IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
March 2023 Vol.:24, Issue:1

Herbicide Resistance and Phosphatase Enzyme Activity of Phosphate Solubilizing Bacteria in Rhizosphere Soil

IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Murugan Karuppasamy*

PG Research Scholar, PG Department of Microbiology, Sri Paramakalyani College, Alwarkurichi – 627 412, Tenkasi District, Tamilnadu, India.

Submitted: 21 February 2023
Accepted: 27 February 2023
Published: 30 March 2023

HUMAN JOURNALS
www.ijsrm.humanjournals.com

Keywords: Rhizosphere Soil, Pikovskaya's Agar Medium, Griseofulvin, Phosphatase Enzyme, Phenolphthalein Diphosphate and Herbicide.

ABSTRACT

Phosphate solubilizing microbes plays an important role in plant nutrition through increase in phosphate uptake buy plants and used as biofertilizers of agricultural crops. Phosphate is one of the most vital macronutrient required for the growth and development of plants. A large number of microorganisms present in the rhizosphere are known to solubilizer and make available the insoluble phosphorus in the available form to the plants. Plant Growth Promoting Rhizobacteria (PGPR) mediate the soil processes such as decomposition, nutrient mobilization, mineralization, solubilization, nitrogen fixation and growth hormone production. Rhizosphere soil of *Oryza sativa* (paddy plant) was collected from cultivated crop field and enumerated for total heterotrophic and phosphate solubilizing bacterial population. In this study, twenty five strains of Phosphate Solubilizing Bacteria (PSB) were isolated by plate assay and characterized biochemically. Mineral Phosphate Solubilizing (MPS) activities of all isolates were tested on tricalcium phosphate of Pikovskaya's agar medium by analyzing the soluble phosphate content after 24 hours of incubation at 37^0C. The aim of the present study was to screen the phosphatase enzymes of phosphate solubilizing bacteria and to access their potential tolerance to herbicides (one shot). The heterotrophic bacterial load was found in the order of 10 CFU/gm and the phosphate solubilizer of 10 CFU/gm. The phosphate solubilizing bacteria was composed of *Bacillus* sp. and *Micrococcus* sp. with a lone of *Pseudomonas* sp. Both *Bacillus* sp. and *Micrococcus* sp. were noted to produce phosphatase at neutral pH and at 45^0C with the exception of *Bacillus* sp. Phosphate Solubilizer (PS) 25. Probably this bacterium is secreting alkaline phosphatase that has temperature optima at 35°C. None of the three representative bacterial strains were found to tolerate the most commonly used herbicide (one shot) at the concentration of 20 ppm. Only *Pseudomonas* sp. was recorded to tolerate this herbicide at 10 ppm. Further understandings on the nature of the enzyme, optimization on its activity would provide basic data for the commercial production of these enzymes.

INTRODUCTION

Soil microorganisms have great potential in providing soil phosphates for plant growth. Phosphorus biofertilizers can help to increase the accessibility of accumulated phosphates for plant growth by solubilization (Goldstein, 1986; Gyaneshwar *et al.,* 2002). Rhizosphere is the soil zone surrounding the plant roots while rhizoplane is directly in contact with the roots (Kennedy, 2005). Plant roots exuded the organic contents in the rhizosphere and subsequently increased the microbial activity and termed as "rhizosphere effect" by Hiltner (1904). Microorganisms exerted beneficial effect on plant growth and development through different means is termed as Plant Growth Promoting Rhizobacteria (PGPR)(Vessey, 2003). Plant growth promoting rhizobacteria accounts for about 2-5% of total the rhizobacteria involved in plant growth promotion (Antoun and Kloepper, 2001).

Phosphate Solubilizing Microorganisms (PSM) have attracted the researchers to exploit their potential to utilize phosphate reserves in semi-arid regions and to enhance the crop yields (Goldstein *et al.,* 1993; Fasim*et al.,* 2002; Khan *et al.,* 2006). Phosphate solubilizing microorganisms have established their role for optimum growth of plants under nutrient imbalance conditions (Glick, 1995; Igual*aet al.*, 2001; Wu *et al.,* 2005).

