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EC PHARMACOLOGY AND TOXICOLOGY

Research Article

Isolation and Biochemical Characterization of Phosphate Solubilizing Bacteria used in Biofertilizer

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Received: September 21, 2021; **Published:** February 25, 2022

Abstract

Microorganisms in the rhizosphere can dissolve and make available the insoluble phosphorus in the form available for plants. This study aimed at isolating phosphate solubilizing bacteria from soil samples. Isolation and characterization were carried out on three soil samples that were collected from three different locations. A total of twelve phosphorus solubilizing microbial colonies were isolated on the pikovaskya agar medium using dilution plate technique. Characterization was done by identification of external colony morphology, colour, halo zone formation and appearance on the PVK agar plates. Six isolates were further sub cultured using agar slants in order to carry out biochemical tests. The isolate tested positive to Gram staining, starch hydrolysis, methyl red test, catalase test and indole test. The soil sample from Amurri farmland and Agbani farmland were moderately saline from the value of the test results while soil sample from Esut farmland was slightly saline. From the test results carried out phosphorus solubilizing bacteria were present in the soil samples collected.

Keywords: *Rhizosphere; Phosphorus; Solubilzing; Bacteria; Isolat; Gram Stain*

Introduction

Biofertilizers are microbial inoculants or carrier dependent formulations consisting of living or latent cells of efficient strains of nitrogen fixing, phosphate-solubilizing and cellulose decomposing microorganisms applied to the seed or soil, to enhance soil fertility and plant growth through its effects on the strength and bioactivity of useful microorganisms in the soil [1,2]. Biofertilizers are composed of living microbes, upon application to the seed, plant surfaces or soil; occupy the rhizosphere or the inner part of the plant. They then promote growth by increasing the availability and supply of basic nutrients to the host plants through nitrogen fixation and phosphate solubilization [3,4], and promoting plant growth through the biosynthesis of growth facors.

It is eco-friendly and cost effective serving as a substitute to chemical fertilizers. Biofertlilzers can mutually associate with plant roots and readily and safely breaking down complex organic material into simple compounds, so that plant can readily absorb them. In its role, it maintains the natural habitat of the soil; conferring resistance against water shortage and some soil-borne disease. Biofertilizer produces a better harvest, safe to human and preserves the environment while offering economic development for the farmers and their country [5].

Biofertilizers are loaded in appropriate carrier such as lignite or peat which helps in maintaining sufficient shelf life and enable their simple usage [6]. The different materials of carriers used are peat, sawdust and charcoal which support high number of plant growth promoting rhizobacteria, phosphate solubilizing bacteria and maintain its survival. For a carrier to be considered suitable, it should be environmentally friendly, soluble in water so that bacteria can be released, and must be able to withstand a harsh environment condition.

The ability of microbial inoculation to enhance growth of plant is greatly influenced by the quantity administered into the soil [7]. Phosphorus solubilizing bacteria is involved phosphorus nutrition via its role in the bioavailability to plants through release from inorganic and organic phosphorus pools. The key process in soil for mineral phosphate solubilization is reduction of soil pH by microbial production of organic acids and mineralization of phosphorus by acid phosphatase. Plant roots thus takes up different forms of phosphorus like H_2PO_4, HPO_4, depending on the pH, temperature, moisture content and other nutrient or mineral present in the soil [8,9].

Phosphorus is important in plant growth and is the major plant growth containing nutrient despite its abundance in soil in both organic and inorganic forms [10]. Energy transfer, signal transduction, nitrogen fixation in legumes, crop quality and resistance to plant disease are the majour characteristics associated with phosphorus nutrition [11]. Phosphorous also plays a vital role in root elongation, proliferation, with its inadequacy affecting root configuration [12]. Deficiency of phosphorous negatively affects grains yield. It is thus highly necessary that farmers makes sure that phosphorous is made available to the plants.

