Isolation of Phosphate Solubilizing Bacteria from the Rhizosphere of *Vigna catjang* Walp (Cow pea)

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Abstract

Isolation of phosphate solubilizing bacteria (PSB) was carried out from the rhizosphere of *Vignacatjang* Walp (Cow pea) cultivated near Hinthada Township, Ayeyarwady Region. Isolation of PSB was undertaken by serial dilution method on Pikovskaya medium. The formation of clear zone was observed for four days of incubation. Phosphate solubilizing bacteria were purified by using streak plate method. They were identified based on colony morphology, cell morphology, gram staining reactions and motility. Total of eight strains were isolated. They showed clear zone on Pikovskaya agar medium and they may be phosphate solubilizing bacteria.

Keywords: Phosphate, Bacteria, Rhizosphere, Cow pea

Introduction

Phosphorus (P) is an essential element for plant development and growth. Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant (Dadarwal *et al*., 1997).

Plants acquire P as phosphate anions. Phosphate anions are extremely reactive and may be immobilized through precipitation with cations. In these forms, P is highly insoluble and unavailable to plants. Despite the high total soil P content, plant P availability is often reported to be limited, particularly in tropical soils (Collavino *et al*., 2010). Of the total soil phosphate, only 1-5% is in a soluble, plant available form and the rest became unavailable due to its fixation in soil as insoluble phosphates of iron, aluminum and calcium (Molla and Chowdhury, 1984).

Poor availability or deficiency P markedly reduced plant size and growth. To satisfy crop nutritional requirement P is usually added to soil as chemical P fertilizer (Kolkar *et al*., 2016). However, only about 25% of the phosphorus applied to the soil is available for the crops and the rest become unavailable due to chemical fixation (Baliahand and Begum, 2015).

Various types of soil microbeslike phosphate solubilizing bacteria (PSB) which can solubilize this fixed form of phosphorus and make it available to plants (Rao, 2004 and Khan *et al*., 2010). Such organisms are called phosphate solubilizers. Large proportions of PBS are found in agricultural land (Kolkar *et al*., 2016). High proportion of phosphate solubilizing microorganisms is concentrated in rhizosphere, and they are metabolically more active than other sources. Population of Phosphate solibilizing bacteria depends on different soil properties and cultural activities. *Pseudomonas* and *Bacillus* are important genera of soil bacteria with promising activity of phosphate solubilisation (Yadav and Tarafdar, 2011).

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These phosphate solubilizing bacteria are known as factors for rising rate of phosphorus absorption that their use in the form of biofertilizers may improve soil nutritional status, section of plant growth regulators, and control of soil-borne diseases and eventually it may lead to better growth and yield in farming plants (Damor and Goswami, 2016).

Their role in increasing the soil nutrient value is of utmost importance. Their application to crop fields has resulted in an increased yield of several crops, such as cereals, legumes, fibers, vegetables, oils, and other crop plants (Silini-Cherif, 2012; Viruel *et al*., 2011 and Khalimi *et al*., 2012). These phosphate solubilizing bacteria can be isolated and propagated and, if use in the form of biofertilizers will improve soil nutritional status and eventually it may lead to better growth and yield in farming plants (Damor and Goswami, 2016).

Therefore, this research was carried out to isolate phosphate solubilizing bacteria as starter culture of biofertilizer, to investigate colony morphology and phosphate solubilizing activity in Pikovskaya medium and to determine the cell morphology and staining reaction of isolated bacteria.

Material and Method

Collection of Samples

The soil adhering to root and plant of Cow pea were collected from cultivated field near Hinthada Township, Ayeyarwaddy Region (17° 39' 46" N and 95º 26' 41" E, fig. 1 and 2) in August 2018. Plants and soil were collected from the depth of approximately 15 cm. Soil pH, soil temperature and weather condition of the environment were also recorded. Rhizospheric soil with roots were put into sterilized polythene bag and carried to the Laboratory of Zoology Department, Pathein University by using ice-box. They were stored in the refrigerator at 4ºC for further study.

Preparation of Glasswares

Glassware were cleaned and sterilized according to methods of Cruickshank (1960). Wire loop and long straight wire were sterilized according to Rao (2005).

