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## Qualitative and Quantitative Estimations of Potassium Solubilized by Two Potassium-Solubilizing Rhizobacteria Isolated from Maize Rhizosphere

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### ABSTRACT

Presently, decline in soil fertility has become a crucial concern to crop farmers in Nigeria. Only about a few percent of farmers can afford the use of chemical fertilizers to substitute in nutrient deficient soils. A study was conducted to isolate potassium-solubilizing rhizobacteria (KSR), a group of plant-growth promoting bacteria, from maize rhizosphere. Root samples were collected from an existing maize plant. Aleksandrov medium was used for the isolation of KSR by serial dilution method. Bacteria colonies observed on Aleksandrov plate were isolated and pure cultures obtained. The results obtained from the molecular analysis, identified two potassium-solubilizing rhizobacteria namely: *Lysinibacillus fusiformis* and *Providencia rettgeri*. Solubilization parameters were determined on colonies which exhibited clear zones of solubilization. The Gram reactions of both bacteria showed that *Lysinibacillus fusiformis* was Gram positive while *Providencia rettgeri* appeared negative. The zones of solubilization of *Lysinibacillus fusiformis* recorded higher solubilization diameter (5.37 cm) and solubilization efficiency (1400.56 %) than *Providencia rettgeri* (5.17 cm, 1291.67 % respectively). Also, the solubilization index was higher in *Lysinibacillus fusiformis* (2.87) than in *Providencia rettgeri* (2.17).

**Keywords:** Potassium-solubilizing rhizobacteria, Rhizosphere, Solubilization, Potassium.

### INTRODUCTION

Soil is a natural habitat for vast microbial communities and a complete understanding of microbial activities is yet to be unraveled (Ritz et al., 2010). However, a significant study has been carried out indicating certain functions of soil microorganisms such as bacteria and fungi in maintaining soil functions (Usman and Kundiri, 2016). The rhizosphere is a specialized microenvironment that accommodates millions of microorganisms whose activities accelerate plant nutrient cycles. It inhabits diverse species and strains of microorganisms. The effort made to harness the potential in mobilizing root systems of plant to increase global food (Zhang et al., 2010). These efforts are usually manipulated on infertile soils mostly in developing countries (Tilman et al., 2002).

Sustainability of crop production is, to a large extent, based on the proper management of biological activities around the rhizosphere of crops, continuous nutrient cycling and biochemical processes that make plant nutrient available for absorption by plants. Yield potentials of crop can be improved with proper management rhizospheric environment (Zhang et al., 2010).

An understanding of the functionality of the resident microorganisms at the rhizosphere is essential for effective soil microbial technology (SMT) that can be applied in the rhizosphere to enhance plant growth, maintain the soil health and influence environmental sustainability. Such environment facilitates and promotes crop development (Nihorimbere et al., 2011). Uptake of mineral nutrients by plant begins at the rhizosphere, where microbe-root exudates interaction

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exists. The level of nutrient available at the rhizosphere is also determined by the level of relationship between beneficial microbes and plant root exudates (Dotaniya and Meena, 2015).

The use of KSR is promising in improving crop production with less use of chemical fertilizers (Setiawati and Mutmainnah, 2016). Certain soil bacteria possess the ability to decompose inorganic potassium, such as potassium aluminosilicate and release it into the soil solution for plant absorption (Biswas and Basak, 2009). Some authors reported that *Bacillus*, *Thiobacillus*, *Pseudomonas* and *Acidithiobacillus* were able to release potassium from K-bearing complex minerals in soil (Sheng, 2005; Liu et al., 2012). The efficiency of potassium-solubilizing bacteria depends much on the nature of bacteria strain or species and condition of the minerals being solubilized (Verma et al., 2016). A wide range of rhizobacteria is able to dissolve insoluble K, from non-soluble materials (Sharma et al., 2013; Muhammad et al., 2018). The soluble K with a positive charge is absorbed by roots and translocated within the plant system (Meena et al., 2016).

## MATERIALS AND METHODS

### Collection of Soil Sample from Maize Rhizosphere

Maize plant was harvested from existing farm in Umuahia North local government area of Abia State. Umuahia North is typically a tropical climate and soil in this location is generally acidic in nature and rich in biodiversity. It records an average annual mean rainfall of about 2202 mm. The parent material of soil in this area is mostly coastal plain sands (Chukwu, 2013). The land resources make the soil rich in nutrient to support cultivation of arable crops such as cassava, maize, yam, cocoyam and vegetables (Abia State Economic Development Report, 2022). A representative soil sample collected aseptically from the rhizosphere into a Ziploc bag, air-dried and properly labeled. The sample was then taken to the Biological Science laboratory of Michael Okpara University of Agriculture, Umudike for isolation of potassium-solubilizing bacteria.

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### Preparation of Aleksandrov Media for KSR

#### Isolation

Potassium solubilizers were isolated using Aleksandrov agar plates. The media is composed of: magnesium sulphate (0.50 g), calcium (0.10 g), potassium aluminosilicate (3.00 g), glucose (5.00 g), Ferric chloride (0.006 g), calcium phosphate (2.00 g), agar (20.00 g) and mix in 1000 ml of distilled water. The final pH was adjusted to about 7.2±0.2 by adding 1 N NaOH (25°C). The mixture was autoclaved at 15 lbs pressure (121°C) for 15

minutes. It was then allowed to cool at about 45°C, before dispensing into sterile Petri dishes.

### Isolation of Rhizobacteria from Maize Roots

The pour plate method was used to isolate potassium-solubilizing bacteria from the soil sample using Aleksandrov medium by serial dilution method (Hu et al., 2006).

#### Serial dilution method

Serial dilution method was used to reduce the bacteria population. From the soil sample, 1 g of the soil was weighed into 9 ml of sterile water and mixed properly to obtain the stock solution. From the stock, 1 ml was collected and serially transferred to the first test tube containing 9 ml of sterile water (10<sup>-1</sup>). The dilution was repeated up to 10<sup>-7</sup> dilution. One milliliter (1 ml) of the 10<sup>-7</sup> dilution was poured in Aleksandrov media plates. The plates were inverted and put in an incubator at 30±2°C for 48 hours to develop colony forming units (CFU). After incubation, plates were observed for colonies of bacteria. Distinct colonies were sub-cultured to obtain pure cultures.

#### Screening of the isolates for K-solubilization

The pure rhizobacteria cultures were spot-inoculated on Aleksandrov media containing potassium aluminosilicate as sole source of K (Sugumaran and Janartham, 2007). The cultures were incubated at 30°C for 72 hours. The plates were checked for formation of clear zone which is an indication of K-solubilization. The diameters of the solubilization zone (clear zone) were measured in centimetre (cm) and the values recorded. The colonies exhibiting clear zones were selected and the streak plate method was used to obtain pure cultures of the isolates. Screened isolates that showed K-solubilization were transferred to nutrient agar slant and stored at 4°C for identification. Their solubilization efficiency and index were also determined. Solubilization efficiency was calculated using the formula below (Yaghoubi et al., 2018):

$$\% \text{ Solubilization Efficiency} = \frac{\text{Solubilization diameter (cm)}}{\text{Colony diameter (cm)}} \times 100$$

Secondary screening was carried out on the basis of zone of activity of the different isolates by using Khandeparkar's selection ratio (Buragohain et al., 2018).

$$\text{Ratio} = \frac{D}{d} = \frac{\text{Diameter of zone of clearance}}{\text{Diameter of colony}}$$

### Identification and Characterization of Potassium Solubilizers

Bacterial isolates with clear zones of K-solubilization were selected for identification and characterization using morphological, biochemical characteristics and molecular methods. Biochemical identification was conducted at Biological Science laboratory, Michael Okpara University of Agriculture, Umudike, while the molecular identification of the selected KSR was carried

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out at Havenwaves laboratory, Ibadan, Oyo State, Nigeria.

