Lafia Journal of Scientific & Industrial Research (LJSIR), Vol. 1(1&2), 2023
e-ISSN:... p-ISSN: 3026 – 9288 pages: 18-24
https://lafiascijournals.org.ng/index.php/ljsir/index ⇒ Published by the Faculty of Science,
Federal University of Lafia, Nasarawa State, Nigeria

Isolation and Optimization of Phosphorus Solubilizing Bacteria from Maize Rhizosphere in Nasarawa State, Nigeria

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Abstract

Phosphorus solubilizing bacteria (PSB) are a type of plant growth-promoting rhizobacteria which has the ability to convert insoluble phosphate into plant available forms. They are used as biofertilizers to restore soil health and fertility. In the present investigation, isolation, characterization and optimization of phosphate solubilizingactivity of these microbes at different pH, temperature and carbon source was carried out. Eight phosphorus solubilizing rhizospheric bacteria (PSRB) isolates were recovered from different farms in six local governmentfrom maize rhizosphere using the spread plate method on Pikovskaya agar. Amongstthese eight isolates, five were recovered from Lafia local government while threewere recovered from Kokona local government with the development of a prominenthalozone. All isolates were morphologically characterised, amplified, and sequencedfor taxonomic identification using 16S primers. The results indicated they belong tomember of the genus *Acinetobacter, Ochrobactrum, Brucella, Curtobacterium,Leifsonia* and *Microbacterium,* respectively. These strains when grown at differentconditions of external carbon sources, temperature and pH showed highestphosphorus solubilizations of 326.23 µg/ml ± 0.21 at 30°C, 325.50 µg/ml ± 1.13 at apH of 7 and 320.30 µg/ml ± 0.36 with glucose as carbon source. The results fromthese findings could indicate the use of these isolate as trial biofertilizers on the fieldwhich will help improve crop yield and enhance plant growth promotion.

Keywords: Phosphorus solubilizing bacteria, inorganic phosphate, phosphorus, solubilization, orthophosphate, optimization, rhizobacteria

Article History
Submitted
August 10, 2023
Revised
October 12, 2023
First Published Online
October 31, 2023

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doi.org/10.62050/ljsir2023.v1n1.233

Introduction

With the demand placed upon intensified farming in an ever-growing population, world fertilizer production will have to increase significantly to meet future demands with an increase of about 50-100% by 2050 [1]. Taking into cognizance the low availability of fertilizer in the global agricultural market, which is followed by a general increase in prices, compounded by fluctuations in oil prices, the market is threatened even more [2].

Soil phosphorus is an essential macronutrient for plant growth and development. It is a component of key molecules such as nucleic acids, phospholipids, and ATP which are necessary for energy transfer, photosynthesis, and building genetic material [3]. Soil phosphorus exists in organic and inorganic forms, with inorganic orthophosphates being the only directly absorbable form for plants. Phosphorus availability is influenced by soil pH, depth, soil type, and organic matter decomposition. Prolonged fertilizer use can lead to phosphorus build-up, which requires prudent management [4]. Phosphorus is converted into orthophosphate through processes like mineralization and hydrolysis for plant uptake. Immobilization converts inorganic phosphorus to organic forms, reducing availability, while mineralization and hydrolysis release it back into the soil solution.

Given that natural sources of Phosphorus (P) fertilizers are finite [5], the environmental policies rationalizing its use and their excessive use, the availability of P becomes even more worrisome hence the need for the selection of microbial strains that effectively promote the growth of major crops becomes essential [6]. These strains can improve the plant fertilization and plant nutrient availability. In addition to improving crop yield and nutrient supply, biofertilization aims at the development of a more sustainable and environmentally friendly agricultural practice [7].

Phosphorus solubilizing bacteria (PSB) are microorganisms that convert insoluble phosphorus in the soil into soluble forms through the process of mineralization and solubilization [8] by making phosphorus available for plant uptake. PSBs have been reportedly isolated from various sources and most belong to genera like *Pseudomonas*, *Bacillus*, and *Micrococcus* [9, 10]. In order to better understand the spread and diversity of PSB around the rhizosphere of different plants, information regarding their identification, characterization and optimization is highly required [11, 12].

The aim of this work was to isolate and optimize PSB from the maize rhizosphere from soil in Nasarawa state.

Materials and Methods

Study area

The current study was conducted in two farms each across six local governments located in Nasarawa State, Nigeria.

