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Isolation and Molecular Characterization of Diazotrophic Bacteria in Arable Soils

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Abstract:- Diazotrophs are nitrogen-fixing micro organisms responsible for promoting plant growth and other living organisms. These groups of organisms are found in soil but most environments have not been explored to ascertain their presence and activity. This study, therefore, intends to assess the availability of diazotrophs in the arable soil within Uzairue community in Edo State with the objective of evaluating the density, populations, and identity of diazotrophic bacteria in soil samples from different cultivable locations using different microbiological approaches. Soil samples from the various arable soil were collected using a composite sampling method specifically from farmlands and physicochemical parameters were determined such as nitrate, phosphate, organic matter, organic, and other properties that can influence microbial activities and density. The soil samples were enumerated the total heterotrophic bacterial population and isolation of nitogen fixation (NF) bacteria was done using N-free media. The samples had trace levels of phosphate, nitrogen, and other varying concentrations of physicochemical parameters. An appreciable population was observed in the samples which ranged from 4.6 x 10^6 to 5.1 x 10^6. Out of a total of thirty (30) bacterial isolates, nine (9) showed potential for nitrogen fixation on the N-free medium and were identified using their morphological features, biochemical reaction, and molecular characterization. The result of the research showed that the identified species have high phenotypic diversity and were identified to belong to four genera: Alcaligenes, Pseudomonas, Priestia, and Providencia. Conclusively the research showed that diazotrophic bacteria with nitrogenase activity are present in arable soil which could be harnessed and exploited for green and sustainable agriculture.

Keywords:- *Diazotrophs, nitrogenase, microorganisms, molecular, agriculture.*

I. INTRODUCTION

Soil as a habitat contains microorganisms with one gram of fertile soil containing up to billion different types of bacteria. Commonest among these microorganisms present in soil include archaea, bacteria, actinomycetes, fungi, algae, protozoa, and their various species of which these organisms present in the soil play a vital role to other living things, especially the plant for nitrogen fixation [1]. Soils are an important source of nutrients in many of the world's agricultural systems due to the large deposits of nitrogen and other mineral contents within them. It is known that in low-input production systems, nitrogen release from soil organic matter turnover is the major part of the crop's nitrogen supply and research suggests that this process is significantly affected by changes in climate [2]. In many production areas around the world, knowledge of the amount of nitrogen responsible for crop nutrition is purely empirical, and data as a foundation for global-scale security change assessment and climate change is scarce, particularly in most local communities of predominant farming culture in Africa. According to Matiru and Dakora [3], nitrogen is an essential plant nutrient that is required for protein, enzyme, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and chlorophyll synthesis, and is thus essential for plant growth and food production. It is a constituent of proteins, enzymes, chlorophyll, and growth regulators in plants, and its deficiency causes reduced growth, leaf yellowing, and leaf trifoliation [4].

There is an abundance of nitrogen in the atmosphere, despite this abundance, this atmospheric nitrogen cannot be utilized directly by the plants and therefore has to combine with elemental hydrogen. There are certain species of bacteria that absorb this nitrogen gas from the environment and create a nodule at the root of plants called nitrogen-fixing bacteria (diazotrophic bacteria). When these bacteria die, the nitrogen consumed by the bacteria is freed for plant utilization. Soil diazotrophs are the dominant source of nitrogen input in primary production environments, as diazotrophic bacteria perform biological nitrogen fixation during nitrogen cycling in natural ecosystems [5]. It is worth noting that all diazotrophs contain nitrogenase, and in order to achieve biological nitrogen fixation, they must employ various techniques to cope with fluctuating oxygen concentrations, which, according to Izquierdo and Nusslein [6,] is lethal to the nitrogenase.

Some bacteria, such as rhizobia, can colonize the rhizosphere, infect legume roots, and use a symbiotic process to fix nitrogen in the soil [7, 8]. Biological rhizobia can dwell on plant wastes (saprophytes), inside plants (endophytes), or in close proximity to plant roots (rhizobacteria) [8, 9]. Rhizobia penetrate the rhizosphere, infect the roots, and fix nitrogen, which leads to improved development of plants and grain output [10]. The ability of rhizobial populations to fix nitrogen is linked to soil fertility, with acidic soils containing less efficient rhizobia strains [11].

