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Isolation and Identification of Phosphate Solubilizing and Nitrogen-Fixing Bacteria from Lake Ol'Bolossat Sediments, Kenya

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Abstract

Phosphate solubilizing and nitrogen-fixing bacteria have a vital role in improving soil fertility and reverting adversely affected soil properties. These bacteria could contribute towards sustainable agriculture with a focus on reducing excessive use of commercial fertilizers. This study aimed at investigating autochthonous populations of phosphate solubilizing and nitrogen-fixing bacteria from Lake Ol'Bolossat sediments. The total microbial counts ranged between 4.8 x 10^3 to 8.5 x 10^5 cfu/ml. A total of 50 bacteria were isolated, 34 were obtained from Pikovskaya's agar medium while 16 were obtained from Norris Glucose Nitrogen free medium. Based on morphological and 16S rRNA gene analyses, the isolates were clustered under the genera *Bacillus*, *Arthrobacter*, *Pseudomonas*, *Paenibacillus*, *Fictibacillus* and *Acinetobacter*. Among potentially novel strains, four strains NFDA2, PKGBC1 (MT799539), PKGB5 and SCEC2 (MT799543) belonged to genus *Bacillus*, three strains NFGA1 (MT799529), NFGA4 and SCDB3 belonged to the genus *Pseudomonas*, two strains NFEB6 (MT799528) and NFDC5 belonged to the genus *Paenibacillus*, one strain PKHC3 (MT7995441) belonged to the genus *Arthrobacte*r while one strain NFDC4, belonged to the genus *Acinetobacter*. Generally, the phosphate solubilizing bacteria were the most diverse and genera *Bacillus*, *Fictibacillus* and *Pseudomonas* were the most dominant, however, nitrogen-fixing bacteria were dominated by genera *Arthrobacter* and *Pseudomonas.*

Keywords: phosphate-solubilizing, nitrogen fixing, sediment, bacteria, lake

1. Introduction

Rapid growth in industrialization along with the increasing population has led to the increase in the demand for crops (Chennappa et al., 2019) which further increases the pressure on the use of land, water and nutrients to increase crop yields. This is affecting food security, poverty eradication and agricultural sustainability of food systems since the world's population is projected to reach nearly 9.8 billion by 2050 (United Nations, 2017). Among the commonly practiced measure to overcome and increase productivity on existing agricultural lands, is the use of commercial fertilizer and pesticides. All growing plants need seventeen essential elements to grow to their full genetic potential. Out of these seventeen, fourteen are absorbed by plants through the soil, while the remaining three come from air and water (Njinga et al., 2013; Vatansever et al., 2017). Nitrogen, phosphorous, and potassium (NPK) are primary nutrient components in commercial fertilizers that play an important role in plant nutrition. Overreliance on these commercial nutrients, however, have a direct effect on soil microbiological aspects, environmental pollution, and health hazards on reaching the soil in significant quantities (Çakmakçi et al., 2006). These results to alterations in the soil microbial composition, soil fertility and crop productivity; altering soil nitrogen balance, interfering with ammonification, and hindering mycorrhizal symbiosis or nodulation in plants, as well as plant growth, soil structure, organic matter decomposition, and nutrients recycling (Chennappa et al., 2019). A recent study by Kaminsky et al. (2018) showed that excessive fertilization of inorganic phosphorous change microbiome composition thus affecting plant growth.

With the widespread public awareness, the negative effects of chemical fertilizers on soils, there has been increasing interest among scientists and engineers to develop environmentally friendly technologies to boost and sustain agricultural production systems. Therefore, innovative methods are needed to protect and enhance natural resources, increase agricultural productivity and sustainability. Several claims have been reported on the positive

effect of organic-rich sediments bio-deposit on plant growth (Grantina-Ievina et al., 2014). The nutrients, microorganisms and enzymes contained in the bio-deposit assist in plant growth by affecting the activity of organic substrates. They can revive dead ground, thus reactivate soil functions, and give it highly fertile properties by forming humus. Further, sediment microorganisms have unique properties since they have to adapt to extreme environmental conditions (John and Salim, 2020).

Conversion of phosphate into readily available form for mineralization and solubilization processes is done by phosphate solubilizing microbes (Behera et al., 2017) making it available for plants uptake. Although soil possesses total phosphorous in organic and inorganic forms, essentially, they remain inactive and or bound to soil constituents making them unavailable for utilization by plants. Even with supplemented phosphorous, about 70–90% of chemical phosphorus fertilizers added to the soil become fixed by forming metal-cation precipitate complexes thus making them unavailable (Kalayu, 2019). Therefore, the insoluble forms of phosphate are converted into soluble forms via phosphate solubilizing microbes through the organic acid production, chelation and exchange reactions (Behera et al., 2017). The process is also achieved by the action of phosphatase enzymes (acid phosphatases) (Liu et al., 2015). The requirements of nitrogenous fertilizer can be reduced by converting inert nitrogen to ammonia through biological nitrogen fixation process using microbes like rhizobia as they form nodules in the roots of leguminous plants and fix atmospheric nitrogen (Singha et al., 2017). They are also known for different plant growth-promoting activities including indole acetic acid (IAA) production, siderophore and ammonia, solubilization of inorganic phosphate among others. Numerous genera such as *Azotobacter*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Arthrobacter*, *Burkholderia*, *Pseudomonas*, *Serratia* among others have been studied and used as biofertilizers as reported by various authors and identified them as plant growth-promoting bacteria (PGPB) (Singh et al., 2019).

Recent studies have also been reported worldwide indicating the importance of phosphate solubilizing and nitrogen-fixing bacteria as biofertilizers, yet scanty information is available on the microorganisms from the Lake sediments in Kenya. Also, many microbes may be indigenous isolates well adapted to a specific ecological niche, local climatic environment conditions, as well as cropping systems (Panda et al., 2016). Undocumented information shows the use of the sediment from Lake Ol'Bolossat by farmers around the lake, which recorded an increase in crop yield. The study aimed at isolating, characterizing, and identifying indigenous nitrogen-fixing bacteria and phosphate solubilizing bacteria from Lake Ol'Bolossat sediments.

2. Method

2.1 Sample Site and Collection

Lake Ol'Bolossat is located in highlands of central Kenya in Nyandarua County (Figure 1) with a catchment area of 340 km^2, it covers the land area of 38 km^2, it is the headwater for the 210, 0000 km^2 Ewaso Ngiro North Basin and it flows through Thompson falls. Sediments from the bottom of the lake were collected from four different locations in lake Ol'Bolossat site 1 (0^o 10'.12.6 S/36^o 26'.48E), site 2 (0^o 08'.24S/36^o 26'.1.8E), site 3 (0^o 4'.45.7S/36^o 24'.51E), and site 4 (0^o 6' 40.2S/36^o 25.51.6E). The sediments were collected in triplicates in a 7.62 cm diameter and 90 cm long aluminium core pipes using Specialty Device Inc (SDI) Sediment Sampler Vibecore and Accessories (Wylie Texas, USA). After collection, the core pipes were divided into three sections (0-30 cm, 30-60 cm and 60-90 cm) each representing a separate independent sample. The core pipes were then split open and the sediments were collected separately in sterile Ziplock plastic bags. They were labelled clearly indicating sample details and collection dates and transported at 4 ^oC to Jomo Kenyatta University of Agriculture and Technology (JKUAT) laboratories for bacterial isolation.