Collection of samples

The rhizospheric soil of maize plant (*Zea mays*) was collected from two different farms across six local governments in Nasarawa state (Kokona, Akwanga, Nasarawa Eggon, Lafia, Obi and Doma) following the protocols put forward by Son *et al.* [13]. Each soil sample was thoroughly mixed and homogenized. Twenty kilograms (20 kg) of three months old maize rhizosphere soil were collected as representative sample from each farm. Soil samples were collected in polyethene bags, sealed and stored prior to analysis.

Isolation of phosphate solubilizing bacteria

Phosphate solubilizing bacteria were isolated using serial dilution method and samples were serially diluted up to (10^{-9}). Spread plate technique was used for isolation and screening by plating 0.1 mL of 10^{-3} and 10^{-6} onto Pikovskaya (PVK) media agar plates consisting of tricalcium phosphate as sole phosphate source, yeast extract 0.5 g/l, dextrose 10.0 g/l, calcium phosphate 5.0 g/l, ammonium sulfate 0.5 g/l, potassium chloride 0.2 g/l, magnesium sulfate 0.5 g/l, manganese sulfate 0.0001 g/l, ferrous sulfate 0.0001g/l, agar 15.0 g/l in 1 L distilled water [14]. Plates were then incubated at 37°C for 7 days to observe the appearance of clear halozones around their colonies. The bacterial colonies producing a distinct halozone around the colony with different morphology were further selected and sub-cultured as described by Aliyat *et al.* [6] to obtain pure cultures which were maintained in 30% glycerol at20°C for further use.

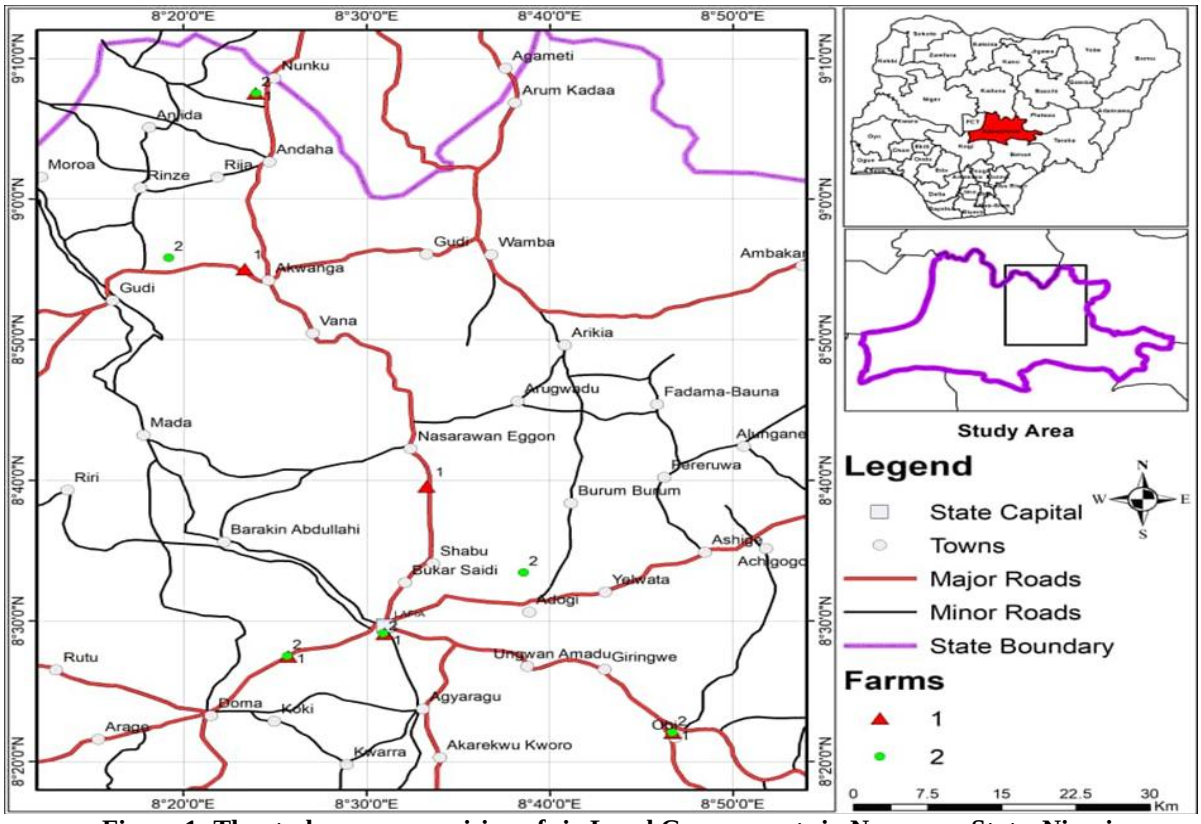


Figure 1: The study area comprising of six Local Governments in Nasarawa State, Nigeria

