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Isolation and Characterization of Phosphate Solubilizing Microorganism (PSM) from the Rhizosphere and Roots of Crops Indigenous to Ihiagwa-Owerri Imo State Nigeria

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Abstract:- The expensive eco-degrading chemical fertilizer demands an affordable sustainable eco-friendly alternative: biofertilizer. Isolates from the rhizosphere and root samples of *Abelmoschus esculentus, Manihot esculenta, Musa paradisiaca* and *Zea mays* were obtained using standard microbiological procedures, screened for phosphate solubilization using Pikovkaya's medium and their phosphate solubilization index(PSI), and efficiency determined. Isolates with PSI ≥ 140 were subjected to morphological and biochemical characterization. Discrete colonies obtained were 145. Total bacterial isolates were 111 and 34 fungal. Phosphate solubilizing isolates were 39 (26.8%), phosphate solubilizing index (PS1) range from 109.1 to 190. Isolates with PSI ≥140 were identified as *Pseudomonas* sp., *Bacillus* sp., *Aspergillus* sp., and *Penicillium* sp. The solubilization efficiency order of the isolates was Pseudomonas> Bacillus> Pencillium> Aspergillus. The application of these biofertilizer-producing microorganisms will deliver an adequate amount of phosphorus plants thereby boosting agro development.

Keywords:- *Biofertilizer, Eco-Friendly, Phosphate Solubilizing Index, Chemical Fertilizer, Phosphate Solubilization Efficiency.*

I. INTRODUCTION

Phosphorus (P) is among the three major plant nutrients required in the optimum amount for proper plant growth. A shortfall in the adequate P imparts negatively on the yield of crops below the maximum economic level. Phosphorus's roles in the metabolism of plants are indispensable. Its biochemical roles extent to photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement, and several other processes in the living plant. An adequate supply of phosphorus in the early stages of plant growth is very essential. It promotes physiological functions including early root formation and is important for the development of the reproductive parts of plants. It is very necessary for seed formation. More phosphorus is found in plants' seeds than found in any other part of the plant. It helps plants to survive stressors like winter rigors and some plants' diseases (Balamurugan et al., 2010). There abound in nature, wide range of microorganisms that can solubilize insoluble phosphate. These microbial solubilizers use several mechanisms which are necessary to maintain the global cycle (Whitelaw, 2000). These unavailable forms of phosphates are solubilized by microbes through excretion of organic acids and or production of phosphatase enzyme (Kucey, 1983). A large number of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium*, and *Serratia* have been reported to enhance plant growth with their different plant growth-promoting activities including phosphate solubilization. Phosphate solubilizing microbes can be used to ensure sustainable organic farming systems and reduce the utilization of agrochemicals in agricultural fields (Widawati, 2011).

The work aims to isolate and characterize phosphate solubilizing microbes from the rhizosphere and roots of indigenous crops to Ihiagwa-Owerri Imo State Nigeria.

II. MATERIALS AND METHODS

2.1 Sampling Collection

Forty rhizospheric soil and 40 roots were sourced from farmlands located at Ihiagwa-Owerri,

latitude 5o25'26" N, longitude 7o1'31" E. Ten rhizospheric samples (soil and root) were sourced from an assortment of *Abelmoschus esculentus*(okro), *Manihot esculenta*(cassava), *Musa paradisiaca* (plantain), and *Zea mays*(maize) plants. The samples were aseptically dug out from the depths 10-30 cm into sterile polythene bags after the crests of the soils were cleared of debris with a clean sterile trowel (Philippot et al., 2012). The samples were transported to the laboratory at 40C temperature.

2.2 Sample Preparation and Microbial Isolation

Composite sample from ten soil samples of each plant type was subjected to ten-fold serial dilution and an aliquot (0.1ml) of dilution 10-2 was each spread plated on nutrient agar (NA) and sabrourand dextrose agar (SDA) plates and incubated at 30 ± 2oC for 2-7days. Discrete colonies were stored in slants for further studies (Philippot et al., 2012).

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Root samples were surface sterilized, macerated, and subjected to ten-fold serial dilution, inoculation, and incubation as the soil samples (Philippot et al., 2012).