Phosphorus (P) is the second most important plant nutrient after nitrogen (Donahue *et al.,* 1990). Phosphorus is an essential macronutrient for growth and development of plants involved in important metabolic pathways like photosynthesis, biological oxidation, nutrient uptake and cell division (Illmer and Schinner, 1992). Worldwide soils are supplemented with inorganic phosphate as chemical fertilizers to support crop production but repeated use of fertilizers deteriorates soil quality (Tewari*et al.*, 2004).

The problem of phosphate fertilization may become serious in the coming years because of the fact that manufacture of phosphatic fertilizers requires the use of non-renewable resources such as high grade rock phosphate which are getting depleted progressively and becoming costlier. It is estimated that about 98% of Indian soils contain insufficient amounts of available phosphorous to support maximum plant growth. So to increase the soil fertility and agricultural productivity phosphatic fertilizers are widely used.

Rock phosphates are the only commercial raw material for the production of phosphatic fertilizers. The total world reserves of rock phosphate are estimated to be about 27,000million tones, of which 80% are located in USA, USSR and Morocco. In India, rock phosphate

deposits are of low grade (contain less than 25% of the P_2O_5) and do not satisfy the requisite specification of fertilizer industry. Beneficiation of such low grade rock phosphates is successful only in acidic soils and in neutral and an alkaline soils, rock phosphate turns out to be rather poor source of phosphorus (Neelima Garg *et al.*, 1989).

Many soil microorganisms including bacteria, fungi, yeast and actinomycetes are capable of solubilizing the insoluble inorganic phosphates (Skujins, 1967). Soil microorganisms play a very significant role in mobilizing phosphorous for the use of plants in bringing about changes in the pH of the soil microenvironment leading to the solubilization of native as well as added insoluble phosphorous.

Sen and Paul (1957) observed the solubilization of calcium and iron phosphate in liquid medium by *Bacillus subtilis, Bacillus megaterium* and *Bacillus mesentericus* with ammonium sulphate as the nitrogen source. They also isolated the bacteria capable of solubilizing tricalcium phosphate from the glands of *Cassia occidentals*.

The soil environment surrounding the plant roots is the zone of intense microbial activity. Swaby and Sperber (1959) reported that population of phosphate dissolving microorganisms are more in the rhizosphere (20–40% of the total population) compared to non-rhizosphere (10–15% of the total population). Solubilization of phosphorous by phosphate solubilizing microorganisms (PSM) is attributed to excretion of organic acids (Sperber, 1957).

Artidave and Patel (1999) worked on inorganic phosphate solubilizing soil Pseudomonads and isolated thirty eight *Pseudomonas* sp. which were examined for their tricalcium phosphate solubilizing activity in Pikovskaya's broth for twenty one days? Though all cultures solubilizer tricalcium phosphate in liquid medium only thirteen cultures showed zone of phosphate dissolution on Pikovskaya's agar medium. The decomposition and mineralization of organic phosphate occurs under the influence of specific enzymes of phosphatase. Hence, it has an essential role to play in the phosphorous cycle in the environment.

Chemical pesticides are commonly used by farmers and corporate in agriculture to protect the crops, but these activities are depleting the soil fertility and soil health. The use of chemical pesticides in Indian agriculture drastically increased in recent years. The word pesticides include a heterogeneous group of chemicals developed to control a variety of pests. Pesticides

are generally categorized as insecticides, herbicides and fungicides according to the type of pest which they have shown efficacious action (Miligi *et al.,* 2006).

As microorganisms play an important role in many soil biological processes, including nitrogen transformations, organic matter decomposition, nutrient release and their availability, as well as stabilize the soil structure and affect its fertility, investigated by (Vyas, 1988; Edwards and Bater, 1990; Khan and Sculion, 2000). However, soil is the most important site of biological interactions. The indiscriminate use of pesticides disturbs the soil environment by affecting flora and fauna including soil microbial flora, soil enzymes and also the physicochemical properties of soil like pH, salinity, alkalinity leading to infertility of soil. The important micro flora, beneficial for the growth of plants includes nitrogen fixing bacteria and phosphate solubilizing bacteria, present in the rhizosphere of the plant.