Isolation of Phosphate Solubilizing Bacteria from the Rhizospheric Soil

Analyzing of PBS population from the rhizospheric soil samples of Cow pea was made according to the following method at the department of Zoology, Pathein University. One gram of rhizospheric soil was weighed with digital balance and put into test tube. Ten ml of autoclaved distilled water was added into soil containing tube and thoroughly shaken. Soil solution was diluted to 10^{-1} to 10^{-10}. And then, 20µL of each dilution was spread on Pikovskaya (PVK) agar medium and incubated at 27-30°C for 7 days. Colonies showing clear zones were picked and purified on PVK medium by streak plate technique.

Preparation of Pikovskaya agar medium(Atlas, 2010)

Ingredients were weighed and thoroughly mixed with 950ml distilled water expect $Ca_3(PO_4)_2$.. $Ca_3(PO_4)_2$ were mixed 50ml distilled water and heated to completely dissolved. Two types of solution were separately sterilized by autoclaving with pressure of 1.05 kg per cm^2 (15 lb per in^2), temperature of 121°C and duration of 15 minutes. After autoclaving these two solutions were mixed at 50ºC and shaken. Then, they were poured into petri dishes aseptically.

Preparation of Nutrient medium(Atlas, 2010)

Chemical ingredients were weighed. Then they were mixed in 100ml of distilled water and sterilized by autoclaving (1.05 kg percm^2, 121ºC for15 minutes)**.**

Isolation of Phosphate Solubilizing Bacteria from Roots

Analyzing of PBS population from the root samples of Cow pea was carried out by the method of Pikovskaya (2010) at the department of Zoology, Pathein University. Roots were washed in tap water. One gram of roots were weighed, cut and rinsed with distilled water and surface sterilized in 75% alcohol. Next, they were washed again with sterilized distilled water for five times. And then, these were ground in sterilized grinder. Ten ml of distilled water were added into ground root pieces and thoroughly shaken. Roots solution was diluted to 10^{-1} to 10^{-10}. Twenty µL of each dilution was spread on Pikovskaya (PVK) agar medium and incubated at 27-30 °C for 7 days. Colonies showing clear zones were picked and purified on PVK medium by streak plate technique.

Measuring of clear zone on PVK medium

Isolated bacteria were inoculated in PVK broth at 27-30°C for 24 hrs. Then a 5 µL drop of bacterial growth suspension of each strain was inoculated and incubated at 27-30 °C for 7 days. After this clear zone and colony diameter were measured.

Study of Colony Morphology

Size, shape, colour, opacity, elevation, consistency and margin of the isolated bacteria colonies were observed and characterized these colonies.

Morphological Characteristic and Gram Staining Reactions of Isolated Bacteria

Gram Staining

A drop of normal saline was placed on a glass slide and mixed with a small amount of isolated bacteria. This mixture was made smear and allowed it to dry. The smear was fixed by passing the slide over a flame. The slide was covered with crystal violet stain and allowed to act for 30-60 seconds. Then, the slide was rinsed with distilled water for a few seconds. It was covered with fresh iodine solution and allowed to act for about 30-60 seconds. And then, we added the alcohol drop by drop and stopped adding alcohol when no more colour flowed out from the smear. For a thin smear, 10-20 seconds may be enough for complete decolourization of gram negative bacteria. As a counter stain, the smear was covered with safranin for about 20-30 seconds and washed with distilled water. Then, the slide was tilted at a 45 degree angle and let to be dry. The stained slide was examined under the oil immersion objective of the microscope (Atlas, 2010).

Motility

Motility of the isolated bacteria can be detected in semi-solid agar medium (Atlas 2010). Sterilized 10ml of semi-solid agar was dispensed in test tubes and they were left to set in the vertical position. A straight wire was inoculated and a single stab down was made in the centre of the tube about half the depth of the medium. After incubation, motile bacteria will spread into the medium and non-motile will confine to the stab.