Arable soil possesses a rich array of free-living diazotrophs as they are obligate anaerobes that live in habitats low in oxygen, such as soils and decaying vegetable matter. Most heterotrophic diazotrophs in soil are prokaryotes [12] including α-, β-, γ-, δ- Proteobacteria, Firmicutes, and Archaea [13]. According to a theory by Lu [14], diazotrophs in symbiosis with legumes provide around

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80% of biological nitrogen-fixing. Diazotrophs are widespread and have a wide range of bacterial and archaeal species. Multiple factors determine the quantity and variety of the diazotrophic community in typical agriculture soils. According to a study conducted on soil types in six Chinese provinces [15]. Their findings revealed that diazotrophic abundance varied by soil type, with *Bradyrhizobium, Azospirillium, Myxobacter, and Mythylobacterium* dominating, while the distribution of free-living diazotrophs was influenced by a variety of factors including crop type, soil features, and climatic conditions. The distribution of free-living diazotrophs differed significantly among the three soil types studied, with *Methylobacterium, Desulfovibrio,* and *Myxobacter* dominating Red soil, *Azospirillum, Myxobacter,* and *Skermanella* dominating Fluvo-aquic soil, and *Myxobacter* and *Azospirillum* dominating Black soil.

Proteobacteria and cyanobacteria have also been found to be among the most numerous diazotrophs in most soils [16]. However, as many studies have shown, diazotroph activity, abundance, and community structure in soil are very variable and are influenced by a variety of environmental parameters, including soil pH [17, 18], soil moisture [19], soil type [20], soil C content [13, 21], and soil N content [22, 23]. Carbon, nitrogen, and phosphorus, on the other hand, were widely acknowledged as essential elements regulating soil diazotrophic abundance in another study [24]. Because the nitrogenase enzyme is sensitive to ammonia, it was thought that high accessible nitrogen levels would harm soil diazotrophs [25, 26].

Because symbiotic nitrogen-fixing bacteria are extremely susceptible to perturbation, fertilization, tillage, soil amendment, soil disturbance, irrigation, and plant community composition can all alter the Microbial community composition [27]. For the populations of these microbial species, environmental parameters such as oxygen, soil ph, temperature, nitrogen, carbon, salinity, soil texture and aggregate size must all be considered [22, 28]. The physicochemical features of soils, rather than the distance between them, influence nifH diversity [28] and thus microbial distributions.

Diazotrophs have a complex method of action and interaction with plants and within the soil that allows them to fix atmospheric nitrogen and produce ammonia, which the plant can use. According to Peoples et al. [29], this method is known as biological nitrogen fixation, which is a cycle in which plants use various elements such as nitrogen, phosphorus, potassium, and carbon while also releasing resources that diazotrophic bacteria can use. As Glick [30] points out, diazotrophic bacteria associate with plant roots in a variety of ways to support plant growth. For example, first N2-fixing bacteria give ammonia for plant growth and boost root nutrient uptakes, such as phosphorus or iron. Second, phytostimulation occurs when microorganisms in the soil produce plant hormones such as cytokinins, gibberellic acids, indole-3-acetic acid (IAA), and some volatile chemical substances. Third, soil bacteria can boost plant immunity, making it possible to distinguish between beneficial and pathogenic diazotrophs during interactions. In addition, distinct stimulatory molecular pathways or metabolic adaptations in host plants are triggered based on the bacterial species or strains, which influence plant growth and development directly or indirectly. As a result, this study aims to identify possible nitrogen-fixing bacteria in agricultural soils within Uzairue.

II. MATERIALS AND METHODS

A. *Study area*

Etsako West Local Government Area of Edo State, Nigeria. The area is defined by latitude 70 20’ and 70 15’ North of the equator and longitude 60 10’ and 60 25’ Wests of the Greenwich meridian. The clan which is made up over thirty (30) villages is situated along Benin-Abuja Highway is characterized by large arable lands for agricultural activities. It borders Uzairue clan in Etsako West LGA to the South, Okene in Kogi State to the North, Atteh and Unemeh clans both of Akoko-Edo LGA to the west and North Ibie clan of the same Etsako West LGA to the West. Maintaining the Integrity of the Specifications.