Various agrochemicals e.g. herbicides when applied intensively and erratically on herbicide resistant non-transgenic and transgenic crops to control the noxious weeds leads to their accumulation in soil to a dangerous level that affects growth, survival, efficiency and quality of beneficial microbial communities of soil (Srinivas *et al.,* 2008; Pereira *et al.,* 2008; Ahemad *et al.,* 2009). The naturally abundant plant growth promoting rhizobacteria are also metabolically inactivated through the uptake of herbicides applied in excess to the soil (Barriuso *et al.,* 2010). In contrast, a few microorganisms found to be tolerant or resistant towards specific herbicides. It is of great concern that how to reduce the effect of herbicides on the beneficial microorganisms and at the same time it is of great interest to screen out microorganisms which are tolerant / resistant to herbicides. Herbicides are applied in modern agricultural practices to offset the plant growth restricting weeds and subsequently to augment the productivity of crops (Ahmed *et al.,* 2009). The intensive application of herbicides leads to their accumulation in soils to a level that adversely affects both the quality and biological composition of soils (Srinivas *et al.,* 2008), the naturally abundant PGPRare metabolically inactivated by taking up excessive herbicides (Ahemad and Khan, 2010; Bellinaso *et al.,*2003).

Many pesticides showed no detectable effects on soil microorganisms at the recommended application rates. However, application at increased rates is often reported as most of the farmers decide based on their own experience of the effective pest control. Repeated and overuse of pesticides in agriculture is a matter of concern because these chemicals are recognized as a source of potential adverse impacts on the metabolic activities of soil

microorganisms as well as their plant growth promoting characteristics (Wani*et al.,* 2005; Ahemad*et al.,* 2009). Some microorganisms (called as phosphate solubilizing microorganisms - PSMs) perform phosphate solubilization. Their growth and phosphate solubilizing activity may also be affected by the pesticides leading to the imbalance phosphorous nutrition for the crop plants. However, the effect of pesticides on microbial growth and their activity especially phosphate solubilization can only be assessed using microorganisms which are tolerant to the pesticide of concern (Oves*et al.*, 2009).

SCOPE AND AIM OF THE STUDY

The present study falls on the following lines:

Bacteriological Examination

❖ To study,.the enumeration of total heterotrophic bacterial population (THBP) in rhizosphere soil sample by total viable count.

❖ To study,.the isolation and enumeration of phosphate solubilizing bacteria (PSB) from the rhizosphere soil sample by spread plate technique.

❖ To maintain the phosphate solubilizing bacterial isolates on nutrient agar slants.

❖ To identify, the phosphate solubilizing bacterial isolates using the scheme of Aiso and Simidu (1962).

Experimental Works

❖ To observe, the determination of phosphatase enzyme activity in phosphate solubilizing bacteria.

- Preparation of the soup of phosphatase enzyme source
- Phosphatase enzyme assay by spectrophotometric method (Spectronic20D)

❖ To study, the culture condition optimization for phosphatase enzyme production.

- Effect of pH on phosphatase enzyme production
- Effect of temperature on phosphatase enzyme production

❖ To evaluate, the herbicide resistance in phosphate solubilizer by plate assay method.

MATERIALS AND METHODS

Sampling area

The sampling area is a crop field in the surroundings of Alwarkurichi village, Tirunelveli Kattabomman district. Here, the surroundings of this area are completely covered by crop field. Most of the people in this village are farmers. They cultivated various types of crops and vegetable plants. The crop field is enriched by applying phosphatic fertilizers and natural phosphate containing manures. So the plants rhizosphere root regions can be expected to load of phosphate solubilizing bacterial flora. Some pests are affected the rhizosphere root region of plants and to retard the growth of plants and to decent the crop yield. So pesticides are very important to needed for the control of insect pests.

Sampling

Rhizosphere acidic soil samples of *Oryza sativa* (paddy plant) was collected from cultivated crop field. The acidic soil samples were collected from various corners and middle regions of the paddy field very important. Because, the content of microbial load is variable from region to region of the paddy field. Where, good growth of paddy plant absorbs high amount of soluble and insoluble phosphates around their rhizosphere root regions. Hence, their rhizoplane of paddy plant root regions would have appreciable concentrations of phosphate solubilizing bacterial flora. So, these selected paddy plant root regions were chosen for the isolation of phosphate solubilizing bacterial flora. The rhizoplane root regions of the paddy plants having higher density of phosphate solubilizing bacterial species and the phosphate solubilizing capacity were highly greater.