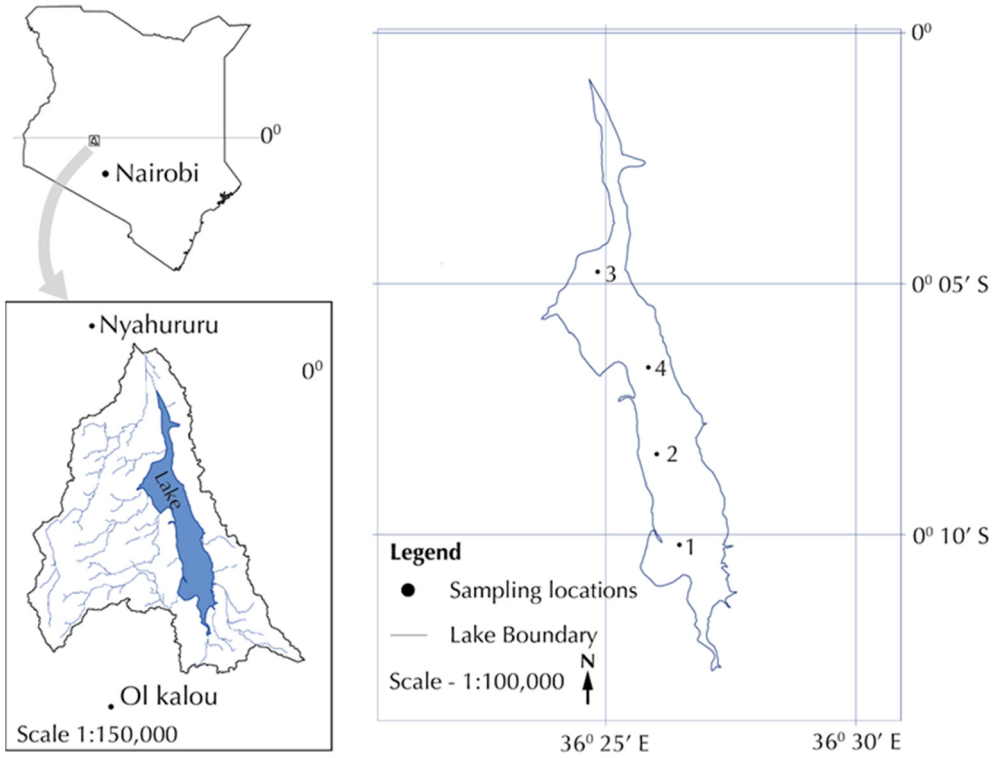

Figure 1. Lake Ol'Bolossat sampling sites in Nyandarua County, Kenya

2.2 Isolation and Enumeration of Phosphate Solubilizing and Nitrogen-Fixing Bacteria

Ten grams of the sediments were suspended in 90 ml of sterile physiological saline (0.85% NaCl) to obtain 10^{-1} dilution, followed by filtration through sterile 125 mm Whatman® qualitative filter paper, Grade 1 (Merck). One ml of the filtrate was transferred to 9 ml of sterile physiological saline to make 10^{-2} dilution. Subsequently, other dilutions were serially made in the range of 10^{-3} to 10^{-8}. The inoculation mixture with serial dilution was then spread in triplicate on the plates containing Pikovskaya's (PKV) agar medium (Pikovskaya, 1948) (Himedia-M520) for the Phosphate solubilizing bacteria (PSB).The medium consisted of yeast extract 0.5 g, dextrose 10.0 g, $Ca_3(PO_4)$ 25.0 g, $(NH_4)_2SO_4$ 0.5 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, $MnSO_4.H_2O$ 0.0001 g, $FeSO_4$ 7H2O 0.0001 g and agar 15.0 in 1 litre distilled water. Nitrogen-fixing bacteria (NFB) were inoculated on a nitrogen-free medium (Norris Glucose Nitrogen free medium (NGNFM), Himedia-M712). NGNFM consisted of glucose 10.0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.2 g, $CaCO_3$ 1.0 g, NaCl 0.2 g, $MoNa_2O_4$ $2H_2O$ 0.005 g, $FeSO_4$ $7H_2O$ 0.1 g and agar 15.0 g in 1 litre distilled water (Ranganayaki and Mohan, 1981). Bacteria counts were determined by plating on both PKV and NGNFM while the total count was carried on Soybean-Casein Digest Agar (Casein Soyabean Digest Agar) (Himedia-MH290). Soybean-Casein Digest Agar medium consisted of tryptone (pancreatic digest of casein) 15.0 g, soya peptone (papaic digest of soyabean (soybean) 5.0 g, NaCl 5.0 g, and agar 15.0 g in 1 litre distilled water. The inoculated plates were incubated at 30 ºC for up to 6 days, and the colony-forming units (CFU) were counted. To measure the survival efficiency the number of bacteria present per gram of original sample were enumerated using the following formula:

$$\text{Viable cell count (CFU/g sample)} = \frac{\text{Number of colonies } (25 - 300 \text{ CFU})}{\text{The volume of inoculum } (0.1 \text{ ml})} \text{ X Dilution factor}$$

The colonies with clear zone on PKV agar were considered positive for phosphate solubilization hence were subcultured on the same media (PKV agar) three times to obtain pure cultures. Colonies that formed on NGNFM agar were considered nitrogen-fixing bacteria thus, were selected and sub-cultured three times by streak method to obtain pure cultures were on the same NGNFM agar media. The pure isolates were cryopreserved in PKV broth for PSB and NGNFM broth for NFB containing 20% glycerol for long term storage at -75 ºC and for further analyses.

2.3 Morphological Characterization of Phosphate Solubilizing and Nitrogen-Fixing Bacteria

Preliminary characterization was performed using morphological and cultural characteristics as described by Benson, (2002). Morphological characterization of the isolates was under the dissecting and compound microscopes to observe cell shape, size and arrangement characteristics after classical Gram-staining and catalase tests were performed as described by Cappuccino and Sherman, (2014) the Gram-reaction was confirmed by 3% (w/v) KOH test (Gregersen, 1978).