2.3 Screening of phosphate solubilizing microorganisms (PSM)

Pikovskaya's agar (PVK) {(grams/liter); Glucose, 10.0gm; Ca3(PO4)2, 5.0 gm; (NH4)4SO4, 0.5 gm; NaCl, 0.2 gm; MgSO4.7H2O, 0.1gm, agar, 15g, pH 7.3±2}was prepared (Oliveira *et al*., 2009), sterilization with an autoclaved and poured into sterile Petri dishes. Loopful of 48 hours old bacterial isolates and 3 days needle scrap of fungal isolates were centrally spot inoculated on sterile PVK agar plates and incubated at 30±2oC (Prajapati and Modi, 2012). Colonies showing halo zones were taken as evidence of P-solubilization. The isolates with a clear halo zone were purified three times on PVK solid medium. The purified bacterial isolates were maintained on nutrient agar slant and fungal isolates on SDA slant.

2.4 Determination of Phosphate Solubilization Index (PSI)

Qualitative estimation of P-solubilization was done by measuring the PSI. Loopful of each isolate (48hours for bacterial and 3 days fungal) was spotted on the PVK solid medium and incubated at 30±2 °C for 5-7 days in three replicates, with the sterile medium serving as a control. PSI formula, PSI = C+H/C, (C = Colony diameter; H =Halo zone diameter) (Pathak *et al*., 2017). Isolates with PSI ≥140 were preserved for preliminary identification and further studies.

2.5 Preliminary Identification of PSM

Preliminary identification was carried using colonial morphology (the colony colour, colony shape, and elevation aided by hand magnifying glass), gram staining, and biochemical test (citrate utilization, catalase, urease, indole, methyl red, vogues Proskauer, H2S, sugar fermentation, and nitrate reduction test) for bacteria; and cultural and microscopic characteristics for fungi. The outcome was matched against Bergey's Manual, 9th edition, and atlas of fungi (Behzadi and Behzadi, 2012, Cheesbrough, 2000, and Willey *et al*., 2017).

III. RESULTS

3.1 Isolates Zone of Phosphate Solubilization

The result of the phosphate solubilization test is presented in Table 1. The diameter of the isolates' zone of clearance on Pikovskaya's agar medium was measured in millimeters. The symbols (+) was employed to represent the diameter of clearance ranging from 0-1.5mm, (++) represent the diameter of clearing ranging from 1.5-3.0 mm, and (+++) represent the diameter of the zone of clearing ranging from 3.0-4.5 mm while above 4.5mm diameter is represented by four pluses (++++). These measured the various abilities of the isolates to solubilize the insoluble tricalcium phosphate component of the medium. The solubilization was the reason for the clearance. Isolates that could not solubilize mica, do not produce a zone of clearance. They are represented by a negative (-) symbol. A total of 145 discrete isolates were obtained from the eighty samples (forty soil samples, forty root samples). Thirty-nine (26.9%) were phosphate solubilizers. Out of the 39 isolates able to solubilize phosphate, 18(46.1%) solubilization ability was represented by one (+), 12(30.8%) solubilization ability was represented by two (++), 6(15.4%) was represented by three (+++) and 3(7.7%) was represented by four (++++).

TABLE 1: Isolates Zone of Phosphate Solubilization

ZONE OF SOLUBILIZATION	ISOLATES
+	PRZS -12, ORZS-11, MRZS-2, CRZS-3, CRZS-12, MRZS-10, ORTS-7, CRTS-7, CRTS-12, PRZS-10, PRZS-11, ORZS-8, ORZS-20, CRZS-2, PRTS-1, ORTS-3, MRTS-1, MRZS-12
++	CRZS-5, PRZS-14, PRZ-38, ORZS-19, MRZS-13, MRTS-3, MRZS-3, MRZS-1, CRZS-10, CRZS-24, ORZS-17, PRZS-30 PRTS-5, CRZS-20, ORZS-3 ORZS-13, MRZS-18, PRZS-21
+++	ORZS-18, CRZS-23, MRZS-14
++++	CRZS-1, 6-9, 11, 13-19,22,25
-	PRZS-1-9, 13, 15-20, 22-29,31-37 ORZS-1,2,4-7,9,10,12,14-16,20-27 MRZS-4-9,11,15-17,18,20 MRTS-2,4,5,6 PRTS-2,3,4 CRTS-1-6,8-11 ORTS-1,2,4-6,8-10