### **Morphological Characterization of K-solubilizing Bacterial Isolates**

All the selected isolates were examined for the morphological characteristics and Gram reaction following standard procedures which involves obtaining pure bacterial culture and inoculating these cultures onto specific media to observe visible changes in the media after incubation.

#### **Gram staining**

Forbes and Sahm (2018) method were used to identify Gram reactions of the isolated bacteria. Bacteria smear was made on a clean grease-free slide, air dried and heat fixed. The slide was flooded with crystal violet for 60 seconds, decanted and rinsed with water. Lugol's iodine (mordant) was applied for 60 seconds and rinsed. Acetone was used in decolorizing and washed immediately then counter stained with safranin for 1 minute. It was then rinsed with water, blotted carefully and air dried. Finally, the slides were observed under the microscope using oil immersion objectives ( $\times 100$ ).

### **Biochemical characterization of K-solubilizing bacteria isolates**

Biochemical characterizations of the isolates were carried out using procedure outlined by Bergey's Manual of Systematic Bacteriology 9<sup>th</sup> Edition (Bergey and Holt, 1993). Biochemical tests such as sugar utilization, indole production, citrate utilization, oxidase, catalase, H<sub>2</sub>S production, methyl red, Voges-Proskauer (VP) and starch hydrolysis tests were performed.

#### **Indole production test**

The isolates were inoculated into a bijoux bottle containing 3 ml of sterile Tryptone broth and incubated at 35-37°C for 48 hours. Indole production was tested by adding 7 drops of Kovac's reagent and examined for formation of a ring of red color in the surface layer within 5 minutes. A ring of red layer indicates a positive test. Absence of a ring of red layer indicates a negative test.

#### **Methyl red test**

The isolates were inoculated into various MR-VP broth bottles and appropriately labeled. The bottles were incubated for five days. After incubation, five drops of methyl red were added to the bottles for methyl red test. A positive test was indicated by appearance of red color. Negative test was indicated by a yellow coloration of the media.

#### **Voges-Proskauer test**

The isolates were inoculated into various MR-VP broth bottles and appropriately labeled. The bottles were incubated for five days. After incubation, 1 ml of the MR-VP broth with test organisms was transferred to sterile test tubes, and 15 drops of VP reagent A was added to the test tubes followed by 5 drops of VP reagent B. A positive test was indicated by appearance of pink-

red color. Negative test was indicated by a light-yellow coloration of the media.

#### **Citrate utilization test**

This was used to detect the ability of each isolate to use citrate as its sole source of carbon and energy. Slopes of Simmons citrate agar were prepared in bijoux bottles following the manufacturer's directives. Using a sterile straight wire, the slope was first streaked with a saline suspension of the isolates and then the butt stabbed and incubated at 35°C for 48 hours. A bright blue color was recorded as a positive test.

#### **Catalase test**

Two (2) drops of 3% hydrogen peroxide was placed on a clean grease-free slide using a pipette and a loopful of pure culture of the isolates was added and emulsified. Formation of visible bubbles was taken as a positive test while absence of bubbles was regarded as a negative test.

#### **Oxidase test**

This test is used to identify cytochrome containing organisms which produce an intracellular oxidase enzyme. A single oxidase test strip was placed in a Petri dish and the test portion of the strip was moistened with water. An applicator stick was used to collect 3-4 colonies from the isolates and smeared on the test region of the strip. Appearance of a blue/purple colour within 30 seconds indicates a positive test. Organisms lacking cytochrome c as part of their respiratory chain do not oxidize the reagent, leaving it colourless and are oxidase negative.

#### **Starch hydrolysis**

Starch Hydrolysis is used to test the ability of an organism to produce certain exo-enzymes including  $\alpha$ -amylase. Starch molecules are too large to enter into bacterial cell, so some bacteria secrete exo-enzymes to degrade starch into subunits that can then be utilized by the organism. An inoculum from a pure culture was streaked on a sterile plate of starch agar and incubated at 35°C for 24 hours, after which iodine was added to flood the growth. Positive result is shown a clear halo surrounding the colonies while negative result is shown by a dark-blue colouration.

#### **Hydrogen Sulphide (H<sub>2</sub>S) production test**

Hydrogen sulphide production test was used to detect presence of hydrogen sulphide gas produced by the organism. An inoculum from each isolate was transferred aseptically to a sterile triple sugar iron agar (TSIA) slant. The inoculated slants were incubated at 35°C for 24 hours and the results observed. Hydrogen sulfide combines with the iron in the media to produce iron sulfide (FeS). The presence of H<sub>2</sub>S producing organism is detected by the turning of the agar slants to black colour.

## Molecular identification of K-solubilizing bacteria isolates

The identities of the potassium solubilizers were determined with the aid of Polymerase Chain Reaction (PCR) analysis. The lengths of the amplicons were also determined by running the Ladder marker simultaneously with the bacterial samples isolated. The obtained sequences were submitted to BLAST to find similar 16Sr DNA homologous sequence. The sequences in the GenBank were compared with the obtained sequences of 16s rDNA gene of the bacterial isolates.

### a. DNA extraction

The genetic identifications were carried out to identify and characterize the potassium solubilizing bacteria. Genomic DNA extraction was carried out with column-based JENA Bioscience Bacteria DNA Preparation Kit following manufacturer's instruction. Bacteria cells were harvested from 500  $\mu$ l aliquot of bacteria broth culture using a microcentrifuge at 10,000 g for 1 minute. Residual pellet was resuspended in 300  $\mu$ l of Resuspension Buffer and 2  $\mu$ l of lysozyme solution. The mixture was homogenized by inverting several times thereafter incubated at 37°C for 1 hour. Resuspended cells were recovered by centrifugation and lysed by adding 300  $\mu$ l of Lysis buffer, 2  $\mu$ l Ribonuclease (RNase) A and 8  $\mu$ l proteinase K solution. Mixture was incubated at 60°C for 10 minutes thereafter cooled at room temperature for 5 minutes. Then, 300  $\mu$ l binding buffer was added to the mixture and vortexed briefly. The mixture was cooled on ice for 5 minutes and centrifuged at 10,000 g for 5 minutes. The supernatant was transferred directly into the spin column and centrifuged at 10,000 g for 1 minute to trap the DNA. Trapped DNA still present in the spin column was washed twice with washing buffer followed by elution of DNA; 50  $\mu$ l elution buffer was added to the tube and the DNA was eluted into sterile eppendorf tube by centrifugation at 10,000 g for 2 minutes.