2.4 Extraction of Genomic DNA, PCR Amplification and Sequencing of 16S rRNA Gene

Total genomic DNA was extracted from overnight cell cultures grown on PKV broth for PSB and NGNFM broth at 30 ^{o}C using QIAamp DNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Polymerase chain reaction (PCR) was performed in Primus 96 advanced thermal cycler (PEQLAB, Erlangen, Germany). The 16S rRNA gene amplification was performed using the universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). PCR was performed in a 50 µl mixture containing 25 µl 3X *Taq* PCR master mix (Qiagen, Germany), 2.5 µl of each primer, 10 µl of DNA template (50 ng) and 10 µl RNase free water. The reaction mixtures were subjected to the following PCR conditions: initial denaturation at 95 °C for 5 min, followed by 32 cycles consisting of 1 minute denaturation at 95 °C, 1 minute annealing at 55 °C, 2 minutes extension at 72 °C, and a final extension step of 10 minutes at 72 °C. The amplified PCR products were resolved in 1.2% agarose gel stained with ethidium bromide (1 µg/ml) and visualized using a Biotec-Fischer Felix 6050 gel documentation system (ProfiLab24, Germany). PCR products were purified using the QIAquick PCR purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified amplicons were Sanger sequenced at Human Genomics Macrogen Europe (Macrogen Europe B.V, Amsterdam, Netherlands).

2.5 Phylogenetic Analysis

The 16S rRNA gene sequences of the bacterial isolates were viewed for quality checks and edited using ChromasPro 2.1.8 software package (http://technelysium.com.au/wp/). They were then compared with available standard sequences of bacteria lineages in the public nucleotide sequence databases in the National Center for Biotechnology Information (NCBI) using nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find closely related bacterial 16S rRNA gene sequences. The 16S rRNA gene sequences of the isolates and those of the unknown closely related bacteria strains were aligned using Clustal W software, phylogenetic trees were constructed using Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with MEGA (Molecular Evolutionary Genetics analysis) 7.0 software package (Kumar et al., 2016). The trees topologies were evaluated using the bootstrap resampling method (Felsenstein, 1985) based on 1000 replicates.

2.6 Statistical Microbial Analysis

The microbial enumeration results were expressed as the mean and standard deviation of triplicate experiments of the counts and sampling sites using two-way ANOVA (GraphPad Prism version 8.4.2 software, GraphPad LLC, San Diego, California, USA), at a significance level of $p<0.05$, multiple range tests were performed using Tukey's test.

3. Results

3.1 Isolation of Phosphate Solubilizing and Nitrogen-Fixing Bacteria from the Sediment

A total of 50 bacteria were isolated from lake Ol'Bolossat sediment out of which 34 were isolated on PKV agar medium (Table 2) and had the potential for phosphate solubilizers due to clear zone around the colonies. Similarly, 16 were isolated on Norris Glucose Nitrogen free medium (Table 1) and thus were considered nitrogen-fixing bacteria (Figure 2).

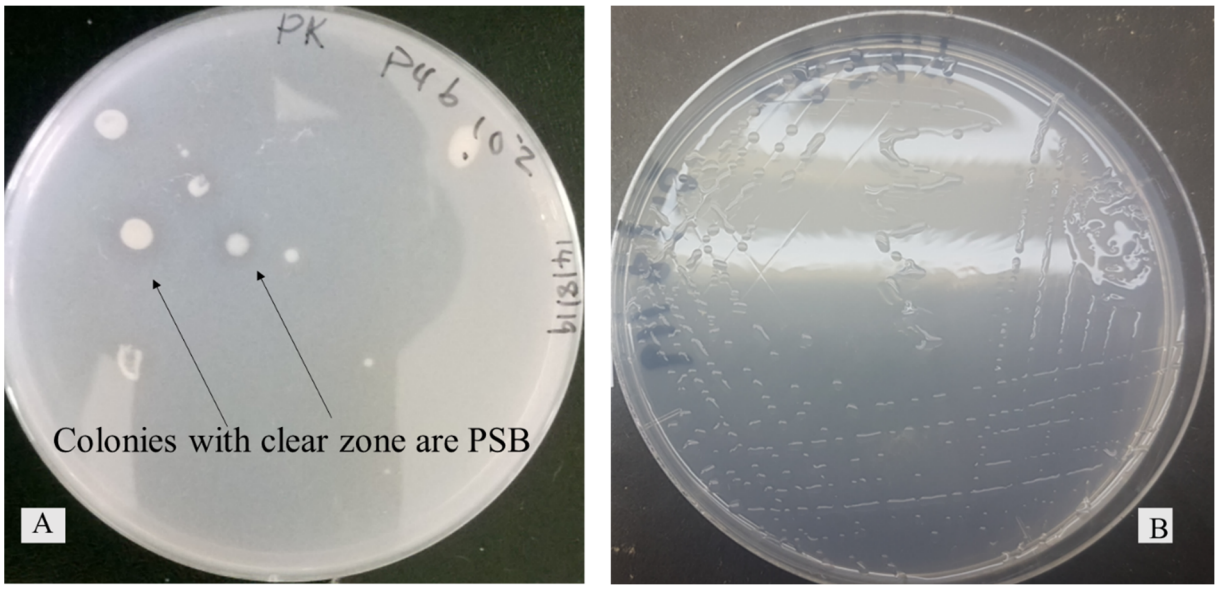

Figure 2. Pure colonies of phosphate solubilizing bacteria with clear zones (A) on Pikovskaya's agar and nitrogen-fixing bacteria (B) on Norris Glucose Nitrogen free medium

3.2 Enumeration of Phosphate Solubilizing, Nitrogen-Fixing and Total Microbial Counts

The microbial counts varied significantly between the sampling site and depth (Figure 3). In general, the highest microbial counts were recorded at 0-30 cm depth for all sampling points, the results showed microbial counts decreased with increase in depth, notably at the depth of 0-30 cm, 30-60 cm and 60-90 cm, for example, phosphate solubilizing bacteria counts ranged between 2.7 x 10^3 to 7.4 x 10^5 cfu/ml, nitrogen-fixing bacteria counts were 1.9 x 10^3 to 4.6 x 10^5 cfu/ml while total microbial counts ranged between 4.8 x 10^3 to 8.5 x 10^5 cfu/ml (Figure 3). The analysis of variance (ANOVA) showed a statistically significant difference between microbial counts and sampling points ($p < 0.05$).