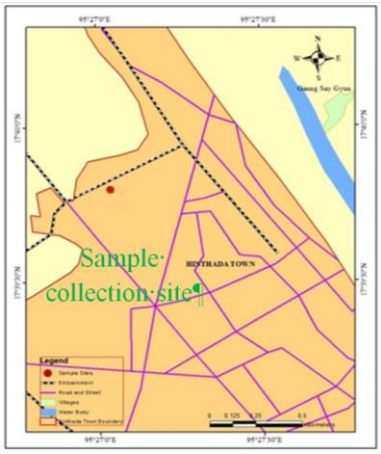


Figure (1). Map of sampling site **(**Source: Department of geography, Pathein University)

Figure (2). Sampling field and sample plants. (A) A plantation of Cow pea (B) A sample plant of Cow pea

Results

Totally eight strains of phosphate solubilizing bacteria were isolated from the rhizosphere of Cow pea. Isolated strains were designated as PSBCPR-1 to 6 (phosphate solubilizing bacteria from roots) and PSBCPS 1 to 2 (phosphate solubilizing bacteria from rhizospheric soils) for Cow pea. They were isolated and identified based on colony morphology, cell morphology, gram staining reactions and motility (Table 1-3, Plate 1-9).

Phosphate Solubilizing Bacteria from Roots of Cow Pea

The clear zone of PSBCPR-1 is 1.6 -1.9 mm and single colony in the clear zone is 1.0-1.3 mm in diameter. Colony facture is circular, yellow-white, entire and raised. Single colony on PVK medium is 1.0-1.5 mm in diameter and on nutrient medium is 1.1-1.6mm in diameter. Cells are rod with 5.0 to 10.0 µm in diameter. They are gram positive, singly or chain and motile. Clear zone of PSBCPR-2 is 1.7-2.0 mm and single colony in the clear zone is 1.1-1.2mm in diameter. Colony facture is circular, yellow-white, entire and raised. Single colony on PVK medium is 1.0-1.8 mm in diameter and on nutrient medium is 1.1-1.9mm in diameter. Cells are rod with 7.5 to 12.5 µm in diameter. Gram negative, singly and chain and motile (Plate 1-2).

Clear zone of PSBCPR-3 is 1.6-2.0mm in diameter and single colony in the clear zone is 1.1-1.4mm in diameter. Colony facture is circular, white, entire and raised. Single colony on PVK medium is 1.0-1.9mm in diameter and on nutrient medium is 1.1-1.8mm in diameter. Cells are ovoid with 5.0 to 12.5 µm in diameter. Gram negative, singly or pair and motile. Clear zone of PSBCPR-4 is 1.6-1.9 mm in diameter and single colony in the clear zone is 1.0-1.1 mm in diameter. Colony facture is circular, white, entire and raised. Single colony on PVK medium is 1.2-1.5 mm in diameter and on nutrient medium is 1.0-1.6mm in diameter. Cells are ovoid with 5.0 to 10.0 µm in diameter. They are gram negative, singly or pair and motile (Plate 3-4).

Clear zone of PSBCPR-5 is 1.6-1.8 mm in diameter and single colony in the clear zone is 1.0-1.1 mm in diameter. Colony facture is circular, creamy-white, entire and convex. Single colony on PVK medium is 1.3-1.9 mm in diameter and on nutrient medium is 1.2-1.7mm in diameter. Cells are ovoid with 5.0 to 10.0 µm in diameter. Gram negative, singly or pair and slightly motile. Clear zone of PSBCPR-6 is 1.4-1.6mm in diameter and single colony

in the clear zone is 1.1-1.3mm in diameter. Colony facture is circular, white, entire and raised. Single colony on PVK medium is 1.3-1.9mm in diameter and on nutrient medium is 1.3-1.8mm in diameter. Cells are ovoid with 5.0 to 10.0 µm in diameter. They are gram negative, singly or pair and motile (Plate 5-6).

Phosphate Solubilizing Bacteria from Rhizospheric soil of Cow Pea

Clear zone of PSBCPS-1 is 1.5-1.7mm in diameter and single colony in the clear zone is 1.1-1.3mm in diameter. Colony facture is circular, white, undulate and raised. Single colony on PVK medium is 1.0-1.4mm in diameter and on nutrient medium is 1.2-1.4mm in diameter. Cells are ovoid with 5.0 to 10.0 µm in diameter. Gram negative, singly or pair and motile. Clear zone of PSBCPS- 2 is 1.6-1.8mm in diameter and single colony in the clear zone is 1.0-1.2 mm in diameter. Colony facture is white, entire and raised. Single colony on PVK medium is 1.3-1.5mm in diameter and on nutrient medium is 1.2-1.6mm in diameter. Cells are ovoid with 5.0 to 10.0 µm in diameter. They are gram negative, singly or pair and slightly motile (Plate 7-8).