### b. 16 rRNA polymerase chain reaction (PCR) amplification

Each PCR mixture consisted of 5  $\mu$ l mastermix (5 $\times$  JENA red load mastermix), 1  $\mu$ l (10 pmol) each of 27 F-AGAGTTTGATCMTGGCTCAG and 1492R-TACGGYTACCTTGTTACGACTT, 1  $\mu$ l DNA template and 17  $\mu$ l sterile nuclease free water to make up a total reaction of 25  $\mu$ l. PCR amplification was carried out in an Applied Biosystem 2720 Thermocycler. The mixture was subjected to an initial denaturation at 94°C for 3 minutes; followed by 35 cycles of 94°C for 45 seconds, 55°C for 60 seconds and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. Analysis of the amplicon was performed on a 1.5% (wt/vol) agarose gel dissolved in 0.5 $\times$  Tris-borate buffer, stained with ethidium bromide as visualized under blue led transilluminator. The incubation study was conducted in the Department of Soil Science and Land Resources Management, Michael Okpara University of

Agriculture, Umudike to quantitatively estimate the amount of K-solubilized by the rhizobacteria.

## Soil Sample Preparation for Incubation Studies

Soil sample was collected from an arable land in Umuahia North Local Government Area of Abia State. The sample was air-dried and sieved through a 2 mm sieve. About 3.5 kg of the sieved soil was autoclaved at 121°C for about 15 minutes and allowed to cool. From the autoclaved soil, 200 g each was weighed into 7 plastic containers of 250 ml capacity and replicated 2 times, to give a total of 14 experimental units. The same procedure was repeated for the non-sterilized soil and arranged in a completely randomized design (CRD). Twenty-eight plastic containers were arranged in the Soil Science laboratory of Michael Okpara University of Agriculture, Umudike for the incubation study, as shown in the layout below (Table 1).

## Preparation of Bacteria Inoculum

The isolated potassium-solubilizing rhizobacteria (*Lysinibacillus fusiformis* and *Providencia rettgeri*) were freshly inoculated into an autoclaved 250 ml nutrient broth using a conical flask, under aseptic condition. After incubation, spectrophotometric analysis was conducted to measure out the different concentrations of bacterial cells which serve as treatments for soil inoculation. The different bacteria cell concentration are,  $1.0 \times 10^8$ ,  $1.5 \times 10^8$  and  $3.0 \times 10^8$  CFU/ml, which indicates the quantity of bacterial cells in each inoculum. Before inoculation of the isolates, 10 grams of potassium aluminosilicate (as source of insoluble K) was thoroughly mixed with each experimental soil unit. The soils were then inoculated with the screened isolates at different concentrations per ml ( $1.5 \times 10^8$ ,  $3.0 \times 10^8$  and  $6.0 \times 10^8$  CFU/ml) using McFarland standard (Aryal, 2021) equivalents (0.5, 1.0 and 2.0) and were allowed to incubate at 28°C for 8 weeks (56 days).

## Preparation of McFarland Standards

McFarland (turbidity) standards are used to estimate the bacteria count in a liquid suspension (inoculum). The McFarland standards were prepared by adjusting the volume of barium chloride ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) and sulphuric acid ( $\text{H}_2\text{SO}_4$ ) to obtain a barium sulphate precipitate. The adjusted volumes of the two reagents were used to prepare standards of varying degrees of turbidity; 0.5, 1.0 and 2.0 which represent different concentrations of bacteria cells;  $1.5 \times 10^8$ ,  $3.0 \times 10^8$  and  $6.0 \times 10^8$  CFU/ml cell counts respectively. The concentrations of the two reagents used for the preparation of McFarland standards are 1% sulphuric acid and 1.175% barium chloride (wt/vol). The method for preparation of McFarland standards is shown in the Table 2 below

## Standardizing the Inoculum (Spectrophotometer Method)

The concentrations of each bacterial inoculum were standardized using the spectrophotometric method (Kipper *et al.*, 2020). The McFarland standard numbers were measured using spectrophotometer to obtain the

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optical densities. The prepared inocula were then standardized using the optical density readings recorded for McFarland Standards (0.5, 1.0 and 2.0). Each concentration of the inoculum was standardized, then, 50 ml each of the different concentrations,  $1.5 \times 10^8$ ,  $3.0 \times 10^8$  and  $6.0 \times 10^8$  CFU/ml, was poured into the plastic containing 200 g of the sterilized and non-sterilized soil samples. The spectrophotometric analysis was used to determine the quantity of bacterial cells in a sample, which is based on the amount of light that is absorbed by the sample (inoculum). It measures the turbidity of a solution. The amount of the light passing through the inoculum was used to measure bacterial cell density of a particular concentration. Increased turbidity (cloudiness) of the inoculum indicates increased bacterial growth and cells numbers. In the spectrophotometric method, the quantity of light transmitted is converted to electrical energy, and is reflected on a galvanometer. The reading on the galvanometer is called the absorbance or optical density. The sample in the spectrophotometer is adjusted to obtain accurate concentrations for the incubation study.

#### Inoculation of K-solubilizers in Soil

The soil samples were air-dried, passed through a 2 mm sieve and 150 g were weighed into each of the 250 ml plastic containers. The laboratory experiment was arranged in a completely randomized design (CRD). Potassium aluminosilicate was ground into powdered form and passed through 2 mm sieve. Ten (10) g of the ground potassium aluminosilicate was weighed into the 150 g soil and mixed properly. The optical densities (0.034, 0.059 and 0.115) of the McFarland standards (0.5, 1.0 and 2.0 respectively) used were obtained using a spectrophotometer. Each of the prepared bacterial

inoculum was then diluted with sterile water to get a corresponding bacterial concentration ( $1.5 \times 10^8$ ,  $3.0 \times 10^8$  and  $6.0 \times 10^8$  CFU/ml) equivalent to the optical densities of the selected McFarland standards. In each of the 250 ml plastic containers, 40 ml of each bacterial concentration was aseptically poured into 150 g soil and the procedure was replicated twice. In other to sustain bacterial activity in the samples, 10 ml of sterile water was added, weekly to prevent dryness of soil.

#### Quantification of K-solubilized by the Isolates

The quantity of available potassium solubilized in the soil by each potassium-solubilizing bacteria strain (*Lysinibacillus fusiformis* and *Providencia rettgeri*) at different concentrations of  $1.5 \times 10^8$ ,  $3.0 \times 10^8$  and  $6.0 \times 10^8$  CFU/ml was monitored to observe the trend of solubilization at 14, 28, 42 and 56 days after incubation (DAI) using flame photometer (Sugumaran and Janarthanam, 2007).