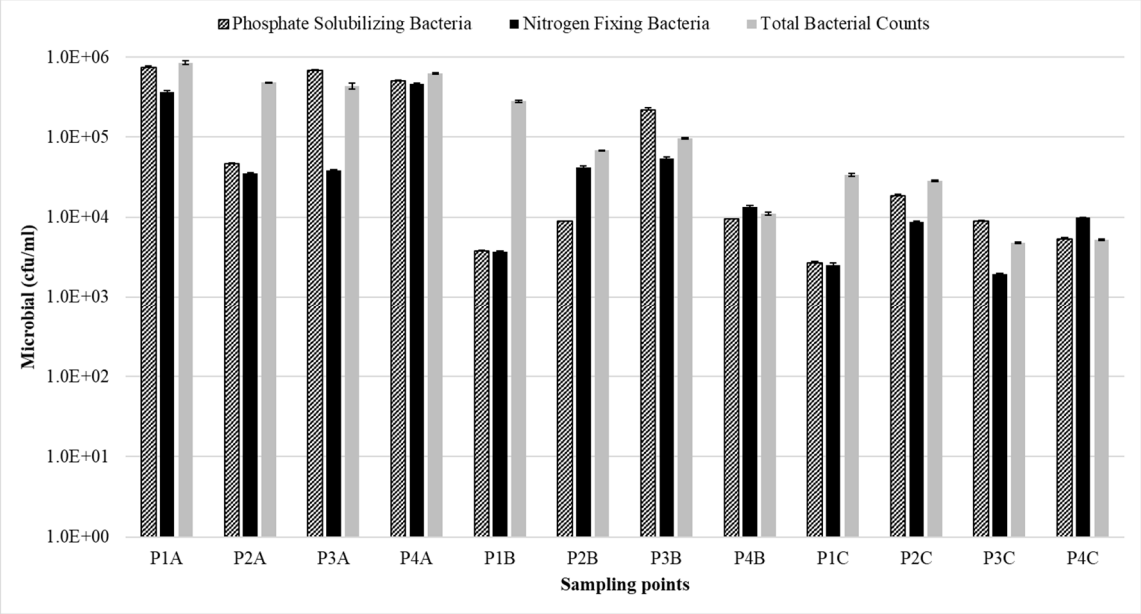

Figure 3. Viable phosphate solubilizing, nitrogen fixing and total bacterial counts from Lake Ol'Bolossat sediment

P1=Sampling point 1 (A=0-30cm depth, B=30-60 cm, C= 60-90 cm), P2=Sampling point 2 (A=0-30cm depth, B=30-60 cm, C= 60-90 cm), P3=Sampling point 3 (A=0-30 cm depth, B=30-60 cm, C= 60-90 cm) P4=Sampling point 4 (A=0-30cm depth, B=30-60 cm, C= 60-90 cm). Counts were determined in triplicates; the standard deviations are indicated.

3.3 Colony Morphologies and Identities of Phosphate Solubilizing and Nitrogen-Fixing Bacterial Strains

Morphological characterization of phosphate solubilizing and nitrogen-fixing bacteria was based on classical macroscopic techniques of colour, form, shape, and elevation of pure colonies. Most colonies were able to grow within 2-6 days of incubation at 30 °C. Bacterial species were further examined for their Gram's reaction, shape and catalase activity. Characteristically, all the isolates were catalase-positive, 86% were Gram-positive, while 14% were Gram-negative and all were rod-shaped. Nitrogen-fixing bacteria strains are shown in Table 1 while phosphate solubilizing bacteria strains are shown in Table 2.

Table 1. Colony morphologies and molecular identities of nitrogen-fixing bacteria strains

	Colony characterization				Cell characterization			Molecular identification		
Sample ID	**Color**	**Form**	**Elevation**	**margin**	**cell shape**	**Gram status**	**Catalase**	**Closest relatives**	**% identity**	**Accession no.**
NFGA3	White	Circular, mucoid	Raised	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.61	MK641666.1
NFGB5	White	Circular, mucoid	Raised	Entire	Rods	+	+	*Arthrobacter oryzae*	99.87	LR216745.1
NFHC4	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.87	LR216745.1
NFEB6	White	Circular ,moist	Flat	Undulate	Rods	+	+	*Paenibacillus graminis*	98.47	KF010806.1
NFGB4	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.39	KY514151.1
NFGB2	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.21	KF387691.1
NFDC5	White	Circular, dry	Flat	Undulate	Rods	+	+	*Paenibacillus caespitis*	97.45	AM745263.1
NFGA1	Brown	Irregular	Raised	Entire	Rods	-	+	*Pseudomonas fluorescens*	96.25	MN511732.1
NFDA2	White	Irregular	Flat	Ciliate	Rods	+	+	*Bacillus subtilis*	96.77	KX109607.1
NFGA4	White	Irregular	Flat	Entire	Rods	-	+	*Pseudomonas moraviensis*	96.25	MN752870.1
NFHC3	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.21	KF387691.1
NFHB1	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.39	KY514151.1
NFHA2	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.61	MK641666.1
NFDC4	Cream	Circular	Entire	Raised	Rods	-	+	*Acinetobacter sp*	85.82	AY961054.1
NFEB5	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter oryzae*	99.90	KY514151.1
NFHB2	Brown	Irregular	Raised	Entire	Rods	+	+	*Arthrobacter oryzae*	99.21	KF387691.1

Table 2. Colony morphologies and molecular identities of phosphate solubilizing bacteria strains