Table (1). Measurement of Clear zone and Colony Diameter of Isolated Phosphate Solubilizing Bacteria

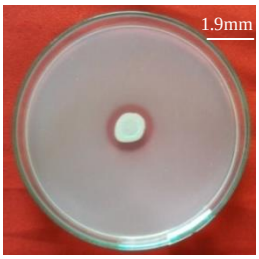
PSB Strain	Diameter of clear zone	Diameter of colony in the clear zone
PSBCPR-1	1.6-1.9 mm	1.0-1.3 mm
PSBCPR-2	1.7-2.0 mm	1.1-1.2 mm
PSBCPR-3	1.6-2.0 mm	1.1-1.4 mm
PSBCPR-4	1.6-1.9 mm	1.0-1.1 mm
PSBCPR-5	1.6-1.8 mm	1.0-1.1 mm
PSBCPR-6	1.4-1.6 mm	1.1-1.3 mm
PSBCPS-1	1.5-1.7 mm	1.1-1.3 mm
PSBCPS-2	1.6-1.8 mm	1.0-1.2 mm

Table (2). Colony Morphology of Isolated Phosphate Solubilizing Bacteria

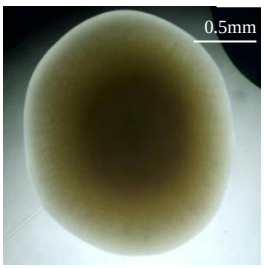
PSB Strain	Size	Shape	Colour	Margin	Elevation
PSBCPR-1	1.0-1.5 mm	circular	Yellow-White	Entire	Raised
PSBCPR-2	1.0-1.8 mm	circular	Yellow-White	Entire	Raised
PSBCPR-3	1.0-1.9 mm	circular	White	Entire	Raised
PSBCPR-4	1.2-1.5 mm	circular	White	Entire	Raised
PSBCPR-5	1.3-1.9 mm	circular	Creamy-White	Entire	Convex
PSBCPR-6	1.3-1.9 mm	circular	White	Entire	Raised
PSBCPS-1	1.0-1.4 mm	circular	White	Undulate	Raised
PSBCPS-2	1.3-1.5 mm	circular	White	Entire	Raised

Table (3). Cells Morphology of IsolatedPhosphate Solubilizing Bacteria

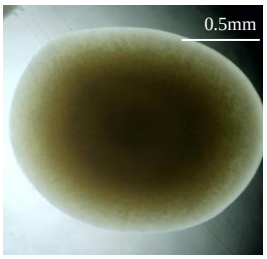
PSB Strain	Cell size	Shape	Arrangement	Gram reaction
PSBCPR-1	5.0 – 10.0 µm	Rod	singly or chain	Positive
PSBCPR-2	7.5 – 12.5 µm	Rod	singly or chain	Negative
PSBCPR-3	5.0 – 12.5 µm	Ovoid	singly or pair	Positive
PSBCPR-4	5.0 – 10.0 µm	Ovoid	singly or pair	Negative
PSBCPR-5	5.0 – 10.0 µm	Ovoid	singly or pair	Negative
PSBCPR-6	5.0 – 10.0 µm	Ovoid	singly or pair	Negative
PSBCPS-1	5.0 – 10.0 µm	Ovoid	singly or pair	Negative
PSBCPS-2	5.0 – 10.0 µm	Ovoid	singly or pair	Negative



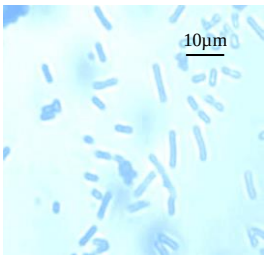
A. Clear zone formation of PSBCPR-1 on PVK medium