B. *Sample Collection*

Soil samples were collected from three different locations within the local government. The samples were obtained at different points, at a depth of 0- 30cm with the aid of a calibrated soil auger, bulked together for homogeneity, and transferred into labeled sterile polyethylene bags. The samples were transported to the laboratory within 6h of collection for physicochemical and microbiological analysis. The soil samples for physicochemical analysis were air-dried and sieved using a two-millimeter (2 mm) micro sieve. The sieved soil samples were stored in tight glass containers for further analysis [31].

C. *Determination of soil physicochemical properties*

Soil samples were analyzed using different physical and chemical characteristics. The physicochemical parameters such as soil color, texture, pH, temperature, organic carbon, and available nitrogen were analyzed based on standard methods.

D. *Enumeration of total culturable heterotrophic bacteria*

The soil samples were taken to the laboratory in sterile plastic bags. A ten-fold serial dilutions technique was used to determine the colony-forming unit (CFU) number of culturable bacteria. The soil samples were taken in five test tubes, each contained 9 ml of normal saline, then a sterile pipette was used in taken 1 ml of properly mixed samples (soil mixture) into the first test tubes (10^{-1}) to make the total volume of 10 ml after which the dilution was mixed by emptying and filling the pipette several times, each pipette was discarded after usage, and a new fresh pipette was used.

Now, 1 ml of the mixture was taken from the first test tube (10^{-1}) dilution and was emptied into the second tube. The second tube now has a total dilution factor of $10^{-2.}$

The same process was repeated for the remaining tubes, taking 1 ml from the previous tube and adding it to the next 9 ml diluents. The final dilution for the bacteria/cells was 10^{-5}. Finally, 0.1ml (aliquot) of an

appropriate diluent (10^{-3} to 10^{-5}) was spread on the surface of nutrient agar plates in duplicate for each sample [32]. Plates were incubated at 37°C for 24 hours and colonies differing in morphological characteristics were enumerated and selected for further studies.

E. Isolation of free-living diazotrophic bacteria

Bacterial colonies were isolated using nitrogen-free media (NFM) with some modifications containing the following compositions per liter: 0.4 g of 129 KH_2PO_4, 0.1 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of NaCl, 0.02 g of $CaCl_2$, 0.01 130 g of $FeCl_3$, 0.002 g of $MoO_4Na.2H_2O$, 5.0 g of sodium malate. The whole media content was adjusted to pH 7 before being sterilized in an autoclave (121°C for 15 minutes). Approximately 20 mL of media was placed in each petri dish and inoculated to obtain pure cultures. Colonies from nutrient agar plate were selected and streaked on the newly fresh nitrogen free media (NFM) plate incubated at 30°C for 5days. Microorganisms with visible growth on the NFM medium were selected as potential diazotrophs, and stored at 4°C for further studies, while those with slow or no growth on the nitrogen free media (NFM) were discarded.

F. Identification of bacterial isolates

a) Colonial morphology of bacterial isolates

Colony morphology was observed on nutrient agar medium. The systematic assessment of the colonies' appearance, focusing on some aspects such as; the size of the colonies (diameter in mm), shape; (circular, irregular, entire, rhizoid or punctiform), color; (white or pigmented), transparency; (clear and transparent or opaque), elevation; (flat, raised, low convex, high convex, umbonate or nodular), as well as its consistency;(brittle, creamy, sticky and dry) and also the changes, produce on the media [33].

b) Biochemical test

Biochemical tests including hydrogen sulfide production, catalase test, oxidase test, urease test, motility test, TSI test, nitrate reduction test, indole tests, Methyl red test (MR), citrate test, etc. were conducted. Biochemical characterization was done by inoculating the isolates in various media. The isolates were tested for their ability to hydrolyze starch, gelatin, and casein and their ability to utilize citrate, to reduce nitrate, and to produce urease and catalase. Kligler Iron Agar (KIA) test is also performed to detect the ability of the isolates to produce H_2S by fermenting sugar.

c) Molecular identification

a. DNA extraction

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 950C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20oC for other downstream reactions.

b. DNA quantifiation

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double-clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitres of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

c. 16S rRNA amplification

The 16SrRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as a template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

d. Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

e. Phylogenetic analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [34].