	Colony characterization				Cell characterization			Molecular identification		
Sample ID	**Color**	**Form**	**Elevation**	**margin**	**cell shape**	**Gram status**	**Catalase**	**Closest relatives**	**% identity**	**Accession no.**
PKGB6	Brown	Circular	Raised	Smooth	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKGBC1	White	Circular	Flat	Ciliate	Rods	+	+	*Bacillus mangrovi*	95.42	MK302238.1
PKDC1	Brown	Irregular	Flat	Entire	Rods	+	+	*Brevibacterium frigoritolerans*	99.79	MN750768.1
PKEB2	Brown	Irregular	Flat	Entire	Rods	+	+	*Bacillus sp.*	96.98	KY457750.1
PKGB5	Brown	Irregular	Raised	Ciliate	Rods	+	+	*Bacillus simplex*	96.82	MK484354.1
PKGB9	Brown	Irregular	Flat	Ciliate	Rods	+	+	*Bacillus simplex*	96.82	MK484354.1
PKEC6	White	Circular	Flat	Smooth	Rods	+	+	*Bacillus simplex*	96.82	MK484354.1
PKGC9	Cream	Irregular	Flat	Smooth	Rods	+	+	*Bacillus simplex*	96.82	MK484354.1
PKECC1	White	Circular, mucoid	Flat	Smooth	Rods	+	+	*Arthrobacter sp.*	96.27	MN596140.1
PKGB4	Brown	Round	Raised	Ciliate	Rods	+	+	*Bacillus zhangzhouensis*	99.50	KY622201.1
PKHBC2	Cream	Irregular	Flat	Smooth	Rods	+	+	*Fictibacillus enclensis*	99.17	MK396594.1
PKGB3	Brown	Irregular	Flat	Smooth	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKEC1	White	Irregular	Flat	Smooth	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKHC3	Cream	Irregular	Flat	Smooth	Rods	+	+	*Arthrobacter scleromae*	97.37	HQ202866.1
PKCC7	Cream	Irregular	Flat	Smooth	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKEA9	Brown	Irregular	Flat	Entire	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKEC3	Brown	Circular	Flat	Entire	Rods	+	+	*Bacillus simplex*	98.84	MK484375.1
PKEC2	Brown	Irregular	Flat	Smooth	Rods	+	+	*Bacillus megaterium*	99.03	KU667114.1
PKEBC3	White	Circular	Entire	Irregular	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKHBC1	White	Circular	Raised	Raised	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKGC4	Brown	Round	Raised	Entire	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKHA9	White	Round	Raised	Entire	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKEB3	Brown	Round	Raised	Smooth	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
PKHC3	White	Circular	Flat	Smooth	Rods	+	+	*Bacillus megaterium*	99.81	MN704496.1
SCHC4	Brown	Circular	Raised	Smooth	Rods	-	+	*Pseudomonas putida*	99.90	MN560132.1
SCGB6	Brown	Circular	Raised	Smooth	Rods	+	+	*Fictibacillus rigui*	99.37	MH910274.1
SCGB9	Cream	Circular	Flat	Smooth	Rods	+	+	*Fictibacillus rigui*	99.37	MH910274.1
SCDC1	Brown	Round	Raised	Entire	Rods	+	+	*Fictibacillus rigui*	99.37	MH910274.1
SCGB7	Brown	Round	Raised	Entire	Rods	+	+	*Bacillus pumilus*	99.90	JF738125.1
SCEC2	White	Irregular	Flat	Entire	Rods	+	+	*Bacillus thuringiensis*	98.06	MG738336.1
SCDB3	Brown	Round	Raised	Smooth	Rods	-	+	*Pseudomonas mendocina*	97.67	DQ178219.1
SCGB13	Brown	Circular	Raised	Smooth	Rods	+	+	*Fictibacillus rigui*	99.59	MH910274.1
SCDB4	Brown	Irregular	Flat	Ciliate	Rods	-	+	*Pseudomonas putida*	99.59	MN560132.1
SCGB4	Brown	Circular	Raised	Smooth	Rods	-	+	*Pseudomonas putida*	99.59	MN560132.1

3.4 Identification and Phylogenetic Analysis of Isolates

Based on the blastn search performed against the GenBank and phylogenetic analysis, sixteen NFB isolates showed similarities of 85.82% to 99.90% between available GenBank entries in which ten isolates, NFGA3 (MT799530), NFGB5 (MT799532), NFHC4 (MT799532), NFGB4 (MT799527), NFGB2 (MT799531), NFHC3 (MT799531), NFHB1, NFHA2 (MT799530), NFEB5 (MT799527) and NFHB2 were affiliated with *Arthrobacter oryzae*, NFEB6 (MT799528) was affiliated with *Paenibacillus graminis*, NFDC5 was affiliated with *Paenibacillus caespitis*. Isolate NFDA2 was affiliated with *Bacillus subtilis*, while two isolates, NFGA1 (MT799529) and NFGA4 were affiliated with *Pseudomonas fluorescens* and *Pseudomonas moraviensis* respectively and isolate NFDC4 belonged to *Acinetobacter* sp (Table 1). Phylogenetically, NFB had four distinct groups corresponding to genera *Arthrobacter* sp*., Bacillus* sp*, Pseudomonas* sp and *Acinetobacter* sp. In the phylogenetic group of genus *Arthrobacter*, isolates NFGA3 (MT799530), NFGB5 (MT799532), NFHC4 (MT799532), NFGB4 (MT799527), NFGB2 (MT799531), NFHC3 (MT799531), NFHB1, NFHA2 (MT799530), NFEB5 (MT799527) and NFHB2 (MT799531) were associated to *Arthrobacter oryzae*, isolate NFDA2 was associated to *Bacillus subtilis,* isolates NFEB6 (MT799528) and NFDC5 were associated to *Paenibacillus graminis* while isolates NFGA1 (MT799529) and NFGA4 formed a cluster associated to *Pseudomonas fluorescens*/*P. moraviensis* and isolate NFDC4 affiliated with *Acinetobacter* sp (Figure 4). These sequences were submitted to the NCBI database and the accession numbers were obtained (MT799527[ACCN]: MT799546[ACCN]).

Additionally, the blast search result performed against the GenBank revealed that thirty-four phosphate solubilizing bacterial isolates had similarities of 95.42% to 99.90% between available GenBank entries in which twelve isolates PKGB3 (MT799537), PKGB6, PKECI, PKCC7, PKEA9, PKEC2 (MT799534), PKEBC3, PKHBC1, PKGC4, PKHA9 and PKEB3, were affiliated with *Bacillus megaterium*, five isolates, PKGB5, PKGB9, PKEC6, PKGC9 and PKEC3 (MT799535) were affiliated with *Bacillus simplex*, four isolates SCGB6, SCGB9 (MT799546), SCDC1 and SCGB13 were affiliated with *Fictibacillus rigui*, three isolates SCDB4, SCHC4 (MT799544) and SCGB4 (MT799544) were affiliated with *Pseudomonas putida*, isolate PKGBC1 (MT799539) was affiliated with *Bacillus mangrovi*, PKDC1 (MT799533) was affiliated with *Brevibacterium frigoritolerans*, PKEB2 was affiliated with *Bacillus* sp., PKECC1 (MT799536) was affiliated with *Arthrobacter* sp., PKGB4 (MT799538) was affiliated with *Bacillus zhangzhouensis*, PKHBC2 (MT799540) was affiliated with *Fictibacillus enclensis*, PKHC3 (MT7995441) was affiliated with *Arthrobacter scleromae*, SCGB7 (MT799545) was affiliated with *Bacillus pumilus*, SCEC2 (MT799543) was affiliated with *Bacillus thuringiensis* and SCDB3 (MT799542) was affiliated with *Pseudomonas mendocina* (Table 2). Three distant phylogenetic groups corresponding to the genera *Bacillus* sp, *Arthrobacter* sp and *Pseudomonas* sp were obtained (Figure 5). In the phylogenetic group of genus *Bacillus*, six different sub-groups were identified, these included five isolates PKGB3 (MT799537), PKEC2 (MT799534), PKHBC1, PKGC4 and PKHA9 were closely related to *Bacillus megaterium*, three isolates PKHBC2 (MT799540), SCGB9 (MT799546) and SCGB13 were closely related to *Fictibacillus* sp*.*, three isolates PKGB9, PKEC3 (MT799535) and PKDC1 (MT799533) formed a sub-cluster closely related to *Bacillus simplex/Brevibacterium frigoritolerans*, two isolates SCGB7 (MT799545) and PKGB4 (MT799538) were closely related to *Bacillus pumilus/B. zhangzhouensis*, isolate SCEC2 (MT799543) clustered together with *Bacillus thuringiensis* and PKGBC1 (MT799539) was closely related to *Bacillus mangrovi.* In the phylogenetic group of genus *Arthrobacter* isolates PKECC1 (MT799536) and PKHC3 (MT7995441) were closely related to *Arthrobacter scleromae* while isolates SCGB4 (MT799544) and SCHC4 (MT799544) formed a cluster closely related to *Pseudomonas putida*, and isolate SCDB3 (MT799542) was closely related to *Pseudomonas mendocina* (Figure 5). These sequences were submitted to the NCBI database and the accession numbers were obtained (MT799527[ACCN]: MT799546[ACCN]).