B. Single colony of PSBCPR-1 on PVK medium

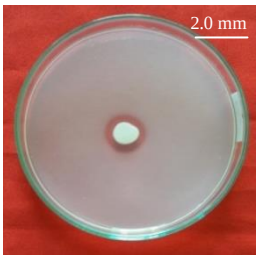


C. Single colony of PSBCPR-1on nutrient medium

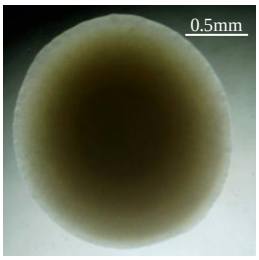


D. Gram staining of PSBCPR-1

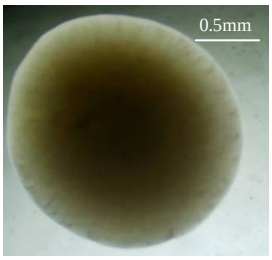
Plate (1). Colony and cell morphology of PSBCPR-1



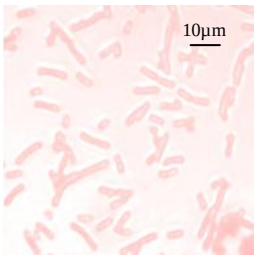
A. Clear zone formation OfPSBCPR-2 on PVK medium



B. Single colony of PSBCPR-2 on PVK medium

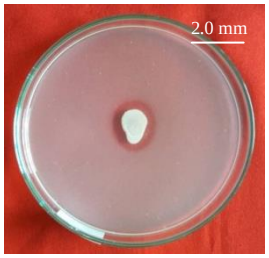


C. Single colony of PSBCPR-2 on nutrient medium

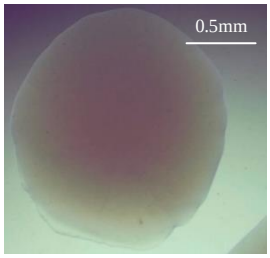


D. Gram staining of PSBCPR-2

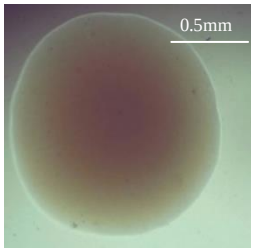
Plate (2). Colony and cell morphology of PSBCPR-2



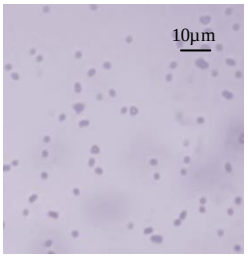
A. Clear zone formation of PSBCPR-3 on PVK medium



B. Single colony of PSBCPR-3 on PVK medium

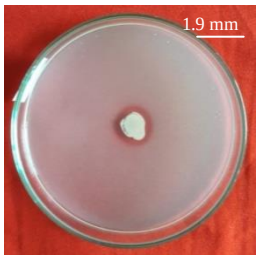


C. Single colony of PSBCPR-3 on nutrient medium

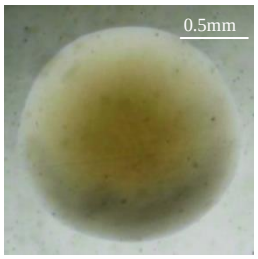


D. Gram staining of PSBCPR-3

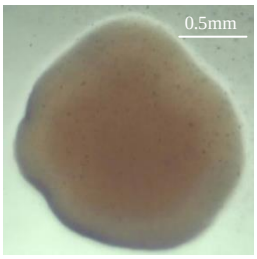
Plate 3. Colony and cell morphology of PSBCPR-3



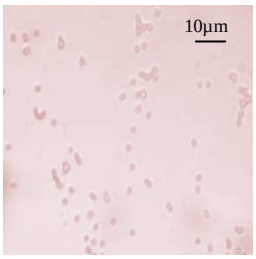
A. Clear zone formation of PSBCPR-4 on PVK medium