During acidic soil sample collection from the paddy crop field, the following care was taken.

1. The rhizosphere acidic soil sample of paddy field were taken and transferred into the sterile conical flask with the help of sterile knife. The acidic soil samples from rhizosphere root regions of the paddy field were taken compulsorily.

2. The sterile conical flask was transferred in an ice pack (>4°C). Because the activity of bacterial flora would not be altered.

3. Then, the soil sample was brought to the laboratory subjected to microbial analysis within six hours to prevent the reduction of bacterial counting.

Bacteriological Examination

Selective culture media employed for the isolation of phosphate solubilizing bacteria

1. Pikovskaya's agar medium (modified by Sundara Rao and Sinha,1963).
2. Hydroxy apatite agar medium (modified by Ayyakkannu and Chandramohan,1970).
3. Soil extract agar medium (modified by Katznelson and Bose,1959).

Enumeration of Total Heterotrophic Bacterial Population (THBP) in rhizosphere soil sample by total viable count

The collected soil sample was allowed to attain room temperature. The soil sample is used to determine the total viable count and the density of phosphate solubilizing bacterial flora.

Steps in serial dilution technique

About ten gm of soil sample was taken from the conical flask and aseptically transferred into a conical flask containing 90 ml of sterile distilled water. It represents 10^{-1} dilution. One ml was pipette out from the conical flask and transferred into a test tube containing 9 ml of sterile distilled water. It represents 10^{-2} dilution. This is repeated up to 10^{-5} dilution. Care was taken to mix the sample solution of each dilution thoroughly in a vortex mixture prior to pipetting out.

Steps in pour plate technique

Petri dishes and pipettes were sterilized in hot-air oven (180°C - 2 hours). Then, the nutrient agar medium was prepared, autoclaved and cooled to 45°C. 15 µl stock solution of antifungal agent griseofulvin (1 mg / 1 ml) was added aseptically to the medium to suppress the growth of fungal flora. From the above serially diluted sample, one ml of three consecutive dilutions to be tested was pipette out on to the sterile Petri dishes in replicates. Then the cooled nutrient agar medium was added to the plates (approximately 20 ml) and mixed thoroughly with the inoculums by rotating the plate in clockwise and counterclockwise directions, taking care not to spill the medium. Care was taken to see that while mixing the nutrient agar medium and

the inoculums, the media does not get solidify while rotating it clockwise or counter clockwise directions. The pour plated materials after solidification were inverted and incubated at 37°C for a period of 24-48 hours. After incubation, plates with colonies ranging from 30 to 300 were selected for counting.

Isolation and enumeration of PSBfrom the rhizosphere soil sample by spread platetechnique

About ten gm of soil sample was weighed aseptically and dissolved in 90 ml of sterile distilled water and mixed thoroughly in a rotator shaker for the maximum recovery of bacterial cells from the soil. This represents 10^{-1} dilution. From the above dilution, the sample was diluted up to 10^{-5} dilution. Then Pikovskaya's agar medium, hydroxy apatite agar medium and soil extract agar medium were prepared, sterilized and cooled, to this 15µ1 stock solution of griseofulvin (1 mg / 1 ml), antifungal agent was added aseptically and dispensed the medium in sterile Petri dishes. From the above 10^{-3}, 10^{-4} and10^{-5} dilutions 0.1 ml of the sample was pipette out and inoculated on the air dried sterile Pikovskaya's agar medium, hydroxy apatite agar medium and soil extract agar medium, these were spread plated. The replicates and control were maintained. One set of plates were incubated at 37°C for 24 hours in an incubator and the another set of plates were incubated at room temperature for 48 hours. After sufficient incubation, the plates were observed for the presence of phosphate solubilizer on the basis of transparent clear zone formation surrounding the colony.

Maintenance of phosphate solubilizing bacterial isolates

Isolations were made at random from Pikovskaya's agar medium or other selective media plates containing countable number of colonies (30 -300 colonies). After recording the morphological characteristics of the colony and pigmentation, they were checked for their purity by repeated streaking on nutrient agar medium plates and were maintained on nutrient agar slants preserved in a refrigerator at 4°C after growth.