The 16S rRNA of NFEB6 (MT799528) shared 98.47% similarity with *Paenibacillus graminis* (KF010806.1), NFDC5 showed 97.45% similarity with *Paenibacillus caespitis* (AM745263.1), NFGA1 (MT799529) shared 96.25% similarity with *Pseudomonas fluorescens* (MN511732.1), NFGA4 shared 96.25% similarity with *Pseudomonas moraviensis* (MN752870.1), NFDA2 shared 96.77% similarity with *Bacillus subtilis* while NFDC4 shared 85.82% similarity with *Acinetobacter* sp (AY961054.1). This study, therefore, suggested that these strains could be potentially novel NFB species. Moreover, the 16S rRNA gene sequences of PKGBC1 (MT799539) exhibited a 95.42% similarity with the *Bacillus mangrovi* (MK302238.1), PKGB5 exhibited a 96.82% similarity with *B. simplex* (MK484354.1), PKHC3 (MT7995441) shared a 97.37% similarity with *Arthrobacter scleromae* (HQ202866.1) and SCEC2 (MT799543) exhibited a 98.02% similarity with *B. thuringiensis* (MG738336.1) thus signifying that they are potentially new PSB species (Kim et al., 2014).

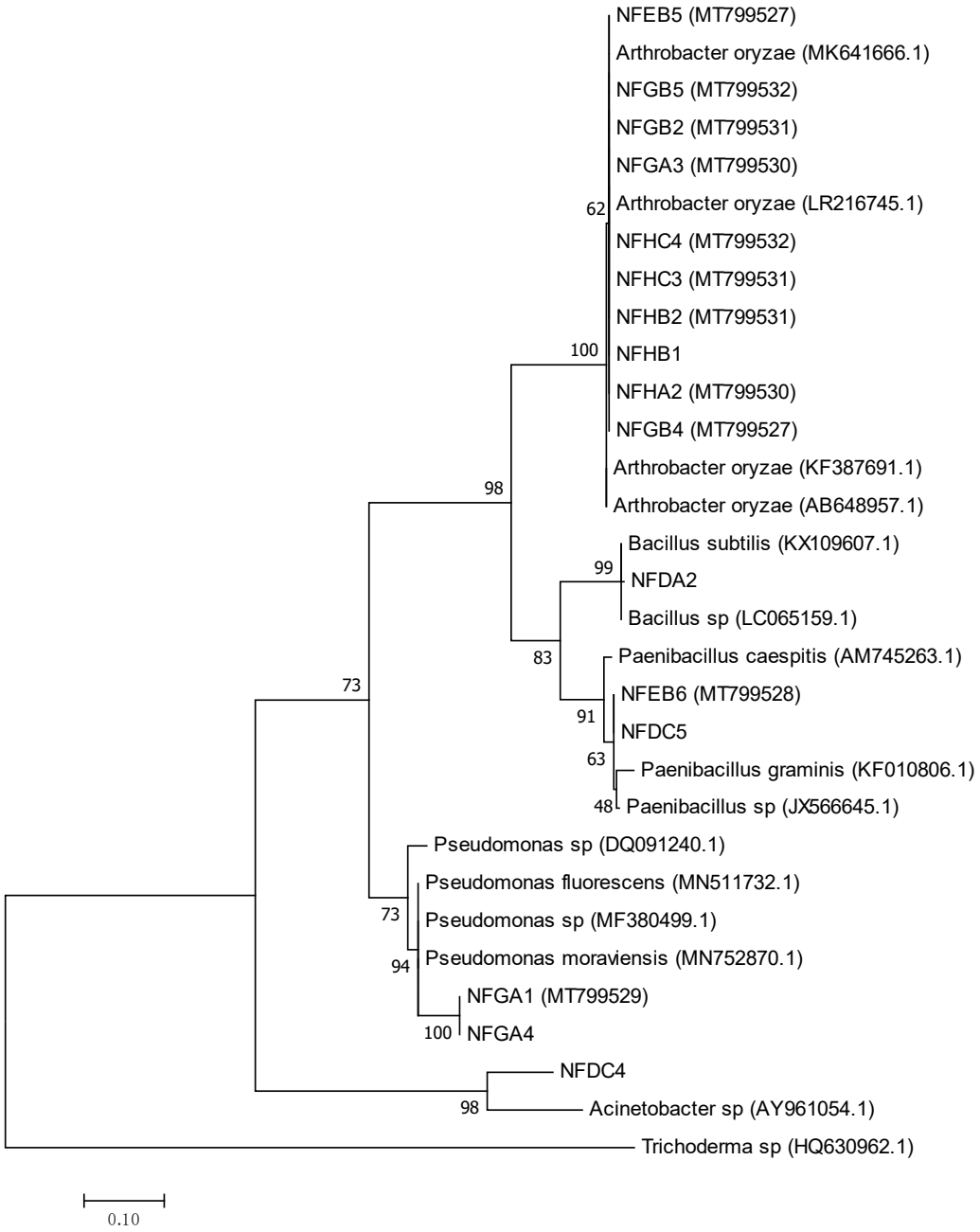

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship among the nitrogen-fixing bacterial isolates and between representatives of other related taxa. The tree was constructed using MEGA 7.0 software package and the distance matrix inferred by the Maximum Likelihood method based on the Tamura-Nei model. The scale bar indicates 0.1 substitutions per nucleotide position. The number beside the node is the statistical bootstrap value. In brackets are the GenBank accession numbers. The gene sequence of Trichoderma sp. (HQ630962.1) was used as an out-group.