B. Single colony of PSBCPR-4 on PVK medium

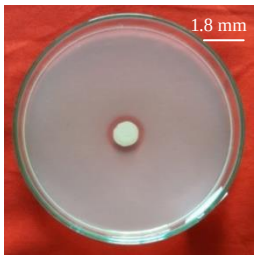


C. Single colony of PSBCPR-4 on nutrient medium

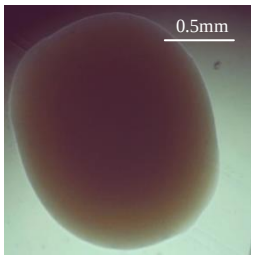


D. Gram staining of PSBCPR-4

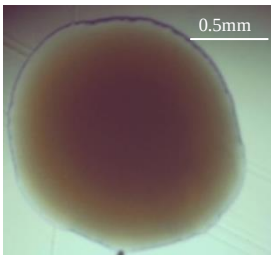
Plate (4). Colony and cell morphology of PSBCPR-4



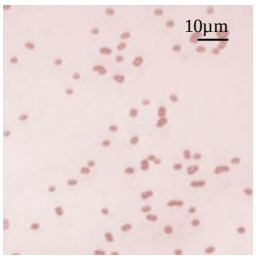
A. Clear zone formation of PSBCPR-5 on PVK medium



B. Single colony of PSBCPR-5 on PVK medium

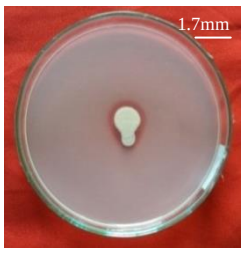


C. Single colony of PSBCPR-5on nutrient medium

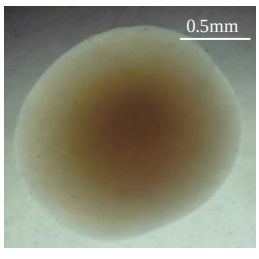


D. Gram staining of PSBCPR-5

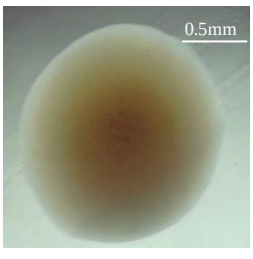
Plate (5). Colony and cell morphology of PSBCPR-5



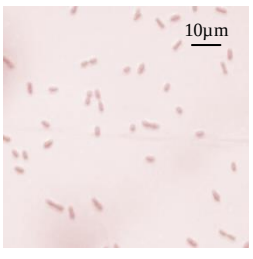
A. Clear zone formation of PSBCPS-6 on PVK medium



B. Single colony of PSBCPS-6 on PVK medium

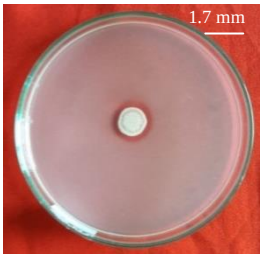


C. Single colony of PSBCPS-6 on nutrient medium

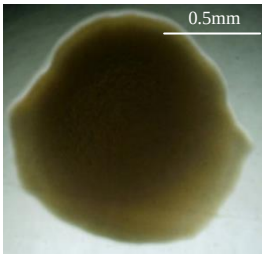


D. Gram staining of PSBCPS-6

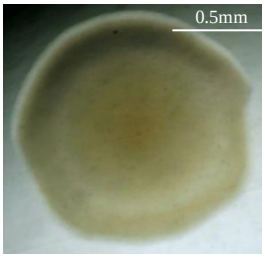
Plate (6). Colony and cell morphology of PSBBGR-6



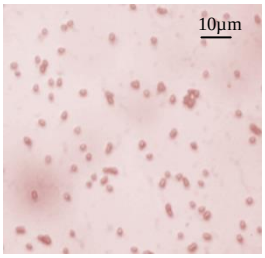
A. Clear zone formation Of PSBCPS-1 on PVK medium



B. Single colon of PSBCPS-1 on PVK medium

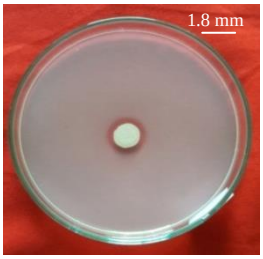


C. Single colony of PSBCPS-1on nutrient medium

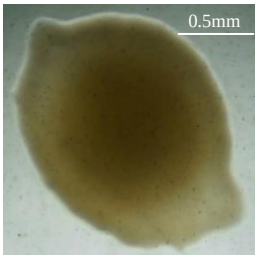


D. Gram staining of PSBCPS-1

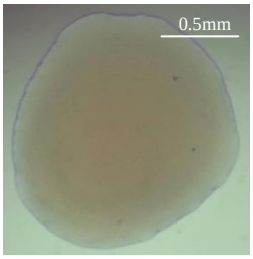
Plate (7). Colony and cell morphology of PSBCPS-1