#### Procedures for Soil Chemical Analysis

Soil samples were air-dried, crushed and passed through a 2 mm sieve. Organic carbon was determined by chromic acid oxidation method of Walkley and Black modified by Nelson and Sommers (1996). Total nitrogen was determined by the semi-micro Kjeldahl distillation method of Bremner and Mulvaney (1982). Available phosphorus was determined by the Bray II extraction and spectrophotometric method of Bray and Kurtz (1945). Potassium was determined by the flame photometric method. All the data were subjected to statistical analysis using R statistic version 4.4.1. Results from the anova were also represented in line graphs

**Table 1: Layout for Incubation Studies of two potassium-solubilizers**

BACTERIA INOCULUM	UNSTERILIZED SOIL		STERILIZED SOIL	
	Rep 1	Rep 2	Rep 1	Rep 2
No bacteria inoculums	Control	Control	Control	Control
<i>Lysinibacillus fusiformis</i>	Lf ( $1.5 \times 10^8$ )	Lf ( $1.5 \times 10^8$ )	Lf ( $1.5 \times 10^8$ )	Lf ( $1.5 \times 10^8$ )
	Lf ( $3.0 \times 10^8$ )	Lf ( $3.0 \times 10^8$ )	Lf ( $3.0 \times 10^8$ )	Lf ( $3.0 \times 10^8$ )
	Lf ( $6.0 \times 10^8$ )	Lf ( $6.0 \times 10^8$ )	Lf ( $6.0 \times 10^8$ )	Lf ( $6.0 \times 10^8$ )
<i>Providencia rettgeri</i>	Pr ( $1.5 \times 10^8$ )	Pr ( $1.5 \times 10^8$ )	Pr ( $1.5 \times 10^8$ )	Pr ( $1.5 \times 10^8$ )
	Pr ( $3.0 \times 10^8$ )	Pr ( $3.0 \times 10^8$ )	Pr ( $3.0 \times 10^8$ )	Pr ( $3.0 \times 10^8$ )
	Pr ( $6.0 \times 10^8$ )	Pr ( $6.0 \times 10^8$ )	Pr ( $6.0 \times 10^8$ )	Pr ( $6.0 \times 10^8$ )

**Table 2: Preparation for McFarland Standards**

Standard Number	Vol/ml		Number of bacteria/ml represented
	BaCl <sub>2</sub> .2H <sub>2</sub> O (1.175%)	H <sub>2</sub> SO <sub>4</sub> (1%)	
0.5	0.5	99.5	$1.5 \times 10^8$
1.0	1.0	99.0	$3.0 \times 10^8$
2.0	2.0	98.0	$6.0 \times 10^8$

Adapted from Chapin and Lauderdale (2003)

## RESULTS

### Biochemical Characteristics of as the Bacterial Isolates

Results on some biochemical tests and the morphology of the potassium-solubilizing rhizobacteria are presented in Table 3. *Lysinibacillus fusiformis* has a long rod-like shape while *Providencia rettgeri* is short and rod-like in shape (Plates 1 and 2). The results of the Gram staining for *Lysinibacillus fusiformis* were positive, while *Providencia rettgeri* appeared negative (Table 3). *Lysinibacillus fusiformis* was positive for arginine, which indicates its ability to produce arginine dihydrolase enzyme for the breakdown of amino acid L-arginine and produce alkaline products such as ammonia. It also showed positive for citrate and indication the bacteria can utilize citrate as a sole source of carbon and energy. Oxidase, catalase and starch hydrolysis showed positive reactions. This makes the bacteria capable of using oxygen as a terminal electron acceptor in its electron transport chain for energy production during aerobic respiration, produce catalase enzyme for the breaking down of hydrogen peroxide into water and oxygen gas, and the ability to produce amylase enzyme for the breakdown of large starch molecules into smaller, usable sugars respectively. Mannitol, triple sugar iron, salign, indole production, citrate utilization, catalase, and methyl red were positive for *Providencia rettgeri*. A positive mannitol reaction makes it possible for *Providencia rettgeri* to ferment the mannitol sugar, which produced acid that lowers the medium's pH resulting to a colour change from red to yellow. The positive reaction of triple sugar iron test showed that *Providencia rettgeri* shows fermentation of sugars or hydrogen sulphide production.

### Molecular Identification of the Bacterial Isolates

The sizes of the bands of both bacterial isolates on Agarose fall within 1500 bp (Plate 3). The BLASTn results showed that KSRa and KSRg possessed 94.90 % and 96.59 % similarities to *Lysinibacillus fusiformis* and *Providencia rettgeri* strains respectively. Table 5 reveals the identity of the potassium solubilizing rhizobacteria by means of 16S rDNA partial sequencing. From results obtained the length of *Lysinibacillus fusiformis* and *Providencia rettgeri* strains are 1003 and 965 respectively. However, the percent identity of *Providencia rettgeri* was higher than that obtained for *Lysinibacillus fusiformis*.

### Phylogenetic Tree of the Isolated Potassium-solubilizing Rhizobacteria Strains

The origins of the potassium-solubilizing rhizobacteria strains were deduced by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). This analysis involved 8 nucleotides for *Lysinibacillus fusiformis* while *Providencia rettgeri* had 19 nucleotides sequences. There were a total of 1418 positions for *Lysinibacillus fusiformis* and 1497 positions *Providencia rettgeri* in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021) (Figures 1 and 2).

### Qualitative Estimation of Solubilization Ability of the Screened Bacterial Isolates

Activities of the rhizobacteria isolates were studied on Alexandrov agar medium and based on observations of zones of solubilization, the qualitative estimate of K-solubilized were determined. The parameters measured for the estimation of K-solubilized by the bacterial isolates include: solubilization diameter, growth diameter, halozone diameter, solubilization index, solubilization efficiency and Khandeparkar's selection ratio. The selected K-solubilizers that showed clear zones of solubilization were isolate KSRa (*Lysinibacillus fusiformis*) and isolate KSRg (*Providencia rettgeri*). From the results obtained for solubilization diameters (cm), the two isolates *Lysinibacillus fusiformis* and *Providencia rettgeri*, showed better abilities to solubilize K than the other that were not selected. *Lysinibacillus fusiformis* gave the highest and significant ( $p < 0.05$ ) solubilization diameter with mean value of 5.37 cm followed by *Providencia rettgeri*, which gave a mean value of 5.17 cm. However, the growth diameters for both isolates were the same, 0.4 cm. *Lysinibacillus fusiformis* recorded a higher halozone diameter of 0.73 cm which was significantly different ( $p < 0.05$ ) from the value obtained for *Providencia rettgeri* (0.47 cm). The solubilization index (SI) results revealed that *Lysinibacillus fusiformis* had higher solubilization index value of 2.87, but was not significantly different ( $p > 0.05$ ) from the lowest mean value obtained from *Providencia rettgeri* (2.17). Solubilization efficiency of *Lysinibacillus fusiformis* recorded a mean value of 1400.56 %, which was significantly ( $P > 0.05$ ) higher than that obtained for *Providencia rettgeri* (1291.67 %). The Khandeparkar's Selection Ratio was calculated and the result shows that *Lysinibacillus fusiformis* which had a mean value of 14.01 was significantly higher ( $p < 0.05$ ) than the Khandeparkar's selection ration of *Providencia rettgeri* (12.92) (Table 4)