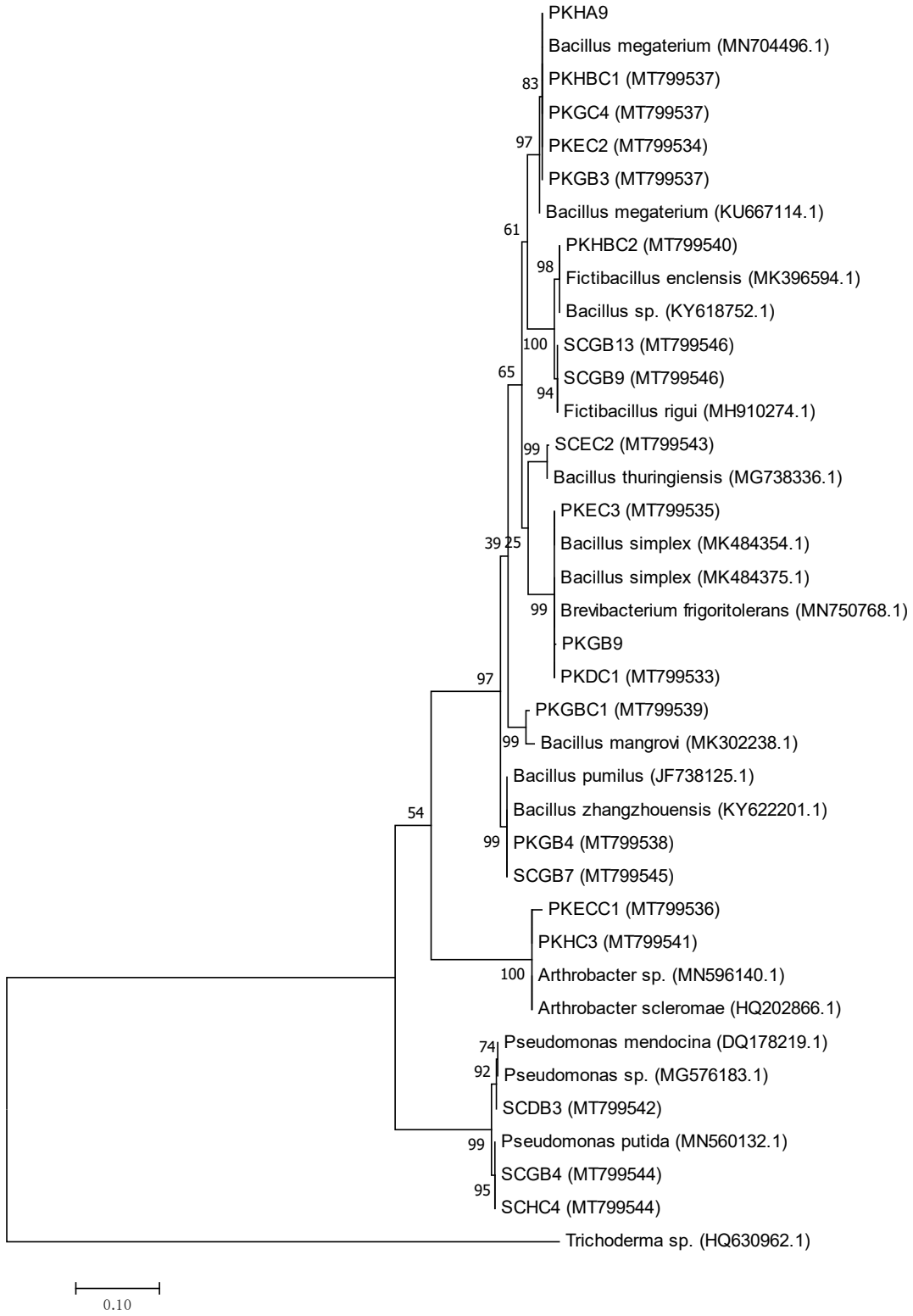

Figure 5. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship among selected phosphate solubilizing bacterial isolates and between representatives of other related taxa. The tree was constructed using MEGA 7.0 software package and the distance matrix inferred by the Maximum Likelihood method based on the Tamura-Nei model. The scale bar indicates 0.1 substitutions per nucleotide position. The number beside the node is the statistical bootstrap value. In brackets are the GenBank accession numbers. The gene sequence of Trichoderma sp. (HQ630962.1) was used as an out-group.

4. Discussion

Lake Ol'Bolossat was selected for the isolation of phosphate solubilizing bacteria (PSB) and nitrogen-fixing bacteria (NFB) because of its greater potential as an alternative source of cheap biofertilizer. Currently, undocumented data indicates that local people living around this lake, are utilizing the sediment in agricultural production with positive results. Phosphate solubilizing bacteria (PSB) are usually screened by a plate assay method on Pikovskaya's medium. The medium allows the bacteria to grow and form clear zones around the colonies as a result of the conversion of tricalcium phosphate in the medium from insoluble to soluble forms (Pikovskaya, 1948). The formation of clear zone around the bacterial colonies (Figure 2) could be attributed to the production of phosphatase enzymes by phosphate solubilizing bacteria (Halder and Chakrabartty, 1993; Paul and Sinha, 2013). The PSB and NFB counts were in line with Stankevica et al. (2015) who showed that freshwater sediments are highly populated with microorganisms ranging between 5.2×10^3 to 6.9×10^6 cfu/g of dry matter. PSB are commonly found in most soils, although their count varies depending upon the conditions of soil and climate with a high concentration in the rhizosphere in comparison with non-rhizosphere soils (Rafi et al., 2019). The quantity of living cells is among the quality parameters within the biofertilizer regulation in international standards for many countries (Malusá & Vassilev, 2014). The standard has different groups of microorganisms (rhizobia, for fast or slow-growing species; N-fixing bacteria; phosphorous solubilizing bacteria (PSB), classified based on the ability to act on organic or inorganic phosphate. However, the quantity of living cells varies from $>0.5 \times 10^9$ cfu/ml to $>0.1 \times 10^9$ cfu/ml and $>1.5 \times 10^9$ cfu/g to $>0.2 \times 10^9$ cfu/g, for liquid and solid products, respectively subject to the kind of bacteria used in the production of the biofertilizer (Malusá & Vassilev, 2014). The difference in the cfu in comparison to the obtained results could be attributed to the fact that microbes may be local isolates that are native to the location, sediment type and climatic conditions (Panda et al., 2016).

Based on morphological characteristics and 16S rRNA sequencing, PSB were grouped under the genus *Bacillus*, *Arthrobacter* and *Pseudomonas* (Figure 5). From table 2 the most dominant phosphate solubilizing bacteria were Gram-positive and catalase-positive belonging to the genus *Bacillus*. *Bacillus* and *Pseudomonas* are common genera for solubilization of phosphate since they can convert the insoluble form of phosphate into soluble one (Rafi et al., 2019). Eleven isolates were affiliated with *Bacillus megaterium*, which have been described by Burgos et al. (2015) and Akinrinlola et al. (2018) as Gram-positive, endospore-forming, rod-shaped plant growth-promoting bacteria. This bacterium has been previously reported to increase the grain yield of rice by approximately 103%–256% in an autoclaved soil either as a single culture or in combination (Khan et al., 2003). According to El-Komy, (2005), wheat inoculated with *B. megaterium* exhibited higher shoot dry weight, total nitrogen yield and phosphate contents. Studies by Nascimento et al. (2020) and Wu et al. (2019) show *B. megaterium* possess genetic elements that play a role in xenobiotic degradation, stress resistance, pathogen antagonistic activities, and other soil rhizosphere colonization traits. Five isolates were closely related to *B. simplex*, a bacterium species reported by Schwartz et al. (2013) to modify the architecture of the roots by enhancing the formation of more lateral roots in pea plants. The bacterium species also enabled the development of larger and highly clustered nodules when pea roots were co-inoculated with either *B. simplex* or *Rhizobium leguminosarum*. According to El-Komy, (2005), *B. simplex* has the abilities to solubilize phosphate and enhancing iron availability to the plants.