Qualitative determination of phosphorus solubilizing bacteria

Strains showing halozones were aseptically spot inoculated on the centre of the Pikovskaya agar plate (PVK) using a sterile toothpick and incubated at 30°C for 7 days. The phosphorus solubilization index (PSI) which is the ratio of total diameter comprising of the colony diameter and halo zone diameter to the colony diameter was calculated [15]. Unstreaked PVK plate served as control.

$$PSI = \frac{colony\ \ diameter\ + halozone\ \ diameter}{colony\ \ diameter}$$

Genomic DNA isolation and molecular characterization

Genomic DNA of the PSB isolate was isolated by following the method of Leonard *et al.* [16]. The electrophoresis of the Genomic DNA was carried out in 1% agarose gel stained with Redsafe (1.25 µl) submerged conditions using 0.5 × TAE buffer as tray buffer.

To each 10 µl of genomic DNA sample, 2 µl of 1× loading dye was added. 1.5 kbp DNA ladder marker was used as standard and the gel was run at 110 V for 45 min until the loading dye reached the gel front. The genomic DNA of the isolates were viewed under the gel documentation system in the form of bands and the image was taken and saved in computer and used for further molecular studies.

Amplification of genomic DNA

The amplification of genomic DNA was carried out using specific oligonucleotide primer sequences. The 50 µl PCR reaction mixture consisted of 0.5 µl of forward (BSF 8/20) 5'AGAGTTTGATCCTGGCTCAG-3' and 0.5 µl of reverse primers (BSR 1541/120) 5'AAGGAGGTCATTCAGCCGCA-3', 5 µl of EconoTaq DNA polymerase, 3 µl sterile distilled water and 1 µl of DNA was used (adjusted to 50 µl) was subjected to amplification which was carried out in Simpli Amp thermal cycler with a total of 35 cycles using a set of forward (AGAGTTTGATCCTGGCTCAG) and reverseprimers (AAGGAGGTCATTCAGCCGCA) under cycling conditions that consisted of an initial denaturation at 95°C for 3 min and then 33 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 120 s. All the amplifications products were then electrophoresed in 1.0% agarose gel and the amplified DNA bands were viewed under thevisualized using BIO-RAD Molecular imager Gel Doc. PCR products were then purified using commercial PCR purification kit (Qiagen, Germany) according to manufacturer's instruction. The gel aliquots with amplified products were sent to Apical Scientific Limited, Malaysia for sequencing using a big dye terminator cycle sequencing kit (Applied BioSystems). Nucleotide sequences were classified using BLAST analysis, edited using the software Bioedit and compared consensus sequence with published sequence in National Centre for Biotechnology Information (NCBI) database for determination of closest strain type.

Morphological characterization of bacterial isolates

Morphological Characterization of bacterial isolates was based on Gram staining and colonial morphology as based on Bergey's Manual of Systematic Bacteriology [17].

***In-vitro* PSB optimization soluble P at different pH, temperature and carbon source in growth media**

In-vitro optimization at different temperatures (20, 25, 30, 35, 40 and 45°C) of selected isolates (LAA1, LAA3, LAA4 and a co-inoculant of LAA1, LAA3 and LAA4) was assessed for phosphate solubilizing ability. Zero-point five millilitre of bacterial culture as described bysome workerswas inoculated into a 250 mL flask containing 50 mL of PVK broth and 2500 µg/mL tricalcium phosphate, incubated at 30°C on rotary shaker incubator (Amsterdam) at 200 rpm for 5 days [15]. Upon incubation, 5 mL of broth culture was withdrawn and supernatant was obtained by centrifugation at 10,000 rpm for 15 min and passed through a 0.45 µM Millipore and then 0.1 mL of the supernatant (filtered) was mixed with 0.25 mL of Barton's reagent and volume was made up to 5 ml with double distilled water (ddw). After 10 min, the intensity of yellow colour was read on spectrophotometer (UV–Visible Spectrophotometer-V-1730) at 430 nm and the amount of P-solubilized was estimated. All experiments were done in triplicate.