KEYS

1. ORTS- OKRO ROOT SAMPLE; CRTS- CASSAVA ROOT SAMPLE; PRTS- PLANTAIN ROOT SAMPE; MRTS- MAZIE ROOT SAMPLE; CRZS- CASSAVA RHIZOSPHERIC SAMPLE; PRZS- PLANTAIN RHIZOSPHERIC SAMPLE; MRZS- MAZIE RHIZOSPHERIC SAMPLE; ORZS- OKRO RHIZOSPHERIC SAMPLE.
2. ZONE OF SOLUBILIZATION RANGING 0.0-1.5mm = +; 1.5-3.0 = ++; 3.0-4.5mm = +++; >4.5mm = ++++;

NO ZONE OF SOLUBILIZATION= -

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3.2 Phosphate Solubilization Index (PSI) of Isolates

The result of the phosphate solubilization index of positive isolates is presented in Table 2. The index is the ratio of the diameter of zone of clearance to the diameter of colonial growth by a hundred. The result values range from 109.1 to 190. The isolate with the lowest index was an isolate from okro rhizosphere sample (ORZS-11). The isolate with the highest index was isolated from the cassava rhizospheric sample (CRZS-23). About twenty percent (8) of the phosphate solubilizers isolated had PSI ≥ 140. These isolates were subjected to further studies.

TABLE 2: Phosphate Solubilization Index (PSI) of Isolates

Isolates	Diameters of zone of Clearance	Diameter of Colonial Growth	PSI ($^{D}/_{d}\,x\,100$)
PRZS-14	9.0	5.0	180.0
MRZS-14	8.5	5.5	154.5
CRZS-23	9.5	5.0	190.0
ORZS-11	6.0	5.5	109.1
CRZS-3	7.5	6.0	125.0
MRZS-10	9.5	7.0	135.7
CRZS-20	10.5	6.5	161.5
CRZS-5	7.0	5.5	127.3
MRZS-2	6.5	5.0	130.0
PRTS-5	7.5	5.0	150.0
ORZS-18	11.5	7.0	164.3
PRZS-21	9.0	5.0	180.0
MRZS-13	6.5	5.0	130.0
MRTS-3	7.5	5.5	136.4
ORZS-19	8.0	6.5	123.1
ORTS-7	8.5	6.5	130.8
MRZS-18	9.0	6.5	138.5
ORZS-3	9.0	6.5	138.5
ORZS-13	12.5	7.5	166.7
PRZS-38	10.0	8.0	125.0
MRZS-3	8.5	6.5	130.8
MRZS-1	7.0	5.5	127.3
CRZS-10	7.5	6.0	125.0
CRZS-24	7.5	5.5	136.4
ORZS-17	8.0	6.0	133.3
PRZS-30	8.5	7.0	121.4
CRTS-12	10.5	8.0	131.3
CRTS-7	9.0	6.5	138.5
PRZS-10	8.5	6.0	130.8
PRZS-11	7.5	5.0	136.4
ORZS-8	9.0	7.0	128.6
ORZS-20	8.5	6.5	130.8
PRTS-1	10.0	7.5	133.3
MRTS-1	7.5	6.0	125.0
ORTS-3	9.0	6.5	138.5
CRZS-2	8.5	7.0	121.5
MRZS-12	9.0	7.0	128.6

3.3 Identification of Bacterial Isolates with PSI≥140 Using Colonial, Morphological and Biosynthetate Characteristics

The morphological and biochemical characteristics of the bacterial isolates with PSI≥ 140 are presented in Table 3. The isolates with PSI≥ 140 were *Pseudomonas* sp. and *Bacillus* sp. Their morphological characteristics were recorded based on their size, shape, margin, elevation, pigmentation, and color. Gram stain test revealed that *Pseudomonas* sp is gram-negative as it retained the color of the counterstain used. Oxidase test was used to differentiate *Pseudomonas* from other gram-negative bacilli. Catalase test was used to test the ability of *Pseudomonas* to grow in the presence of oxygen. Other biochemical tests carried out showed that *Pseudomonas* was positive for the following test: "citrate, gelatin hydrolysis, nitrate oxidase, and test". On the other hand, *Pseudomonas* was negative for indole, methyred urease, voges proskauer, maltose, glucose and sucrose. *Pseudomonas* does not produce gas and does not hydrolyze starch. The spore test revealed that *Pseudomonas* is a non-spore former.