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d) Statistical analysis
All data generated were analyzed and presented using tables, graphs, and appropriate statistical tools such as ANOVA.

III. RESULTS

A. *Soil Physicochemical analysis*

The representative samples from different sites were analysed for their physicochemical attributes and the results are presented in Table 1.

Parameters	Iyamho	Auchi	Igbira-Camp
Moisture Content (%)	8.9	9.9	9.4
pH	6.86	6.66	6.68
Electrical conductivity (**mbo/cm**)	375	328	188
Organic carbon (%)	3.13	0.87	0.35
Organic matter **(%)**	5.4	1.53	0.6
Pb **(mg/kg)**	15.24	8.44	10.7
Ni **(mg/kg)**	25.6	11.39	14.56
Fe **(mg/kg)**	1.47	1.272	1.16
Zn **(mg/kg)**	69.73	85.47	42.7
NO^{3-} **(mg/kg)**	10.79	12.33	22.73
PO_4^{-2} **(mg/kg)**	2.42	9.08	9.92

Table 1: Physicochemical parameters of soil samples

B. *Total culturable heterotrophiccount of bacterial isolates*

The total heterotrophic bacteria count were 4.6 x 10^6 CFU/g for soil sample 1, 4.4 x 10^6 CFU/g for soil sample 2 and 5.1 x10^6 CFU/g from soil sample 3 respectively. The bacteria population varied across all samples but however all counts were in the same order of magnitude 10^6 and no significant difference was observed at $P \leq 0.05$. The results are presented in Table 2.

Sample	Mean Value (CFU/g)
S1	4.6x 10^6
S2	4.4 x 10^6
S3	5.1x10^6

Table 2: Total Heterotrophic count from the three samples

A total of 25 isolates were obtained from all samples collected and 10, 8 and 7 bacteria were isolated from S1, S2 and S3 respectively (Table 3). Out of which, 9 isoaltes were selected as diazotrophs based on microbiological analysis. Sample 2 had the highest population of diazotrophs (50%) which was closely followed by S1 (30%) and S3 (28.57%). The phenotypic characterization based on morphological and biochemistry reaction revealed that most of the diazotrophs were gram negative although others were gram positive. The most abundant genus is Pseudomonas.

Samples	Number of isolates	Number of Diazotrophs	Percentage of diazotrophs (%)
S1	10	3	30
S2	8	4	50
S3	7	2	28.57

Table 3: Distribution of diazotrophs in various samples

Bacterial isolates	Shape	Size	Color	Elevation	Transparency	Consistency/texture
S1C	Rhizoid	Big	Cream	Umbonate	Opaque	Dry
S1D	Round	Big	Yellow	Raised	Opaque	Shiny
S1E	Irregular	Medium	Pink	Raised	Transparent	Slimy
S2G	Round	Small	Yellow	Flat	Transparent	Slimy
S2D	Round	Small	Yellow	Flat	Opaque	Dry
S2E	Rhizoid	Big	Cream	Umbonate	Transparent	Moist
S2H	Round	Big	Pink	Flat	Transparent	Sticky
S3B	Round	Small	Cream	Raised	Transparent	Shiny
S3J	Round	Big	White	Raised	Transparent	Shiny