**Table 3: Some Biochemical Test for Selected Isolates**

Bacterial isolates	Morphology		Biochemical tests																			
	Cell arrangement	Gram stain	Mannitol	Triple	Fructose	Galactose	Raffinose	Glucose	Sorbitol	Arginine	Mannose	Arabinose	Lactose	Saligen	Indole	Citrate	Oxidase	Catalase	H <sub>2</sub> S production	Methyl red	Voges-Proskauer	Starch hydrolysis
KSRa	Long rods	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	+
KSRg	Rods	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	-	-

- = negative (Absent), += (Present), KSR = Potassium Solubilizing Rhizobacteria

**Table 4: Parameters of the clear zones of potassium solubilization of four potassium-solubilizing rhizobacteria from maize roots**

Bacterial Isolates	Solubilization Diameter (cm)	Growth Diameter (cm)	Halozone Diameter (cm)	Solubilization Index	Solubilization Efficiency	Khandeparkar's selection ratio
<b>*A</b>	<b>5.37</b>	<b>0.4</b>	<b>0.73</b>	<b>2.87</b>	<b>1400.56</b>	<b>14.01</b>
F	0.95	0.3	-	-	316.67	3.17
<b>*G</b>	<b>5.17</b>	<b>0.4</b>	<b>0.47</b>	<b>2.17</b>	<b>1291.67</b>	<b>12.92</b>
I	5.1	0.6	0.95	2.58	850	8.5
p>0.05	0.000***	0.005**	0.022*	ns	0.007**	0.007**

\*A = *Lysinibacillus fusiformis* \*G = *Providencia rettgeri*

**Table 5: Identification of Selected KSR by 16s rDNA Partial Sequencing**

Isolates	Length of 16s rDNA gene sequenced	Most closely related bacteria strain	Percent Identity (%)
KSR_a	1003	<i>Lysinibacillus fusiformis</i>	94.90
KSR_g	965	<i>Providencia rettgeri</i>	96.59

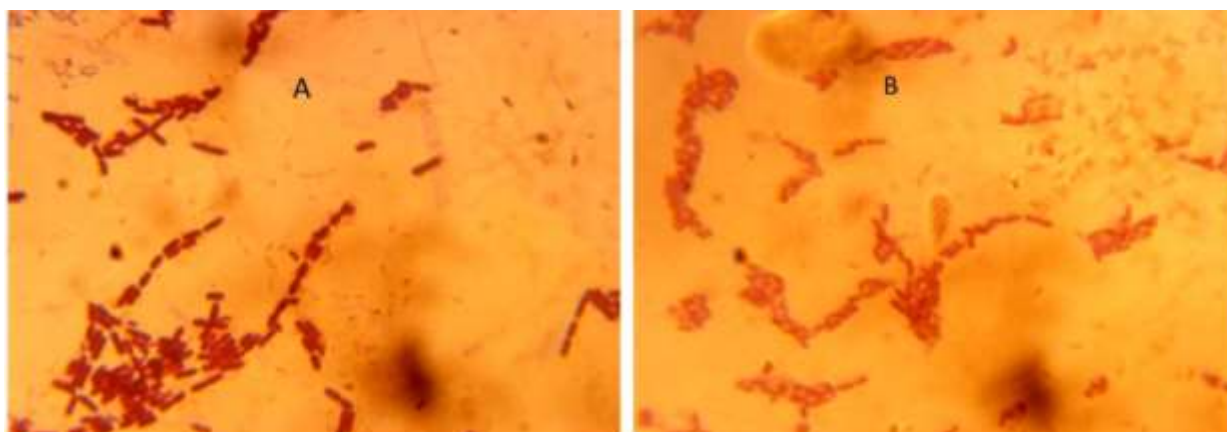


Plate 1: A) *Lysinibacillus fusiformis* and B) *Providencia rettgeri*

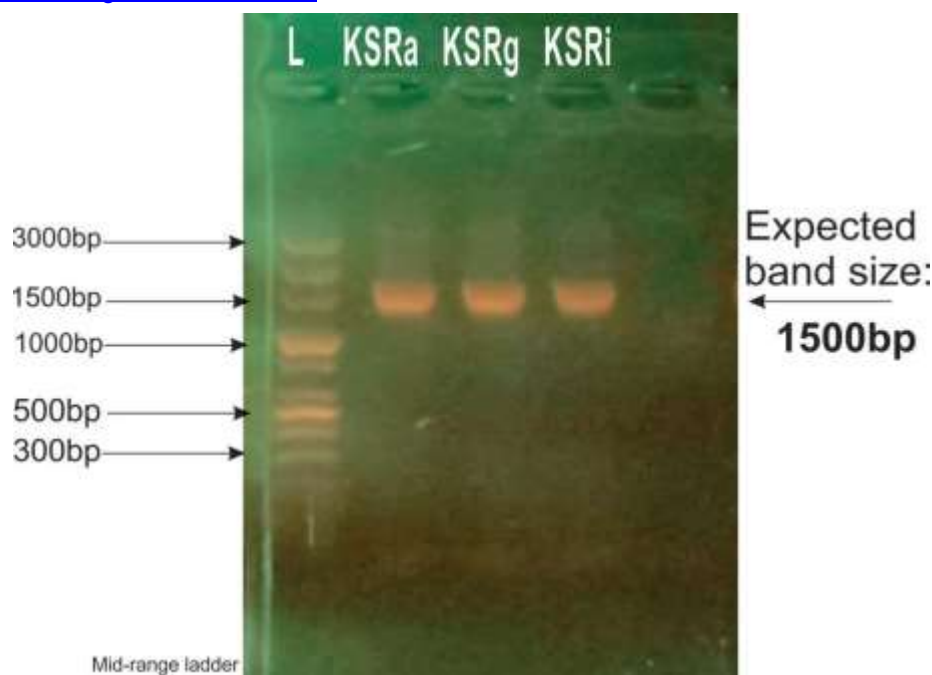


Plate 2: Agarose Gel Image from Polymerization Chain Reaction (PCR)

### Quantitative Estimation of Solubilization Ability of the Screened Bacterial Isolates

#### Effect of bacteria concentration and time of incubation on nitrogen content (%) in non-sterilized and sterilized soils.

The influence of different concentrations and time of incubation of the potassium solubilizers on nitrogen content of the soils is presented in Figure 3. No significant ( $p > 0.05$ ) effect was observed on nitrogen content among the different bacteria concentrations of both *L. fusiformis* and *P. rettgeri* in the non-sterilized soil. Nitrogen contents impacted by *Lysinibacillus fusiformis*, in the sterilized soil, at  $1.5 \times 10^8$  CFUml<sup>-1</sup>,  $3.0 \times 10^8$  CFUml<sup>-1</sup> and  $6.0 \times 10^8$  CFUml<sup>-1</sup> concentrations were statistically the same but significantly ( $p < 0.01$ ) higher (0.203 %, 0.187 % and 0.204 %, respectively) than the control (0.137 %). In the sterilized soil, *Providencia rettgeri* at  $3.0 \times 10^8$  CFUml<sup>-1</sup> concentration, had significantly ( $p < 0.05$ ) higher nitrogen content (0.182 %) at two weeks after incubation (WAI). The lowest nitrogen content (0.158 %) was obtained at  $6.0 \times 10^8$  CFUml<sup>-1</sup> concentration of *Providencia rettgeri* inoculants.