From the results, five isolates were affiliated with genus *Fictibacillus* with four associated with *Fictibacillus rigui* (formerly known as *Bacillus rigui*) while one was associated with *F. enclensis* (Table 2 and Figure 4). Just like other members of the genus they are Gram-positive, aerobic and endospore-forming commonly isolated from varied habitats like freshwater wetlands, marine sediments, hot springs and industrial wastes (Baik et al., 2010; Glaeser et al., 2013). Members of genus *Fictibacillus* are known to produce siderophores, indole acetic acid as well as participate in phosphate solubilization and nitrogen-fixation (Battini et al., 2016). Studies by Ansari et al. (2019), have shown that *B. pumilus* can produce strong biofilm with enhanced indole acetic acid, exopolysaccharides, deaminase and phosphate solubilization activities. *Bacillus zhangzhouensis* was phylogenetically clustered together with *B. pumilus* (Figure 5), this bacterium is mainly isolated from aquaculture water and sea sediments (Liu et al., 2016). *B. zhangzhouensis* has been reported as a good plant growth-promoting bacteria through phosphate solubilization (Emami et al., 2019). One isolate was identified and clustered together with *B. thuringiensis* (Figure 5). According to Wang et al. (2014), *B. thuringiensis* is an excellent phosphate solubilizing bacteria since when inoculated with the soil, *B. thuringiensis* had a positive effect on the plant growth characteristics of peanuts (*Arachis hypogeae*) as well as seed weight and crude protein content.

An isolate PKGBC1 (MT799539) clustered together with *B. mangrovi*, was isolated from sediments of the

mangrove forest in India (Gupta et al., 2017). Although it belongs to PGPB there is limited information on the exact role it plays in plant growth promotion. Members of the genus *Pseudomonas,* are usually Gram-negative, rod-shaped bacteria commonly found in soils, sediments, plant rhizosphere and freshwater as saprophytes (Bossis et al., 2000). *Pseudomonas putida* (Table 2), is an excellent plant growth-enhancing bacteria through the production of indole acetic acid (Bharucha et al., 2013). The study by Tiwari et al. (2016), has demonstrated the ability of the *P. putida* to ameliorate drought resistance stress through the production of abscisic acids (ABA) in chicken pea. *P. mendocina* has been reported as important nitrogen-fixing bacteria for improving soil nitrogen content (Sharma and Singh, 2020). *Pseudomonas fluorescens* was reported to produce, indole acetic acid, ABA and the gibberellins with potential as biocontrol, enhancement in the length of stem and roots, the germination rate of various plants (Salomon et al., 2014).

Various species of *Arthrobacter* are found in varied environments like freshwater, soil, plant rhizosphere and marine habitats and are implicated in the promotion of plant growth. Ten isolates phylogenetically clustered with *A. oryzae* (Figure 4), were isolated. Recent study indicate that this bacterium is responsible for the degradation of heavy metals from contaminated lake sediments (Cho et al., 2019). *A. koreensis* has shown to benefit plants withstand desiccation due production of xeroprotectants (Manzanera et al., 2015), while *Arthrobacter pokkalii* promotes plant growth under high salt concentrations in agricultural soils (Krishnan et al., 2016) thus protecting them from abiotic stress while improving plant health, nutrition and yield. Evidence shows some species of *Arthrobacter* can degrade various xenobiotic compounds like 4-chlorophenol and 4-nitrophenol (Sahoo et al., 2011; Arora and Jain, 2013).

Phylogenetic analysis showed two isolates formed a cluster with *Paenibacillus* sp (Figure 4). Members of this group are designated as nitrogen-fixing bacteria and are widely distributed in nature such as plant rhizosphere, soil, animal and humans (Grady et al., 2016). Members of genus *Paenibacillus* are usually Gram-positive, rod-shaped, facultatively anaerobic, endospore-forming bacteria (Liu et al., 2019). According to Liu et al. (2019), most *Paenibacillus* species possess *nifH* genes for encoding Fe protein with nitrogenase activities important in fixation of atmospheric nitrogen, production of siderophore and indole-acetic acid. There is also strong evidence that *Paenibacillus polymyxa* produces various antimicrobial and insecticidal compounds against different pathogenic fungi, bacteria and nematodes in cucumber, wheat and tomatoes (Liu et al., 2019; Grady et al., 2016).

Bacillus subtilis is an important model organism commonly used in agriculture as plant growth-promoting rhizobium (PGPR), its crucial in solubilizing soil phosphates, increase nitrogen fixation, facilitates iron absorption by plants via siderophores production and promotes plant growth by suppressing fungal pathogens (Adam et al., 2014; Hashem et al., 2019). Protection of plants by this bacterium is thought to involve biofilms formation on the plant roots (Chen et al., 2012). According to Bais et al. (2004), *B. subtilis* is an excellent biocontrol agent against pathogenic *Pseudomonas syringae* in tomatoes. A study on kiwi fruit showed that the bacteria improves the root growth mass through the production of indole-acetic acid (Erturk et al., 2010). Another important PGPR isolated was clustered together with the genus *Acinetobacter* (Figure 4). Members of this species are associated with plant growth promotion via mineral solubilization, siderophore production, inhibition of phytopathogenic fungi such as *Fusarium oxysporum*. They have also been reported to enhance plant growth characteristics in pearl millet (Rokhbakhsh-Zamin et al., 2011). Additionally, studies by Wafula et al. (2012) isolated *Acinetobacter* spp from tea soils in Kenya which showed promise as potential biofertilizers and indicators of soil health.

5. Conclusion

In total 50 bacteria were isolated and identified from the sediments of Lake Ol'Bolossat. Based on 16S rRNA gene analyses, the identified bacterial strains belonged to the genera *Bacillus*, *Arthrobacter*, *Pseudomonas*, P*aenibacillus*, *Fictibacillus* and *Acinetobacter*. The study has demonstrated that Lake Ol'Bolossat harbors diverse bacteria species with potential for plant growth promotion. The results highlighted the significance of the isolated strains in phosphate solubilization and nitrogen-fixation and could be used as potential biofertilizers. Owing to complex factors of lake Ol'Bolossat sediments, production of a clear zone on a PKV solid agar medium alone cannot be used as a definitive proof for phosphate solubilization or growth on NGNFM as nitrogen-fixing properties. Therefore, further studies are needed to assess the effects of the isolates on the plant growth promotion characteristics under field and greenhouse conditions.

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