The result of the gram stain revealed that *Bacillus* sp was a gram-positive organism because it retained the color of the primary stain (crystal violet) and was not decolorized by alcohol. It was found to be catalase, citrate, nitrate, voges

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proskauer , mannitol, maltose, glucose, sucrose and gelatin hydrolysis positive. *Bacillus* sp. showed a negative result for indole, methyl red, oxidase, and urease. The result of the spore stain revealed that it is a spore former.

TABLE 3: Identification of Bacterial Isolates with PSI ≥ 140 using Colonial, Morphological and Biochemical Characteristics

Isolates	PRZS-14	MRZS-14	CRZS-23	CRZS-20	PRZS-21	PRZS-5	ORZS-18	ORZS-13
Colonial and Morphological characteristics								
Size	2mm	3mm	3mm	3mm	3mm	2mm	3mm	3mm
Shape	Rods	Spherical	Rods	Rods		Rods	Rods	Rods
Margin	smooth edge	Entire	Irregular Edge	Irregular edge	Entire	smooth edge Convex	Irregular edge	Irregular edge
Elevation	convex	convex	Flat	Flat	Convex		Slightly Convex	Flat
Colour	bluish green	Yellow	Glassy Appearance	Glassy appearance	Pale orange	Bluish green +	Glassy appearance	Glassy appearance
						-	-	-
Pigmentation	+	-	-	-	-		+	+
Gram reaction	-	+	+	+				
Biochemical Test						+		
						+	+	+
Catalase	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	-	-
Gas	+	-	-	-	-		+	+
Gelatin hydrolysis	+	+	+	+	+	-		
						-		-
Indole	-	-	-	-		+	-	-
Methyle red	-	-	-	-	-		-	+
Nitrate reduction	+	+	+	+	+	+	+	
						-		-
Oxidase	+	-	-	+	+	-	+	+
Sporer	-	+	+	+	+	-	+	-
Urease	-	-	-	-	-		-	+
Voges Proskauer	-	+	+	+	+	+	+	
						-		+
Mannitol	+	+	+	+	+	-		+
Maltose	-	+	+	+	+	-	+	+
Glucose	-	+	+	+	+	+	+	+
Surcrose	-	+	+	+	+	-	+	+
Motality	+	+	+	+	+	***Pseudomonas***	+	+
Starch	-	+	+	+	+	**sp.**	+	***Bacillus***
Preliminary Identification	***Pseudomonas*** **sp.**	***Bacillus sp.***	***Bacillus sp.***	***Bacillus sp.***	***Bacillus sp.***		+ + ***Bacillus*** **sp.**	**sp.**

3.4 Identification of Fungal Isolates with PSI≥140 Using Cultural and Microscopic Characteristics

The result of the identification of fungal isolates with PSI≥ 140 is as presented in Table 4. The isolates were *Aspergillus* sp. and *Penicillium* sp. *Aspergillus* sp. was found to be a powdery colony, with dark brown front colour. The reverse colour was also brown. It had a flatty spread on the surface of the solid medium. Microscopically, *Aspergillus* sp. had septate and branched hyphae with conidia that appeared in chains. *Penicillium*s sp. front colour was found to be grey with a large white border and white reverse. Microscopically, *Penicillium* sp. has long branched septate conidiophores consisting of brown-like conidia in chains at the tips of the phialides.