#### Effect of bacteria concentration and time of incubation on phosphorus content (mgkg<sup>-1</sup>) in non-sterilized and sterilized soils

Results shown in Figure 4 represent the effect of bacteria concentration and time of incubation on phosphorus content in the soils. In both non-sterilized and sterilized soils, K-solubilizers did not have significant ( $p > 0.05$ ) effect on phosphorus content at 4, 6 and 8 WAI. At 2 WAI, the bacterial strains at different concentrations significantly ( $p < 0.05$ ) affected phosphorus content in the non-sterilized and sterilized soils. In the non-sterilized soil, *Providencia rettgeri* at  $3.0 \times 10^8$  CFUml<sup>-1</sup> concentration gave the highest phosphorus content with mean value of 36.80 mgkg<sup>-1</sup>, while the lowest value was obtained at a concentration of  $1.5 \times 10^8$  CFUml<sup>-1</sup> (25.30 mgkg<sup>-1</sup>). Similarly, in the sterilized soil, *Providencia rettgeri* at  $3.0 \times 10^8$  CFUml<sup>-1</sup> concentration also recorded the highest phosphorus content (41.45 mgkg<sup>-1</sup>). This result indicates that *Providencia rettgeri* may have the ability to solubilize insoluble phosphorus and make it available for plant uptake. The lowest phosphorus content was obtained in the control soil, where no bacterial strain was inoculated.