Similarly, *in-vitro* optimization at different pH (3, 4, 5, 6, 7 and 8) of selected isolates (LAA1, LAA3, LAA4 and a co-inoculant of LAA1, LAA3 and LAA4) adjusted with 1M HCl and 1M NaOH was assessed for phosphate solubilizing ability. The amount of soluble phosphate was determined as described above. Lastly, *in-vitro* optimization at different carbon source (glucose, sucrose, fructose and lactose) of selected isolates (LAA1, LAA3, LAA4 and a co-inoculant of LAA1, LAA3 and LAA4) was assessed for phosphate solubilizing ability. The amount of soluble phosphate was determined as described above.

Data analysis

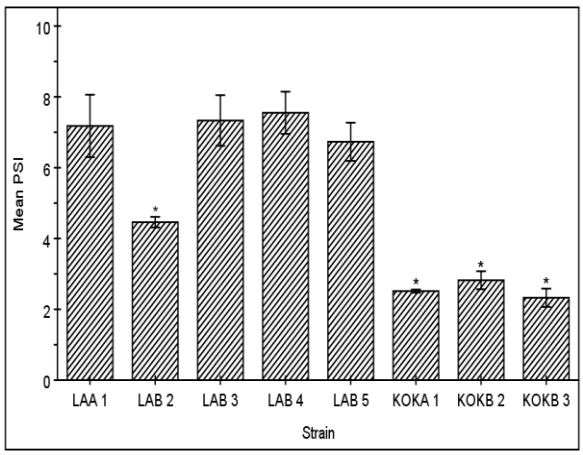
Statistical analyses were performed with the Statistical Package for the Social Sciences (IBM SPSS Incorp., Illinois, Chicago, USA) version 27 for Windows. Statistical significance was set at P<5% level and statistical analyses conducted were two-tailed. Whenever statistical significance was noted, it was followed by Tukey post-hoc tests for pairwise comparison against the control group.

Results and Discussion

Isolation and screening of PSB strains

From the rhizosphere soil sample of maize plant collected from two farms in six local governments, a total of 8 PSB were recovered showing prominent halozones (Plate 1). Out of 8, 5 isolates were recovered from maize rhizosphere of two farms in Lafia local government while the other three were from Kokona local government. The isolates were designated as LAA1, LAA2, LAA3, LAA4, LAB5, KOK1, KOK2 and KOK3 for easy identification.

Plate 1: PSB strain from soil showing halozone in Pikovskaya agar from Lafia Local Government



*Indicates statistical significance at P <0.05 for pairwise comparison

Figure 2: Qualitative measurement of phosphate solubilizing bacteria efficiency after 7 days of incubation

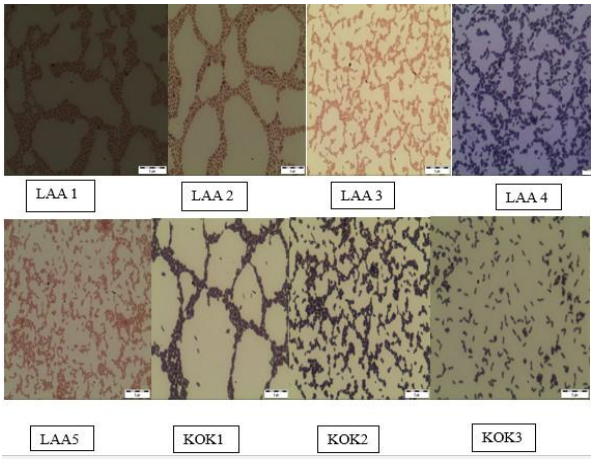


Plate 2: A morphology of LAA1, LAA 2, LAA 3, LAA 4, LAA5, KOK1, KOK2 and KOK3 under light microscopy observation (1000×) Bar 5 µm

Qualitative determination of phosphorus solubilizing bacteria

Phosphate solubilization index (PSI) was determined by dividing colony diameter and halozone diameter by colony diameter (cm) for growth on Pikovskaya agar. All strains demonstrated phosphate solubilization activity (Fig. 2) with LAA1, LAA3 and LAA4 recording the highest PSI with 8.33cm±0.57, 8.30cm±0.52, 8.33cm±0.52, respectively while the least PSI was recorded by KOK1 with 2.58cm±0.38.

