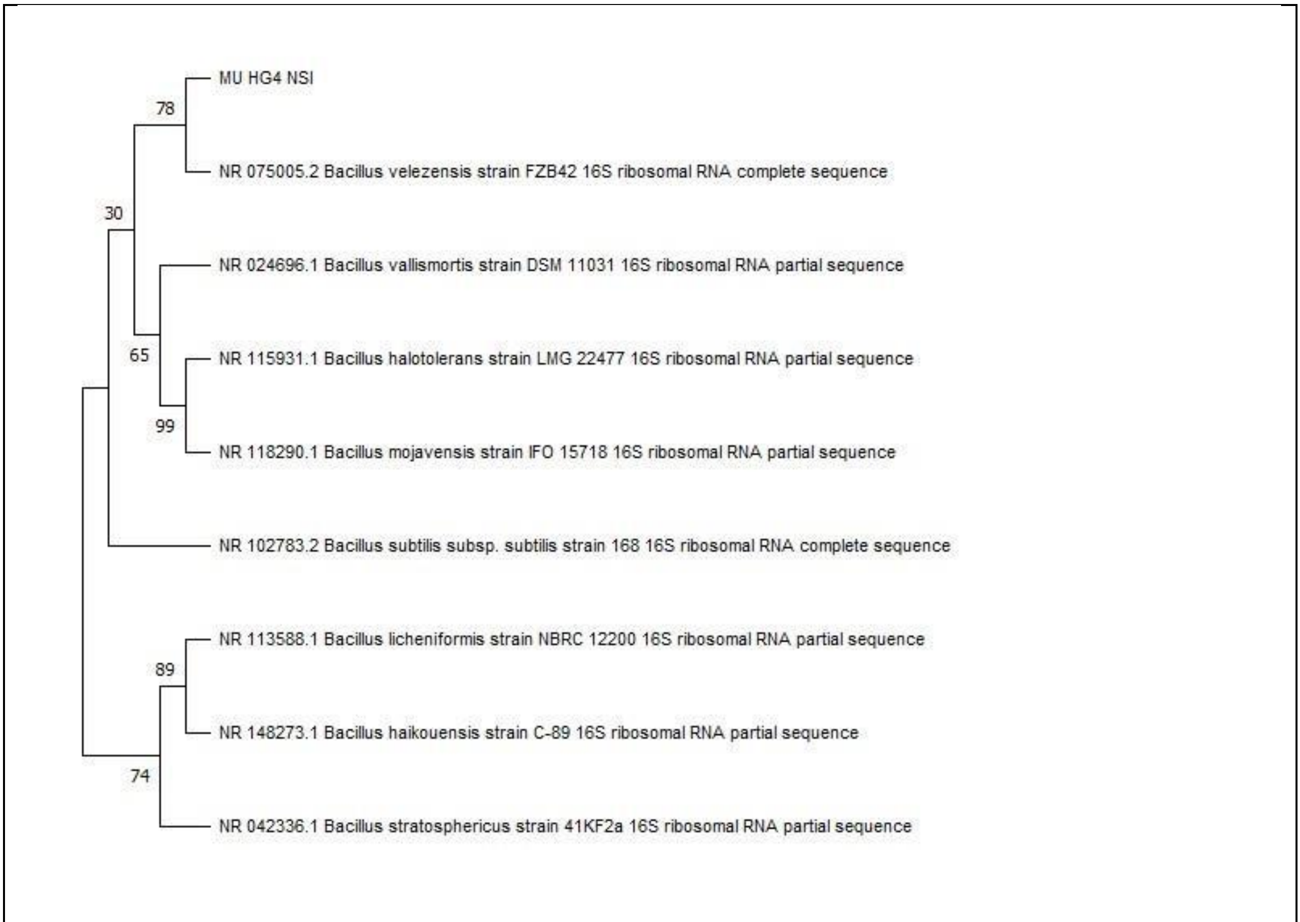

Figure 3 Phylogenetic tree based on 16S rRNA gene sequences of strain MUHG4NSI from the root nodule of *Macrotyloma uniflorum*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA 11