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Table 4: Identification of Fungal Isolates with PSI ≥140 Using cultural and Microscopic characteristics

Isolates	Cultural Characteristics	Microscopic Characteristics	Preliminary Identification
MRTS-3	Powdery, dark brown, flatty spread and brown reverse.	Septate and branched hyphae with conidia in chains.	*Aspergillus* sp.
MRZS-18	Grey colony with large white border and white reverse.	long conidiophores consisting of brown like conidia in chains.	*Penicillium* sp.
ORZS-3	Grey colony with large white border and white reverse.	long conidiophores consisting of brown like conidia in chains	*Aspergillus* sp.
PRZS-38	Powdery, dark brown, flatty spread on the surface of the solid medium and brown reverse.	Septate and branched hyphae with conidia in chains.	*Penicillium* sp.

3.5 Phosphate Solubilization Efficiency of Isolates on Pikovskaya's Agar

The result of the Phosphate Solubilization Efficiency of Isolates on Pikovskaya's Agar is presented in Fig 1. The bar chart is the mean of triplicate solubilization indexes of the isolates in two days intervals. The bar chart reveals that bacterial isolates are more phosphate solubilizing efficiency than fungal isolates. There were two bacterial genera isolated: namely *Pseudomonas*, and *Bacillus*, with efficiency order as follows: *Pseudomonas*> *Bacillus*. Among fungal isolates, *Penicillium* sp. is more phosphate solubilizing efficiency than *Aspergillus* sp.

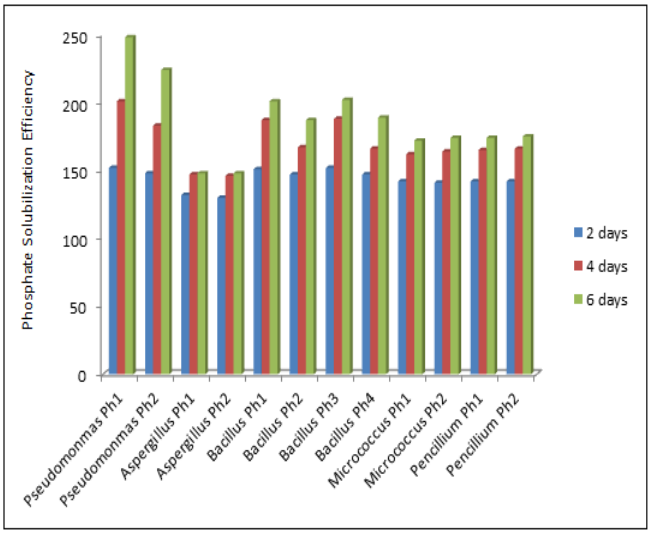

Fig. 1: Phosphate Solubilization Efficiency of Isolates on Pikovskaya's Agar

IV. DISCUSSION

Phosphate plays an indispensable metabolic role in plant biochemistry. It is of great importance in photosynthesis, respiration, energy storage, and transfer, cell division and enlargement, and in several other processes in living plants. Below optimal P availability, plant yield is impaired below economic level. The packaging of P and other plant primary nutrients (nitrogen and potassium) into chemical fertilizer, which was responsible for the success of the Green Revolution that save a billion people (Pingali, 2012), is discovered to be inefficient. This is because 75-90% of P fertilizer applied to soil is immobilized in precipitation reaction with Ca2+, Al3+ or Fe3+ depending on the soil pH (Goldstein, 1994). The high cost of the fertilizer and its environmental degradation ability demands the development of a sustainable biological-based source of P to avert the danger of permanently destroying the fertility of soil through prolonged use of P fertilizer (Aliyat et al., 2020). This study, which isolated and characterized Phosphate solubilizing microorganisms from the rhizosphere and the roots of crops indigenous to Ihiagw-Owerri , Imo State Nigeria, was inclining to the new trend of sourcing P biologically.

Whitelaw (2000) reported the importance of microbial solubilizers in the maintenance of the global cycle. The mechanisms used by these microbes include the excretion of organic acids or the production of phosphatase enzymes (Kucey, 1983). The medium used for the screening of the isolates for phosphate solubilizing ability, Pikovskaya's agar, contains tricalcium phosphate (Ca3(PO4)2) an insoluble phosphate. The ability of the isolates to solubilize it produces a positive result which manifests as the zone of clearance by 26.9% (39) of the 145 isolates screened. The production of organic acids by these solubilizers was the reason for the zone of clearance. The carboxylate of the organic acids produced, through chelation and ligand exchange, brought about the solubilization. The diameter of clearance varied between 1.5-4.5mm among the isolates; with 46.1% producing ≤ 1.5mm, 30.8%≤3.0mm, 15.4% ≤ 4.5, and 7.7% ≥ 4.5mm. The diameter could be proportional to the concentration of organic acid produced.