In the present study, 8 strains were isolated from different maize rhizosphere on the formation of clear halo zone formation around bacterial colonies on Pikovskaya agar plates. The phosphorus solubilization index (PSI) of these strains was also qualitatively determined. The result was in accordance with other works previously reported by many scholars who also selected PSB on the basis of halo zone formation and measured solubilizing index of 1.85–2.88 PSI, 3–4 PSI and 4–7 PSI, respectively [8, 9, 18]. The phosphate solubilization index of PSB strains increased with increase in number of days in the present study. The increase could be as result of elaboration of the enzyme phosphatase and production of organic acids [4, 8]. The study further corroborates with works done by some scholars who reported strains with high solubilization index [6, 19].

Cultural, morphological and molecular characterization of PSB isolates

The isolated PSB strains consist of four Gram- positive and four Gram-negative (Plate 2). Five of the bacterial strains were creamy while two were yellowish, smooth and opaque.

Table 1: Strain names with accession numbers deposited at National Centre for Biotechnology Information (NCBI) database

Strain code	Strain name	Accession number
LAA1	*Acinetobacter_pittii*_GUP-1	OP159675
LAA2	*Acinetobacter_sp.*_GUP-2	OP159676
LAA3	*Brucella_haematophila*_GUP-3	OP159677
LAA4	*Brucella_haematophila*_GUP-4	OP159678
LAA5	*Ochrobactrum_soli*_GUP-5	OP159679
KOK1	*Curtobacterium_citreum*_GUP-6	OP159680
KOK2	*Leifsonia_shinshuensis*_GUP-7	OP159681
KOK3	*Microbacterium_testaceum*_GUP-8	OP159682

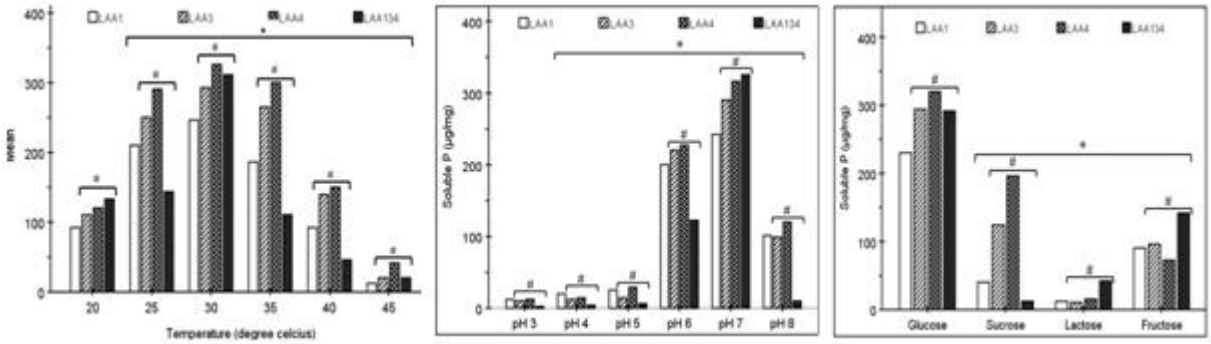
The molecular characterizations of the eight PSB strains, based on 16S rRNA sequences are presented in Table 3. Six genera were identified based on partial sequencing of their 16S rRNA gene: *Acinetobacter, Ochrobactrum, Brucella, Curtobacterium, Leifsonia* and *Microbacterium*. Strains were deposited with National Centre for Biotechnology Information (NCBI) database and given accession numbers (Table 1). The PSB strains were culturally and morphologically characterized, revealing four Gram positive rods (LAA4, KOK1, KOK2 and KOK3) and four Gram negative rods (LAA1, LAA2, LAA3 and LAA5). These findings fail to corroborate the studies of [5, 20, 21] who found dominance of gram negative over gram positive PSB strains.

Partial sequencing of 16S rRNA gene of bacterial isolates showed they belong to member of the genera *Acinetobacter, Ochrobactrum, Brucella, Curtobacterium, Leifsonia* and *Microbacterium*. The occurrence of *Acinetobacter* has previously been reported by scholars [7, 22] from rhizosphere grasses in Spain. *Bacillus* and *Pseudomonas* species have been reported to be efficient phosphate solubilizers [23, 24].

***In-vitro* optimization for PSB growth at different pH, temperature and carbon source in growth media**

Single inoculum of LAA1, LAA3, LAA4 and a combination of (LAA1, LAA3 and LAA4) were grown at different pH, temperature and carbon source. The results showed that both single and mixed inoculation were able to solubilize phosphate from calcium tri phosphate after 48 h. There was a significant difference ($p<0.05$) in phosphate concentration at the different incubation temperatures. The highest phosphate concentration of 326.23 µg/ml ± 0.21 at 30°C was observed with medium inoculated with LAA134 while the least was 12.47 µg/ml ± 0.23 at 45°C with medium inoculated with LAA1 (Fig 3). The optimum temperature for phosphorus solubilization for all tested strains was 30°C.