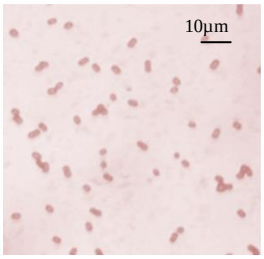
Clear zone formation of PSBCPS-2 on PVK medium



B. Single colony of PSBCPS-2 on PVK medium



C. Single colony of PSBCPS-2 on nutrient medium



D. Gram staining of PSBCPS-2

Plate (8). Colony and cell morphology of PSBCPS-2

A. Motility of PSBCPR-1

B. Motility of PSBCPR-2

C. Motility of PSBCPR-3

D. Motility of PSBCPR-4

E. Motility of PSBCPR-5

F. Motility of PSBCPR-6

G. Motility of PSBCPS-1

H. Motility of PSBCPS-2

Plate (9). Motility test of PSB isolated from the roots andrhizospheric soil of Cow pea.

Discussion and Conclision

In this research, total of eight strains of phosphate solubilizing bacteria from the rhizosphere of Cow pea was isolated. Serial dilution method and PVK medium was used for the isolation of PSB. Culture temperature was 27-30ºC and streak plate method was used for pure culture. Among eight strains PSBCPR-2 was the largest in forming clear zone of phosphate solubilization and PSBCPR-6 was the smallest.

Balamurugan *et al.*,(2010) reported the isolation of phosphate solubilizing bacteria from tea garden soil using dilution plate technique on Petri plates containing Pikovaskaya's media. Damor and Gaswami (2016) isolated PSB from the soil samples by serial dilution

method and plated on PVK medium and incubated at 28 ºC for 7 days. In this work, serial dilution method and PVK medium was also used for the isolation of PSB.

Damar and Gaswami (2016) isolated PSB from soil samples by serially diluted and plated on PVK medium and incubated at 28ºC for seven days. Koklkar *et al.,* (2016) isolated PSB from soil samples by using PVK medium and incubated at 27-30ºC for 7 days.

Rfaki*et al.* (2014) studied the PSB from the rhizosphere of three cultivated legumes (fababean, chickpea and green peas). They isolated PSB by serial dilution using spread plating on NBRIP medium with tricalcium phosphate and incubated at 27ºC for 72-120 hrs. Colonies showing clear zone of phosphate solubilizing were counted as PSB. Baliah and Begum (2015) suggested that PSB strains preferred temperature ranging from 20ºC to 35ºC and above and below which the growth was retarded. PSB strains could grow well at the temperature of 28ºC to 35ºC. So, in this study, culture temperatures of PSB were 27-30 ºC and were agreement with the culture temperatures of above studies.

Baliah and Begum (2015) isolated eight strains of PSB from crop plants and stated the diameters of clear zone of PSB ranging from 2 to 5mm.In this observation, clear zone of isolated PSB ranging from 1.0 to 2.0mm and slightly smaller than the clear zone of above work. This may be due to the different in culture media i.e., Balih and Begum (2015) used hydroxyl apatite medium and in this research PVK medium was used.

Kudu *et al*. (2002) isolated 73 strains from the rhizosphere of different crops. Out of 73 PSB isolates, 11 isolates showed better zone of P- solubilization on solid medium. Gaind (1987) reported that the PSB strains were isolated using the Pikovskaya's medium based on the formation of halo zone around these microorganisms. Sanjotha and Sudheer (2016) also isolated microbial colonies from soil of Karwar Costal Region, which showed cleared zone on PVK medium were considered as phosphate solubilization. In this investigation, all isolates showed clear zone on PVK medium.

Therefore, isolated bacteria of this research can solubilize phosphate and could be useful as starter culture for the production of biofertilizer.

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