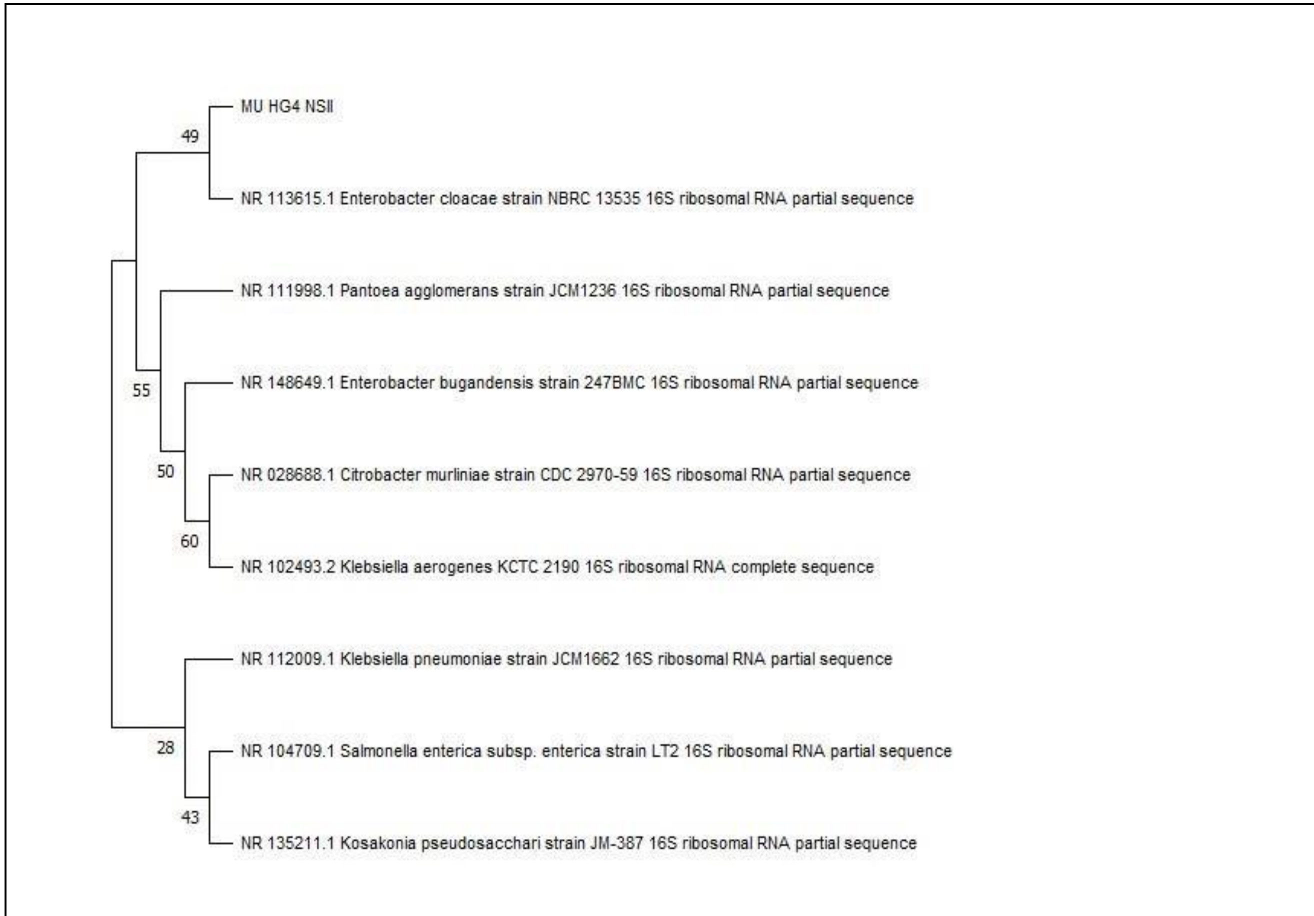

Figure 4 Phylogenetic tree based on 16S rRNA gene sequences of strain MUHG4NSII. from root nodule if *Macrotyloma uniflorum*. The evolutionary history was inferred by using the Maximum Likelihood Method based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA 11

4. Discussion

The present study is expected to reveal the diversity of some very rare native bacterial strains. Isolation and characterization of native bacterial populations can be valuable biological resource for the application of novel bacteria towards enhancement of agricultural productivity. The initial phase of this experiment was to isolate and purify the bacterial isolates collected from *Macrotyloma uniflorum* (var. HG4) which is grown in two different soils i.e., sterile and non sterile soils. The test strains were identified and classified on the basis of their cultural properties, biochemistry etc. The strains of bacteria were found to be diverse in several properties. The growth rate of the strains and their behavior on different media were noted.

A total of 4 isolates were isolated from the root nodules of *Macrotyloma uniflorum*. All the 4 isolates were morphologically characterized on YEMA media plates. The bacterial colony size ranged from pin point to small to medium. The isolates were white and off white translucent in colour, circular in shape and colony diameter was 2 to 3 mm with entire margin, raised (convex) and sticky mucilage. The 3 isolates were found to be gram positive and one isolate is gram negative in the reactions.

All the isolates showed pink colour in Congo Red YEMA media. The appearance of pink colour is due to poor absorption of the dye Congo Red present in the medium.

The results of biochemical characteristics for the isolates were presented in Table 1 and 2. Starch Hydrolysis test was found positive in 2 isolates, Casein Hydrolysis Test was found positive in 2 isolates, Gelatin Hydrolysis test was found positive in 2 isolates, Nitrate Reduction test was found positive in 3 isolates, all isolates were found positive for Catalase test and methyl Red Test and all isolates were found negative for Triple Sugar Iron Agar Test, H2S Production test, Indole test and Voges Praskauer test.

Based on the above morphological and biochemical characteristics, and 16S rRNA sequencing the isolates were identified as *Bacillus velezensis* and *Enterobacter cloacae.*

5. Conclusion

Through this research work identification of local bacterial strains can be done. Indigenous bacteria as plant growth promoter can mitigate the threat to the environment which may be posed by various non-native and invasive species of bacteria. Superior strains can be selected to fix nitrogen in the legumes and that can improve the security for food and nutrition in a very sustainable manner. Also correct identification of nitrogen fixing bacteria is very important in the area of research because the application of effective nitrogen fixing bacteria has the tendency to improve crop production. Effective nitrogen fixing bacteria can also maintain the fertility of the soil.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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