Strain LAA 134 solubilized 325.50 µg/ml ± 1.13 phosphate from tricalcium phosphate at pH 7. LAA134 was also able to solubilize the least phosphate with 2.44 µg/ml ± 0.02 liberated at pH 3. There was a significant difference ($p<0.05$) in phosphate concentration at the different incubation pH (Fig 3). It was observed that in medium supplemented with glucose as carbon source, solubilization was highest. LAA4 was able to liberate 320.30 µg/ml ± 0.36 when inoculated in media consisting of glucose as only carbon source while LAA3 liberated the least soluble phosphate of 10.45 µg/ml ± 0.03 in medium containing only lactose (Fig. 3).



*Indicates statistical significance at P <0.05 for pairwise comparison with LAA1 as control
#indicates statistical significance at P <0.05 for pairwise comparison each group against control

Figure 3: *In-vitro* optimization of phosphate solubilization by PSB at different temperature, pH and carbon sources for 48 h of incubation

PSB have been reportedly found in wide variety of soil types but their function is greatly influenced by different environmental factors which include organic matter, cation exchange capacity, temperature, salinity, pH and soil composition [14, 21, 25]. Thus, ability to withstand intrinsic and extrinsic stress is of great importance for microorganism growth, survival and establishment in the rhizospheric soils which was tested in the current research at laboratory conditions by optimizing the performances of the selected strains at different pH, temperature and sugar utilization.

The present study revealed that the best temperature and pH for phosphorus solubilization *in-vitro* were 30°C and 7, respectively. The highest phosphate concentration of 326.23 µg/ml ± 0.21 at 30°C was observed with medium inoculated with the mixed culture. This works corroborates findings of some scholars [6, 26] who also found the highest solubilization at same pH and temperature as in the case of this study even though more soluble P was liberated in their study. Scholars have also reported highest amount of soluble phosphate at pH 7 [15, 25]. This works contradicts a rather similar work done by Agboola *et al.* who reported temperature of 35°C and pH 9 for phosphorus solubilization although they used a different strain of bacteria [22].

Glucose has been reported to be the ideal carbon source for phosphorus solubilizing bacteria [27]. It was observed that in medium supplemented with glucose as carbon source, solubilization was highest. LAA4 was able to liberate 320.30 µg/ml ± 0.36 in medium containing glucose when compared with other carbon sources. Chen and Liualso reported that by using glucose, sucrose, starch, fructose, lactose, mannitol, or glycerine as the carbon source, the P solubilization efficiency of *Pantoea* spp S32 strain was highest with glucose as carbon source [27].

Conclusion

Phosphate solubilizing bacteria (PSB) of the genera *Acinetobacter, Ochrobactrum, Brucella, Curtobacterium, Leifsonia* and *Microbacterium* were isolated in this study. The PSB isolates were able to solubilize inorganic phosphate in the form of calcium tri phosphate in liquid medium at different pH, temperatures and carbon sources. It can be concluded that from this research, the best carbon source for PSB solubilization was found to be glucose and the highest solubilization was attained at a pH of 7, temperature of 30°C for 48 h of incubation. Applying these isolates for field study in enhancing plant growth can prove a vital tool for the production of biofertilizers.

Conflict of interests: The authors declare no conflict of interest.

Funding statement: This work was funded by the Tertiary Education Trust Fund, Nigeria and grants provided by Prof. Sudesh Kumar in the Ecobiomaterial Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Malaysia.

Acknowledgments: The authors are grateful to the Management of Federal University of Lafia, Nasarawa State, Nigeria for providing the platform necessary for accessing the fund. I also want to acknowledge Prof. Abioye, Dr. Bala, Dr. Philip for their thorough supervision throughout this work and lastly Prof. Sudesh and members of the Ecobiomaterial laboratory for their support.

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Citing this Article

Uyi, O. G., Abioye, O. P., Bala, J., Tsado, P. A., Kumar, S. & Ahmad, S. (2023). Isolation and optimization of phosphorus solubilizing bacteria from maize rhizosphere in Nasarawa State, Nigeria. *Lafia Journal of Scientific and Industrial Research*, *1*(1&2), 18 – 24. https://doi.org/10.62050/ljsir2023.v1n1.233