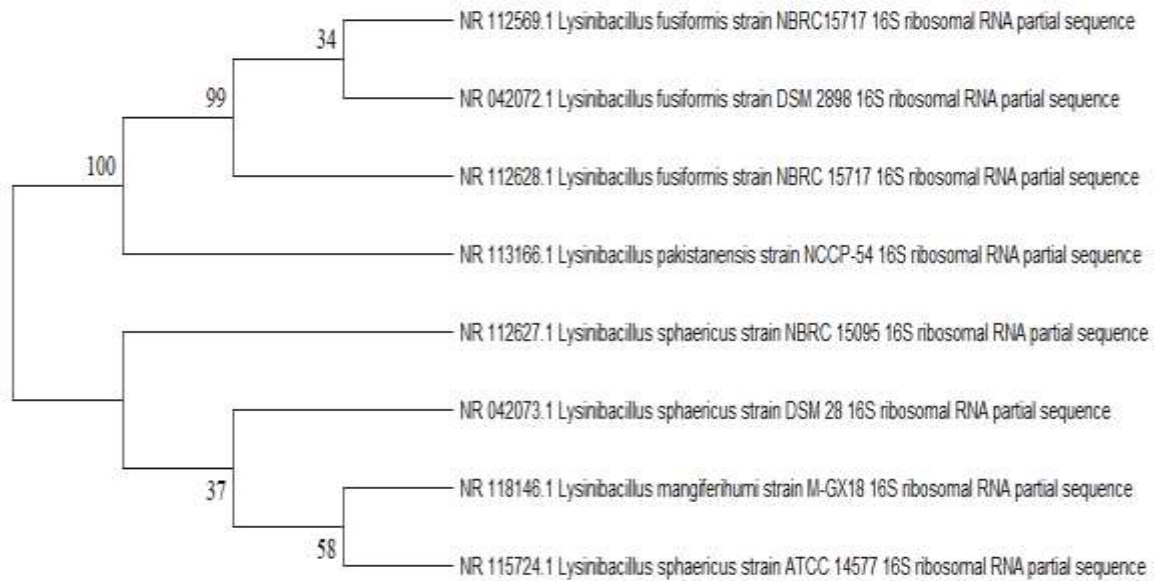


Figure 1: Phylogenetic tree of *Lysinibacillus fusiformis*

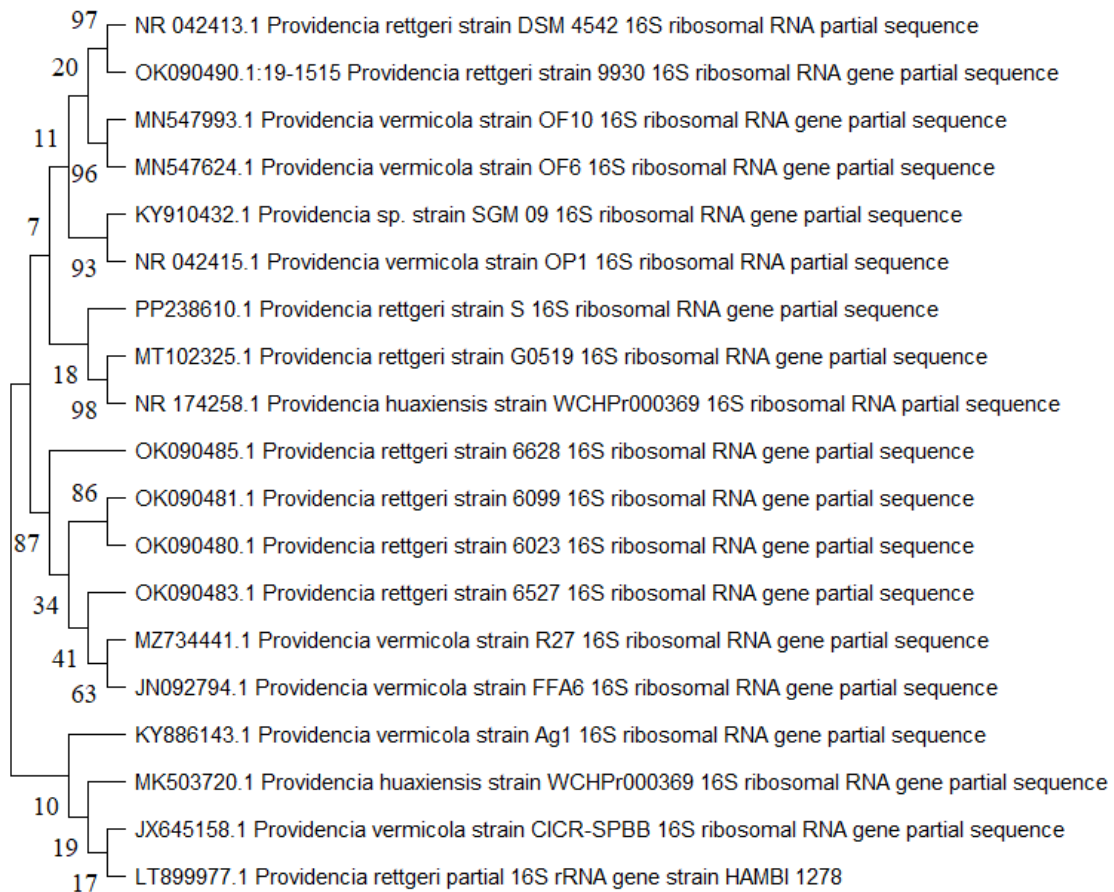


Figure 2: Phylogenetic tree of *Providencia rettgeri*

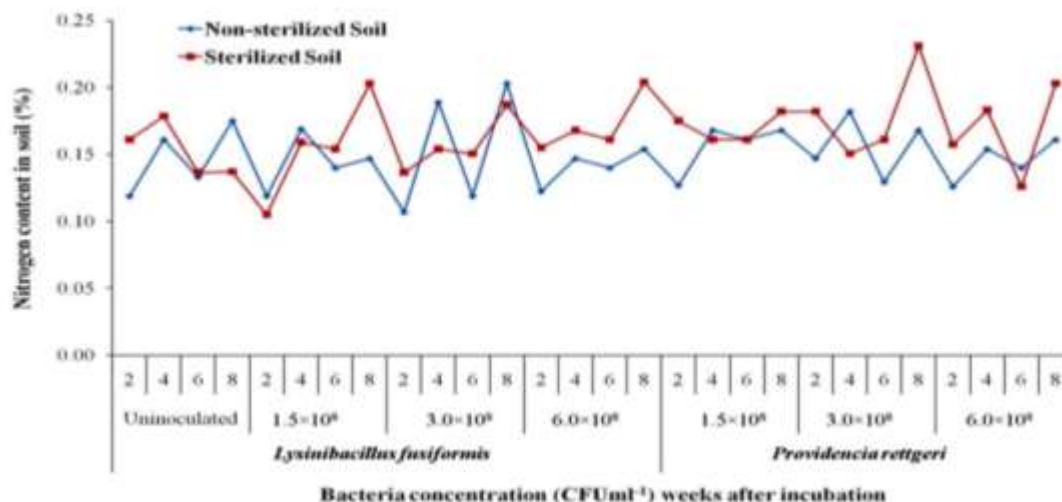


Figure 3: Effect of bacteria concentration and time of incubation on phosphorus content (%) in non-sterilized and sterilized Soils

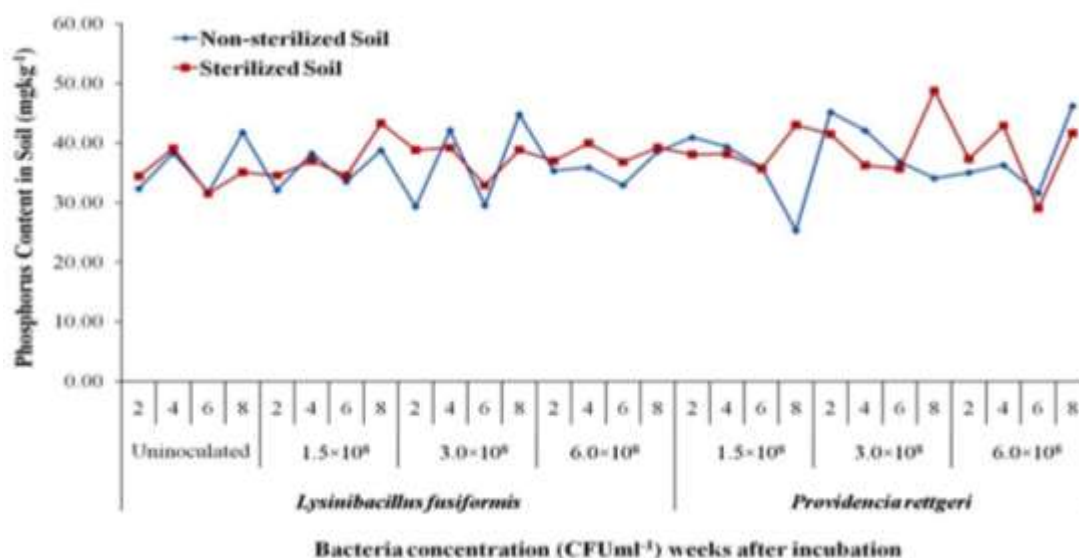


Figure 4: Effect of bacteria concentration and time of incubation on nitrogen content (%) in non-sterilized and sterilized Soils

**Effect of bacteria concentration and time of incubation on potassium content (cmolk<sup>-1</sup>) in non-sterilized and sterilized soils**

The results of the impact of the potassium solubilizers on potassium content in the soils are presented in Figure 5. The rhizobacteria concentrations impacted significantly ( $p < 0.01$ ) on potassium in soil. *L. fusiformis* at 2 WAI, at  $3.0 \times 10^8$  CFUml<sup>-1</sup>,  $6.0 \times 10^8$  CFUml<sup>-1</sup> and the control, in the non-sterilized soil, solubilized a significantly ( $p < 0.01$ ) higher amount of potassium (0.242 cmolk<sup>-1</sup>, 0.247 cmolk<sup>-1</sup> and 0.248 cmolk<sup>-1</sup> respectively) than at  $1.5 \times 10^8$  CFUml<sup>-1</sup> (0.212 cmolk<sup>-1</sup>). However, in the sterilized soil, the control treatment recorded the highest amount (0.253 cmolk<sup>-1</sup>) of soluble potassium which was significantly ( $p < 0.001$ ) different from the concentrations of *L. fusiformis*. The lowest means value

was observed at  $1.5 \times 10^8$  CFUml<sup>-1</sup> (0.162 cmolk<sup>-1</sup>). This result suggests that in the control soil, soluble potassium was not utilized by microbes, however, the bacteria in the other soil utilized some of the solubilized potassium. At 4 WAI, *L. fusiformis* concentrations at  $3.0 \times 10^8$  CFUml<sup>-1</sup> and  $6.0 \times 10^8$  CFUml<sup>-1</sup> were significantly the same (0.236 cmolk<sup>-1</sup> and 0.261 cmolk<sup>-1</sup> respectively), but significantly ( $p < 0.01$ ) higher than the control and  $1.5 \times 10^8$  CFUml<sup>-1</sup> samples (0.181 cmolk<sup>-1</sup> and 0.193 cmolk<sup>-1</sup> respectively) in the sterilized soil. *Providencia rettgeri*, in unsterilized soil, the control sample at 2 WAI recorded highest amount of potassium (0.248 cmolk<sup>-1</sup>) which was significantly ( $p < 0.001$ ) different from other concentrations, while  $6.0 \times 10^8$  CFUml<sup>-1</sup> concentrations recorded the least potassium content of 0.170 cmolk<sup>-1</sup>. In the sterilized soil, the concentrations of *Providencia rettgeri* affected potassium content significantly at 2 and 4 WAI. The  $3.0 \times 10^8$  CFUml<sup>-1</sup> concentration at 2 WAI was significantly ( $p < 0.01$ ) higher (0.259 cmolk<sup>-1</sup>) than

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other concentrations, while  $1.5 \times 10^8$  CFUml<sup>-1</sup> concentration recorded the least amount of potassium (0.241 cmolkg<sup>-1</sup>) in soil. However, at 4 WAI, all the three concentrations ( $1.5 \times 10^8$  CFUml<sup>-1</sup>,  $3.0 \times 10^8$  CFUml<sup>-1</sup> and

$6.0 \times 10^8$  CFUml<sup>-1</sup>) had potassium contents that were statistically the same, but significantly ( $p < 0.01$ ) higher (0.248 cmolkg<sup>-1</sup>, 0.234 cmolkg<sup>-1</sup> and 0.248 cmolkg<sup>-1</sup> respectively) than the control soil (0.181 cmolkg<sup>-1</sup>).