Methods

Collection of soil samples

Soil sample were collected from ESUT school farm, Amurri, and Agbani farmlands in clean sterile polythene bags at 5 - 10 cm depth. Pebbles and plant residue were removed from the samples and were labeled properly with masking tape. The samples collected were taken the Laboratory for analysis.

Media preparation

Preparationof pikovaskya broth

Pikovaskya broth was prepared for production media of PSB bacteria. This media is composed of: glucose -10 gm/ml, yeast extract - 0.5 g m/ml, ammonium sulphate- 0.5 gm/ml, magnesium sulphate-0.1 gm/ml, calcium phosphate - 5 gm/ml, sodium chloride - 0.2 gm/ml, potassium chloride - 0.2 gm/ml, manganese sulphate - 0.002 gm/ml, ferrous sulphate - 0.002 gm/ml and distilled water - 1000 ml. The constituents were dissolved in 1000 ml of distilled water in a conical flask, sealed properly and autoclaved at 121 ^{o}C 15/psi for 15mins.

Preparation of sucrose broth

A quantity, 25.7 g of sucrose broth was dissolved in 1 L of distilled water in a conical flask and 10 ml of the suspension was dispensed into clean test tubes, sealed properly and sterilized in an autoclave at 121 ^{0}C 15 psi for 15mins.

Preparation of simmon's citrate agar

A quantity, 24.28 g of simmon's citate agar was dissolved in 1000 ml of distilled water in a conical flask sealed properly and autoclaved at 120 ^{0}C 15 psi for 15 mins.

Preparation of pikovaskya agar slant

A volume, 10 ml of already prepared pikovaskya agar was dispensed into clean bijou bottles sealed properly and were utilized at 121 ^{0}C of 15 psi for 15 mins. Thereafter the bottles containing the sterilized medium were placed in slant position and were allowed to solidify.

Preparation of simmon's citrate slant

A volume, 10 ml of the already prepared simmon's citrate was dispensed into clean bijou bottles, sealed properly, sterilized by autoclaving at 121 ^{0}C 15 psi for 15 mins and placed in slant position for solidification.

Determination of soil pH

The electrodes were removed from the solution rinsed, blotted dry with soft tissue and placed in the initial buffer solution and standardized the pH meter. The electrodes were removed from the first buffer solution, rinsed thoroughly with distilled water, blot dried and immersed in the second buffer solution of the pH within 2 pH units of the soil sample. The pH was read which was within 0.1 unit of the second buffer. The pH of the three sampling points was determined after the establishment of the equilibrium between the three samples and electrodes with poorly diluted buffer solutions. The electrodes were equilibrated by immersing them in four successive portions of the samples. A fresh sample was stirred gently while the pH was taken to insure homogeneity.

Determination of electric conductivity

The cell was rinsed with one or more portion of the samples. The level of the sample aliquot was above the vent hole in the cell and air bubbles were not allowed inside the cell. The temperature of the sample was adjusted to 20 ^{0}C and the sample conductivity was read and the temperature was also noted to the nearest 0.1^{0}C. The cell was rinsed thoroughly in distilled water after measurement calculation. If the sample's conductivity is measured with instrument having temperature compensation, the result automatically is corrected to 25^{0}C. The calculation is shown below.

$$K_c = \frac{1412}{C_{kcl}} \times [0.019(t - 25) + 1]$$

Where K_c = the cell constant 1/cm

C_{kd} = Measured conductance, µmho

t = Observed temperature of standard kd solution.

Serial dilution of soil samples

A known quantity, 1 g of homogenous of the soil samples collected were diluted to 10 ml of water in a test-tube which served as stock solution. Remaining 9 test tubes were filled with 9 ml of water. A volume of 1 ml from the stock solution transferred was to 9 ml of sterilized distilled water with the help of pipettes yielded 10^{-1}dilutions and the series continued up to 10^{-9} dilutions.