The phosphate solubilization index (PSI) of the isolates ranged from 109.1 to 190. Isolates with PSI ≥ 140 were 20% of the 39 isolates that could solubilize phosphate. Microbial identification protocol carried out on these isolates revealed that 66.7% were bacterial while 33.3% were fungal. The genera of microbes were *Bacillus* sp. were 75% of the bacterial isolates while *Pseudomonas* sp. was 25%. Fungal isolates were *Aspergillus* sp and *Penicillium* sp with an equal frequency of occurrence.

Bhattacharyya and Jha (2012) report of most significant bacterial solubilizers as Azotobacter, *Bacillus (B. megaterium, B. circulans, B. subtilis, B. polymyxa*, *Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas (P. striata),* and *Serratia. Bacillus* sp. are among their report. The genera were 75% of the bacterial isolated as phosphate solubilizer. In the report of Oteino *et al.*, (2015) *Pseudomonas*, *Acinetobacter*, *Pantoea*, and *Enterobacter*, and *Bacillus* were among the major solubilizers of phosphate. The two bacterial genera isolated in this work, *Pseudomonas* and *Bacillus*, were commonly reported as been among effective phosphate solubilizing bacteria. These microbes were able to solubilize inorganic soil phosphates by the production of organic acids, siderophores, and hydroxyl and carboxyl groups that chelate the cation of the phosphate thereby converted the latter into soluble forms (Sharma *et al.*, 2013). The report of Krishnananda and Dipika (2017) is in line with the result that the fungal isolates of the genera *Aspergillus* sp. and *Penicillium* sp. solubilize phosphate. In the soil, phosphate exists as inorganic (IP) and organic forms (OP). The insoluble IP can be dissolved by low molecular weight organic acids (e.g., citric and gluconic acids) produced and released by both phosphorus solubilizing bacteria (PSB) and fungi (Khan *et al.*, 2014), and OP can be digested by extracellular enzymes (e.g., phosphatase and phytase) mainly synthesized and secreted by microbes (Alori*et al.*, 2017). The application of phosphorus solubilizing microbes provides a new approach to improve soil quality, this will help in achieving sustainability in agriculture. Phosphorus solubilizing microbes especially PSB are widely distributed in soils, freshwater, seawater, and sediments. These organisms are responsible for the cycling of insoluble P to soluble PO43- ion.

The solubilization efficiency of the isolates identified reveals that *Pseudomonas* sp. had the highest mean value of 248.1±0.1, followed by *Bacillus* sp., 201.7±0.1, then, *Penicillium*, 175.0±0.4, and the least was *Aspergillus* sp. with 148.3±0.9. This implies that the bacterial isolates are better phosphate solubilizers than the fungal. The order of the efficiency is *Pseudomonas* > *Bacillus*>*Penicillium* >*Aspergillus*. This result corroborates, in part with the phosphate efficiency reported by Selvi et al.(2017), in which *Pseudomonas* sp. had an efficiency upper limit of 71.43%, *Bacillus* sp., 61.54%, *Penicillium* sp., 25%, and *Aspergillus* sp. 33.33%. The variation is, while *Aspergillus* sp. upper limit was more than that of *Penicillium* sp. in their report, the reverse is the case in our result. But, if the mean value of the range of their result is considered, which is 22.12%, then the variation does not exist

V. CONCLUSION

There is the possibility of biological sourcing of P for the enhanced crop productivity using isolates from the rhizosphere and roots of crops indigenous to Ihiagwa-Owerri Imo State Nigeria. These phosphate solubilizing isolates, identified as *Penicillium* sp. *Aspergillus* sp., *Pseudomonas* sp., and *Bacillus* sp. can be optimized and developed into biofertilizer products for use as an alternative to chemical fertilizer. This will ensure sustainable agro development in Nigeria and open an agro channel for entrepreneurial microbiology.

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