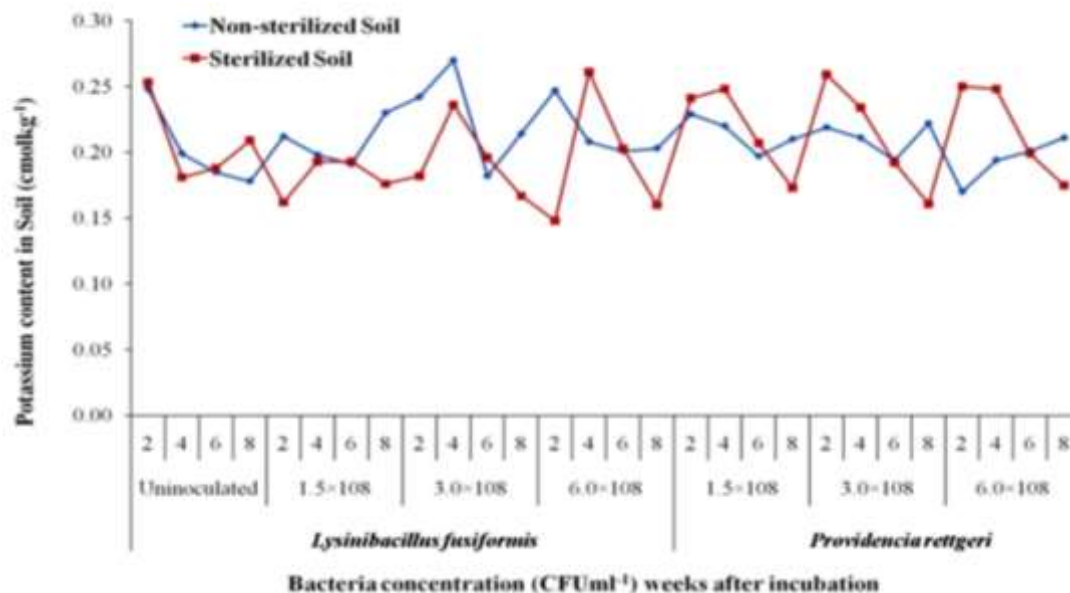


Figure 5: Effect of bacteria concentration and time of incubation on phosphorus content (%) in non-sterilized and sterilized Soils

## DISCUSSION

In this study, two plant-growth promoting bacteria species were isolated and identified as potassium solubilizers and both are rod-like in shape. The Gram staining results showed that *Lysinibacillus fusiformis* gave a positive reaction, while *Providencia rettgeri* was negative. These reports are also in concordance with what was reported by Hashmi et al. (2020). The findings of Riaño Castillo et al. (2024) supported that under microscopic characteristics, seven (7) strains of *Lysinibacillus* species were Gram positive and rod-shaped bacteria. All these findings agree that most *Lysinibacillus* species are similar in shape, Gram reaction and cell arrangements. Findings on the Gram reaction of *Providencia rettgeri* in this study was similar to that reported by Ayyal Al-Gburi (2020) where he observed that four (4) *Providencia* species isolated from raw cow milk showed Gram negative, which is in agreement with the result of this study. Among all other tests carried out for *Lysinibacillus fusiformis*, only five (5) showed positive reactions (arginine, citrate utilization, oxidase, catalase and citrate utilization tests). Mahendran et al. (2013) reported similar findings, where *Lysinibacillus fusiformis* isolated from soil was Gram positive and the cell shape appeared rod-like. They also reported that *Lysinibacillus fusiformis* showed positive in citrate utilization, oxidase, catalase and starch hydrolysis tests, which is similar to this present study. The ability of potassium solubilizers to produce varieties of enzymes and amino acids gives them the abilities to solubilize insoluble potassium (Prajapati et al., 2013; Maurya et al.,

2014; Meena et al., 2014). Biochemically, some authors documented that some *Providencia* species showed positive for oxidase and positive for catalase (Ayyal Al-Gburi, 2020, Rushabh et al., 2020), however in the present findings, *Providencia rettgeri* was negative for oxidase and positive for catalase test.

The qualitative estimate of potassium solubilized was determined by measuring solubilization indices. In this study, *Lysinibacillus fusiformis* gave a higher halozone diameter (0.73 cm) when compared with *Providencia rettgeri* (0.47 cm). The solubilization index of *Lysinibacillus fusiformis* (2.87) from this study was similar to the value reported by Nihala and Syed (2017) for three *Bacillus* species ranging between 2.85 to 3.28. However, the solubilization efficiency of *Lysinibacillus fusiformis* (1400.56) was higher than the value (ranged from 185 to 228.5 %) reported by Nihala and Syed (2017). In another study, six potential potassium solubilizing microorganisms isolated from cotton rhizosphere of rainfed vertisols showed that the potassium solubilization index ranged from 1.3 to 4.0, but notably, *Lysinibacillus fusiformis* isolated from the cotton rhizosphere recorded solubilization index of 1.30 which is lower than what was reported in this study (Santosh et al., 2022). The differences observed in the values may be due to the individual abilities of different *Bacilli* class to produce more organic acid which is their mechanism of solubilization. The type of organic acid produced by the bacteria may also well influence the amount of potassium solubilized (Melero et al., 2008; Saiyad et al., 2015; Meena et al., 2014). Soil pH and temperature

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may affect the activities of bacteria, which invariably affect the amount potassium of solubilized (Buragohain et al., 2018).

The incubation study performed in this study showed that *Lysinibacillus fusiformis* solubilized potassium at 2 weeks after inoculation (WAI). As number of weeks increased, the amount of potassium solubilized reduced. Differently, another study indicated that the amount of K released from mineral K by potassium solubilizers isolated from ceramic industries of the Kadi, North Gujarat Region, India increased with increase in incubation time and reached its maximum at 20 days after incubation (Prajapati, 2016). In this present findings, *Providencia rettgeri* solubilized more potassium at two weeks after incubation. The trend suggests that as bacterial cells increased, they utilize available potassium that has been solubilized, thereby reducing the potassium content in the soil. Babar et al. (2024) reported that application of K-solubilizing bacteria improved K availability and other plant nutrient contents. The amount of organic acids released by resident potassium solubilizers may also determine the amount of insoluble potassium solubilized (Hu et al., 2018; Sarikhani et al., 2018). Four *Bacillus* species which produced varying types and amount of organic acids were able to achieve potassium solubilization (Bakhshandeh, 2015), which agrees with the present findings.

## CONCLUSION

Potassium solubilization by rhizobacteria is a good indication that bacteria are capable of improving nutrient uptake and enhance soil fertility. *Lysinibacillus fusiformis* and *Providencia rettgeri* qualitatively solubilized potassium and quantitatively increased the amount potassium in soil. However, *Lysinibacillus fusiformis* performed better than *Providencia rettgeri* based on potassium solubilization indices. This development offers a sustainable approach to agriculture and supports environmentally friendly food system by reducing the need for chemical fertilizers, making them a crucial tool for improving soil health and food security.

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## Data availability

Not applicable

## Consent For Publication

Not applicable

## Competing interests

The authors declare no conflict of interest

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