Bacterial colony identification and external morphology study

A volume, 100 ml of nutrient agar media was prepared for four petri plates. The nutrient agar media was autoclaved and then poured in four petri dishes which were also sterilized by autoclave. Then the serial dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}were chosen and from that 0.1 ml of culture was transferred from each serially diluted test tubes and spread on the petri plates by means of the spreader. Then the petri

dishes were kept in the incubator at 37 ^{0}C for 24 hrs for the incubation and growth of bacteria. After 24 hrs of incubation the petri dishes were taken out of the incubators and the bacterial external morphology were examined.

Pure culture isolation of bacteria

Fully developed and distinct colonies identified on the nutrient agar plates were marked and then these separated colonies were selected and using inoculating needle the colonies were transferred and streaked on six different test tubes having nutrient agar slants for the growth of the single colonies of bacterial cultures from the mixed culture of bacteria that were grown in the petri plates. The test tubes were marked after the strains of selected colonies from petri plates and were placed in the incubator at 37° C for growth and incubation. Following the incubation of the pure cultures overnight, the different single species of bacterial culture which developed in the test tubes were further selected and purified.

Gram staining of the bacterial species strains from the pure culture slants

The pure cultures of different colonies that were obtained in test tubes were put for gram staining for more specific identification of the colonies. The gram staining was done in laminar airflow hood. For this purpose the six slides were taken from slide rack. The slides were washed with ethanol. Each colony was then marked on the slides. Then loopful strains were picked from each test tube using inoculating needles and a smear made on the slides and heat fixed. The slides were then taken in the staining room for staining the smears. Then smears were stained in following steps, first applied was crystal violet on each six slides and kept for 30 secs. This was distilled with distilled water and iodine was then added on the slides as mordant followed by 95% alcohol wash and then washed with distilled water. Safranin was applied on the slides and then washed with distilled water and the slides air dried.

Screening of bacterial strains

The bacterial colonies were picked from the pure culture slants by the help of the inoculating needle and were streaked in the PVK agar media plates and were incubated at 37° C overnight. In the next day the bacterial colonies showed a clear halo zone formation which confirmed them to be PSB bacterial species. For further studies these colonies were again grown in nutrient agar media and several biochemical tests were performed.

Biochemical analysis of the isolates

The starch hydrolysis involved a 24 hour old isolates which was inoculated on pikovaskya agar plate containing 10% starch and it was incubated at 37^{0}C for 24 hours. After incubation period, the plate was flooded with iodine solution. A clear zone indicates starch hydrolysis was observed.

Catalase test

In this test, a 24 hour old culture colony was picked and emulsified in a few drops of hydrogen peroxide on a clean slide. Air bubbles, is a positive test for catalase.

Indole test

A volume 5ml of tryptone water was dispensed into test tubes and was sterilized by autoclaving at 121^{0}C 15 psi for 15mins. A loopful was inoculated into test tubes and incubated for 3 days at 37^{0}C. After incubation, 3 drops of kovac's reagent was added into the test tubes. A cherry red color indicated a positive result.

Methyl red test

In this test, a volume 5 ml of tryptone water was dispensed into the test tubes and was sterilized by autoclaving at 121 ^{0}C 15 psi for 15 mins. A loopful of the isolates was inoculated into the test tubes and was incubated for 3 days. Thereafter, 3 drops of methyl red reagent was added into the tubes. A pink red color indicated a positive result of this test.

Results

Isolation and characterization

The result of the isolation and characterization of the bacteria indicated the growth of phosphobacter species on the plates, which is distinguished by the formation of halo zones

Colony identification and color appearance on PVK agar plate.

The results indicated that the organism present in plates 10^{-2} 10^{-6} and 10^{-8} according to their physical features gave the following results.