Table 4: Morphological properties of the bacterial isolates

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Isolates	Gram strain	Shape	Oxidase	H2S	Catalase test	Sucrose	Citrate	Urease	Glucose	Lactose	Maltose	Fructose	TSI	Tentative Identification
S1D	-	R	+	-	+	-	+	-	-	-	-	-	+	*Pseudomonas sp*
S1E	-	R	-	-	+	-	+	+	+	-	+	+	+	*Serratia sp.*
S1C	-	R	+	-	-	-	+	+	-	-	-	-	-	*Pseudomonas sp.*
S2G	-	R	-	+	+	+	+	+	+	+	-	+	+	*Azotobacter sp.*
S2H	-	R	+	-	+	+	+	+	+	-	+	+	-	*Serratia sp.*
S2E	+	R	+	+	+	+	+	+	-	+	-	+	+	*Bacillus sp.*
S2D	+	R	+	-	+	-	+	+	-	+	-	+	+	*Bacillus sp.*
S3B	-	R	+	-	+	-	-	-	+	-	-	-	+	*Alcaligenes sp*
S3J	-	R	+	-	+	-	+	-	-	-	-	-	-	*Pseudomonas sp.*

Table 5: Gram reaction and biochemical characteristics of bacterial isolates

Keywords:- *R= rod, C = cocci, - = gram negative, += gram positive.*

C. Molcular Identifiation

PCR amplification of the 16S rRNA gene fragment was obtained for all isolates indicating that DNA was successfully extracted. The extracted DNA all yielded PCR products of 1500bp. The products were visualized in 1.5% tris acetate EDTA (TAE) agarose gel with ethidium bromide. The 16S rRNA gel electrophoresis picture is a representative of the gel pictures of the 4 isolates.

Amplified PCR products (16S rRNA) as shown in Fig. 2 were purified and sequenced. Identification of bacterial 16S rRNA sequences were aligned with BLAST search facility of National Centre for Biotechnology information (NCBI) databases. The sequences aligned gave 95 - 100% similarity with those deposited in GenBank and as such considered close relatives and assigned identities.

Samples	Accession Number	Molecular identity of bacterial isolates
S1D	OK426387	*Priestia flexa*
S2D	MK641351	*Providencia stuartii*
S3B	OK083684	*Alcaligenes fecalis*
S3J	HM209783	*Pseudomonas plecoglosscida*