Plate	Colony/Strain	Form	Elevation	Margin	Color	Transparency	Size
10^2	6	Punctiform	Raised	Undulate	Brownish	Opaque	Small
10^6	4	Circular	Flat	Entire	Greenish	Translucent	Medium
	5	Circular	punctifom	Entire	Offwhite	Opaque	Medium
10^8	1	Circular	Raised	Entire	Greenish	Opaque	Entire
	2	Rhizoid	Raised	Undulate	Offwhite	Opaque	Medium
	3	Punctifom	Flat	Curly	Clear white	Opaque	Small

Table 1: *Colony morphology and color appearance on the PVK agar plates.*

Biochemical characterization of bacterial strains shows that

Table 2 and 3 different biochemical tests carried out on PSB bacterial strains both for *Bacillus* spp and *Pseudomonas* spp and they showed either positive results or negative results.

Sl.no	Tests	*Bacillus*	*Pseudomona*
1	Methyl red test	Positive	Positive
2.	Starch Hydrolysis	Positive	Positive
3.	Catalase test	Positive	Positive
4.	Indole	Positive	Positive
5.	Gram reaction	Positive	Negative

Table 2: *Biochemical test of bacterial strains.*

S/N	Sample I.D	pH M±SD	Conductivity M±SD
1.	Agbani farmland	8.3135 8.981	2.50 2.50
2	Amurri farmland	6.065 6.072	1.60 1.60
3.	Esut farmland	5.99 6.032	1.00 1.00

Table 3: *pH and electrical conductivity of Agbani, Amurri and Esut farmlands.*

The result of this research work shows that both bacteria strains gave the same result to each biochemical test carried out.

pH and electrical conductivity of the soil samples

The result of this works shows that the soil pH of Agbani was (8.981) which shows strong alkalinity, Amurri (6.072) shows a moderate acidity and ESUT farmland (6.032) show moderate acidity. Agbani farmland recorded (2.50), Amurri farmland (1.60) and ESUT farmland (1.00). The result from the sample showed that ESUT farmland and Amurri farmland are slightly saline and are best suited for cultivation because most minerals and nutrients are more soluble in acidic soil than in neutral or slightly alkaline soils.

Discussion

Soil samples were collected to ascertain the possibility of occurrence of phosphorus solubilizing bacteria. Sampling was done at different sites in order to ensure uniform representation of the microflora in PVK media, which is a selective and differential media. Halo zone formation is useflu identifying phosphate solubilising bacteria [13]. In this research, about twelve colonies were identified in the culture plate distinguished by halo zone formation as shown in plate 1 to 3, signifying the presence of phosphate solubilising bacteria. The identification of bacteria strains was carried out on the basis of external colony morphology and color as appeared in the PVK agar plates and gram staining of microorganisms [14].

Plate 1: *Pure culture slants of phosphate solubilising bacteria.*

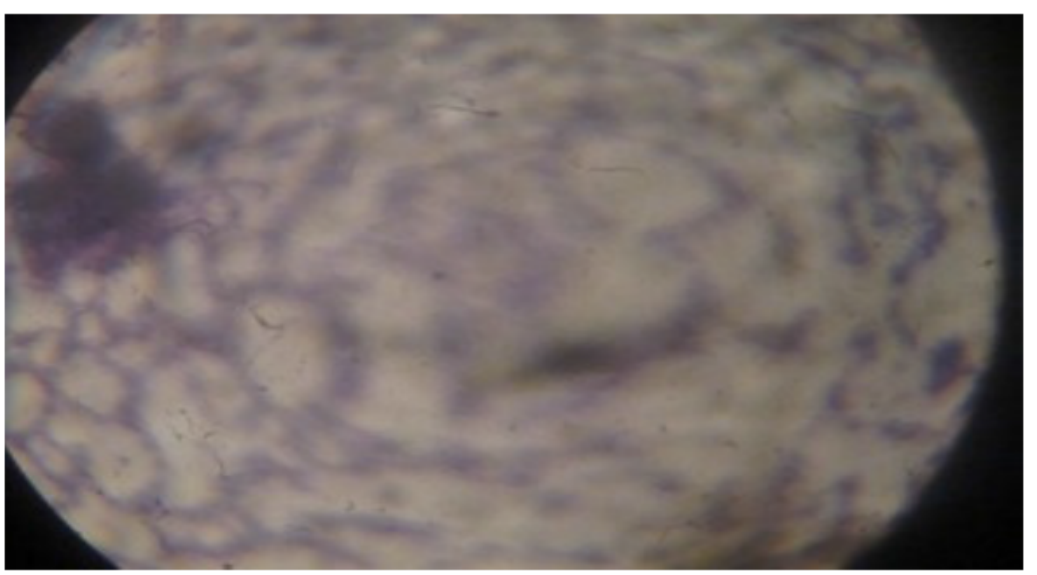

Plate 2a: *Gram staining identification of both Bacillus spp and Pseudomonas spp strains.*

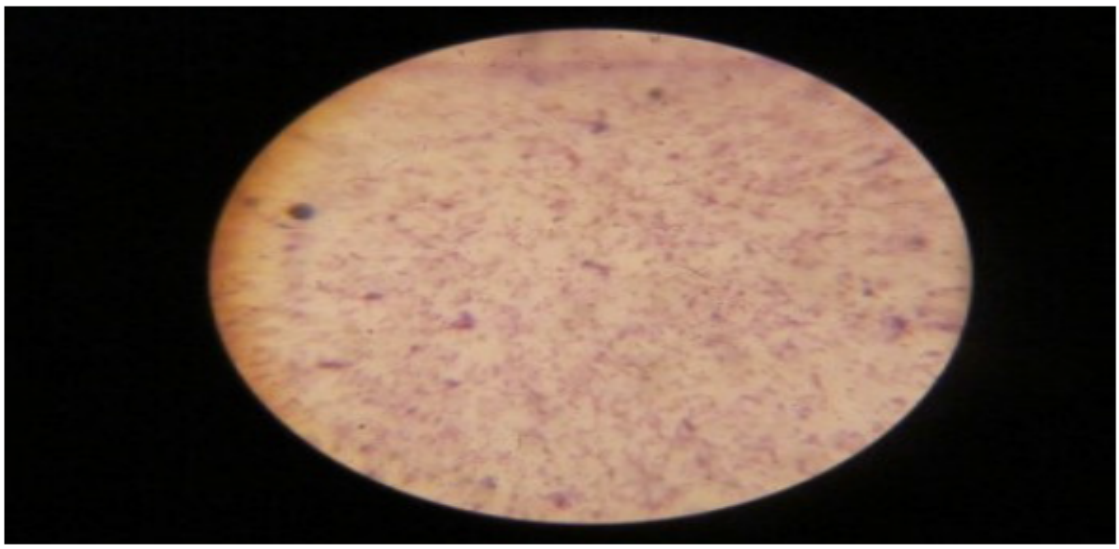

Plate 2b: *Psudomonassp.*

Plate 3: *Halozone formation of PSB bacteria.*

The biochemical test such as indole test, catalase test, methyl red test, and starch hydrolysis were carried out was to ascertain the mechanism of survival and microbial activities of the bacteria in phosphorus solubilization. The essence of starch hydrolysis is to ascertain if the isolate can use starch a complex carbohydrate made from glucose as a carbon and energy source for growth. Methyl red test is carried out to determine the fermentation pathway used to utilize glucose. In mixed acid fermentation glucose is fermented to produce several organic acids example, lactic acid, formic acid as well as glucoronic acid. The catalase test shows that the isolates possess the ability to breakdown hydrogen peroxide by producing enzyme catalase. The Indole test further shows that the organisms can degrade the amino acid tryptophan to produce indole. Tryptophan is oxidized by bacteria with the release of enzymes and by converting it into metabolic product.