Table 6: Molecular Identification using 16SrRNA Amplification

D. Phylogeneticanalysis

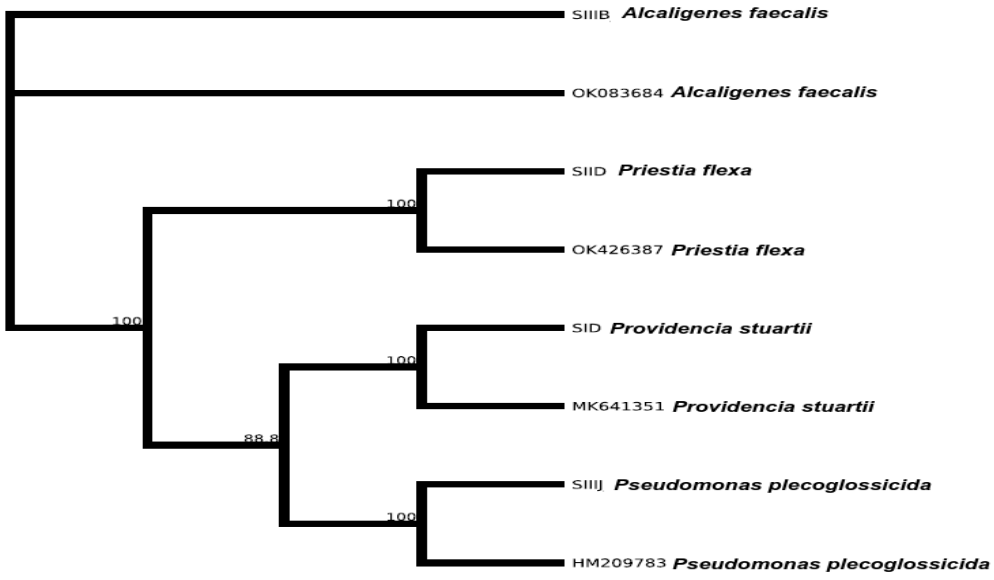

Fig 2: Evolutionary distances between the bacterial isolates

ISSN No:-2456-2165

IV. DISCUSSION

Based on the physicochemical parameters of the soil sample from the three locations (Igbira-camp (S1), Iyamho (S2) and Auchi (S3)), it is evident that the organic content of Iyamho soil is very high which encouraged the growth of these bacteria (diazotroph) and these result correspond with the result of the experiment which also showed that Iyamho has the highest number of the diazotrophs. Other parameters such as Organic carbon, pH, Electrical conductivity were also checked and the concentrations varied across the different samples which could be as a result of the different inputs into the soils which forms the basis for the physicochemical properties and which consequently shapens the microbial community. The organic matter content in samples from Igbira-camp and Auchi environment were lower than sample from Iyamho community which could attributed to the exposure the soils to agro-industrial related activities and chemicals inputs compared to Iyamho. The application of too many synthetic chemicals may also contribute to the reduction of these microorganisms (diazotrophic bacteria) within the location of minimal count which agrees with a similar observation by [35]. The Gram reaction revealed that the bacteria isolated were both Gram negative (-ve), and Gram positive (+ve), and also results from microscopic observation showed that all isolates were either cocci (singled and clustered) or rod shaped. All

The use of 16S rRNA in the characterization of bacterial isolates from environmental samples has been demonstrated by previous authors to be more reliable and sensitive than conventional phenotypic systems. In this study, bacterial isolates with diazotrophic potentials were characterized using I6S rRNA molecular typing and only four were successfully identified. The bacteria identities were *Priestia flexa* (OK426387), *Providencia stuartii* (MK641351), *Pseudomonas plecoglosscida* (HM209783) and *Alcaligenes faecalis* (OK083684). In this research about 50% of the isolates happened to be from the genera of *Pseudomonas* based on biochemical characterization and this result corroborates with the findings of Rodriguez-Blanco et al. [37] in which *Pseudomonas* and *Enterobacter* were reported the most abundant diazotrophic bacteria. However this shows that the microbial community within the plant root region and free living microbes in arable soils could differ in their diversity, abundance and function because of the different chemical and physical conditions within both environments which shapens microbial communities.

Notably, all isolates from this study with potential ability as nitrogen fixers have been identified and characterized as diazotrophs in documented literatures. *Priestia flexa* was isolated from the soil environment alongside *Bacillus* and Bacillus related organisms which belongs to the same class as *Priestia*. *Priestia* is a mesophilic bacteria usually isolated from the soil environment and it was formerly *Bacillus flexus* until recently when the nomenclature was changed to *P. flexa*. Although there are scanty reports are associating *P. flexa* to nitrogen fixation and nitrogen uptake, however, several species of *Bacillus* have been documented to be diazotrophs (38). *Providencia stuarti* among 88 N2-fixing bacteria isolated from various farm areas showed the highest rate of acetylene reduction and its effectiveness as microbial inoculant using pot trial. *P stuarti* gave significant increase over inoculated control and in terms of nitrogen content uptake and greater amounts was observed in plants grown with P. stuarti (39). The presence of nitrogen fixers within this genus Pseudomonas has been established in literature. Diverse strains of *Pseudomonas* are known plant growth promoting microorganisms especially in terms of nitrogen fixation and nutrient uptake (40, 41). *Alcaligenes fecalis* genes has also been documented as a known diazotroph based on literature (43).

The perspective on microbial diversity has improved enormously over the past few decades. In large part, this can be due to molecular phylogenetic studies that objectively relate organisms. Also, the phylogenetic trees based on gene sequences are maps which aid in describing the concept of evolutionary biodiversity (Yang *et al*., 2015; Thomas *et al*., 2016). In this study, the phylogenetic analysis of small-subunit (16S rRNA) sequences show that the bacterial community from all samples fall into two phyla, Proteobacteria and Bacillota. Generally, the bacterial isolates were affiliated to two phyla (Proteobacteria and Bacillota) and three classes (Bacilli, Gammaproteobacteria and Betaproteobacteria). However, Proteobacteria and Gammaproteobacteria were the dominant phylum and class, respectively and this corresponds with the findings of Smith *et al*. (2015).

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