The pH and the electrical conductivity of the soil influence the microbial activities of microorganisms, thus the need to determine them. The pH of Amurri farmland was (6.072) and ESUT farmland was (6.032), both soil samples shows moderate acidity, whereas Agbani farmland has a pH of (8.981) shows strong alkalinity. The soil's pH is known to influence plant growth by its effects on the activity of beneficial microorganism and plant growth; it prevents organic matter from being decomposed by bacteria, resulting in the accumulation of organic matter which causes soil toxicity. A pH range of 6-7 promotes availability of plants nutrients and survival of microorganisms. From the research work Amurri farmland and ESUT farmland are best soil for agricultural practices because from their pH values it will favour the growth of microorganisms such as phosphobacter that mobilize nutrients required for plant's growth [15].

The soil pH affects plant growth by its effect on activity of useful microorganism. Bacteria that break down organic matter are limited in strong acid soils and can prevent the availability of nutrients particularly nitrogen, stored in the organic matter. Most minerals and nutrients are more soluble or available in acid soils than in neutral or slightly alkaline soils.

From the result of electrical conductivity (EC) Amurri farmland has EC of (1.60), ESUT farmland (1.00) and Agbani farmland (2.50) showing that the soil samples has different degrees of salinity which affects microbial activities such as decomposition and respiration [16], showed that the pH and electrical conductivity of soil samples as the most important soil analysis. When electrical conductivity is less than 1 ds/m, the soil is considered non-saline and do not encourage most crops and soil microbial processes to take place. As electrical conductivity increases, soil microorganism activity declines. When the electrical conductivity is greater than 1 ds/m the soil is considered saline and enhance microbial activity.

Use of phosphate solubilizing microbes can increase crop yield up 70%, and combined inoculation of arbuscular mycorrizha gives better uptake of both native phosphorus from the soil and phosphorus coming from the phosphate rock results to a higher crop yield. Microorganisms with phosphate solubilizing potential promote the release of soluble phosphate and enhance growth by improving biological nitrogen fixation. *Pseudomonas spp* increased the number of nodules, dry weight of nodules, yield components, grain yield and nutrient availability [17], while the inoculation of phosphate solubilizing bacteria and plant growth rhizobacteria reduces phosphorus application by 50% without affecting corn yield.

The PSB are present everywhere in different forms and number in different soils, these numbers of PSB rely on different soil features and cultural activities [18]. Strains from bacterial genera *Pseudoninase Bacillus, Rhizobium* along with *Penicillium* and *Aspergillus* are the most powerful phosphorus solubilizers [19].

Most PSB have the capacity to scavenge Fe from mineral complex into soluble form that is taken up by active transport processes [20]. Sideorophore production by PSB has indirect potential to improve phosphorous availability as these ligands can also extract Fe from ferric citrate and ferric phosphate [21].

The phosphate solubilizing bacteria in addition to lowering pH of rhizosphere can dissolve the soil phosphate through the release of low molecular weight organic acids such as glucoronic and keto glucoronic acids [22]. The pH of rhizosphere is lowered by biotical production of proton and bicarbonate release. A wide range of organic acids such as glucoronic acid, 2.kelogulcoronoe acid, oxalic acid, citric acid, acetic acid, fumaric acid, pyruvic acid, succinic acid, tartaric acid, lactic acid, malic acid as well as propanoic acid facilitate insoluble phosphate solubilization [23,24]. Among all the organic acid, glucoronic acid is the most effective in solubilizing mineral phosphate [13]. Glucoronic acid is further oxidized to 2. keloglucononic acid, an effective chelating agent for calcium ions and the strongest monobasic carboxylic acid for dissolution of hydroxylaptite [25]. Glucoronic acid is also known to have a bio control activity. According to [26], D-gluconoic acid is the most significant antifungal agent in bio-control.

Conclusion

From the result of this research work, phosphorus solubilizing bacteria are present in the soil samples collected in different location, and could be further exploited in biofertilizer production.

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