Identification of phosphate solubilizing bacterial isolates

The isolates were identified on the basis of their morphological, physiological and biochemical characters. The bacterial cultures from the stored nutrient agar slants were sub cultured in the nutrient broth and the broth cultures were subjected to various identification

tests. The bacterial cultures were identified using the scheme of Aiso and Simidu (1962). The Bergey's manual (1984) was also referred in the identification procedure.

Biochemical tests employed for the identification of phosphate solubilizing bacteria

1) Gram's staining technique

A loopful of the broth fresh culture was subjected to Gram staining procedure and observed microscopically under oil immersion objectives.

2) Motility test

The inoculation needle was dipped into the bacterial culture and stabbed into the SIM medium to test for the motility after incubation at 37°C for 24hours.

3) Penicillin sensitivity test (Kirby-Bouertest)

Overnight fresh cultures were streaked on the penicillin sensitive nutrient agar plates. The plates were incubated at 37°C for 24 hours. Then plates were observed for the presence of penicillin resistant isolates.

4) King's media for pigment production test (King's *et al.*,1954)

From the overnight test bacterial culture, a loopful culture was streaked on the pigment production medium and incubated for 24 hours at 37°C. The results were observed for the pigment production of bacterial isolates.

5) Oxidation-Fermentation (OF) test (Hugh and Leifson test)

The oxidation-fermentation basal medium is used for differentiating Gram-negative organisms on the basis of fermentative and oxidative metabolisms of carbohydrates. The oxidative-fermentative basal medium was incorporated with lactose. Culture tube with 2 ml of sterile medium was stabbed with the inoculation needle of dipped bacterial culture and the culture tubes were incubated at 37°C for 24-48 hours. Acid and gas production was observed from the change in the medium colouration and cracking of the medium in the culture tube.

6) Luminescencetest

A loopful of test bacterial culture was inoculated into the *Photobacterium* broth and incubated at 37°C for 24 hours. The results were observed for the luminescence of bacterial isolates.

7) Kovac's oxidasetest

To place the oxidase disc strips into an empty Petri dish and with a sterile platinum wire loop or sterile glass rod smear fresh bacterial culture cells were thoroughly into the oxidase disc. The oxidase test is positive if the transferred cells turn into dark purple colour within 5-10 seconds.

Growth of selective isolates on selective media

To confirm the identify and purity of the isolates they were streaked on air dried selective agar medium plates such as Pikovskaya's agar medium and hydroxy appetite agar medium and incubated appropriately. Their growth pattern was observed and recorded.

Experimental Works

a) Determination of phosphatase enzyme activity in phosphate solubilizing bacteria

i) Preparation of the soup of phosphatase enzyme source

Overnight fresh cultures of phosphate solubilizing bacterial isolates were withdrawn in a 1.5 ml eppendorf and centrifuged at 10,000 rpm for 10 minutes. After centrifugation, the pellet was taken and the soup was discarded. The pellet was resuspended in 1.5 ml of sterile saline. Then this was centrifuged at 10,000 rpm for 10 minutes and the soup was discarded. The pellet was resuspended in 1.5 ml of sterile saline and vortexed well. The enzyme soup was stored at 4°C under refrigeration for the determination of phosphatase enzyme activity.

ii) Assay of phosphatase enzyme on diphosphate by spectrophotometric method
Phenolphthalein diphosphate (sodium salt), at 0.5% concentration was prepared and filter sterilized. Then, the nutrient broth was prepared and 10ml of this nutrient broth was transferred into test tubes. The nutrient broth test tubes were sterilized by autoclaving. After sterilization, one ml of filter sterilized phenolphthalein diphosphate stock solution was added into each 10 ml of nutrient broth containing test tubes under aseptically and then mixed well.

To this, 10 µl of saline suspension of the phosphate solubilizing culture was inoculated aseptically. The test tubes were incubated at 35°C for 24-48 hours. After incubation, 1 ml of 1 N sodium hydroxide (NaOH) solution was added to this incubated test tube cultures. A bright pink red colouration was observed. This colouration indicates the liberation of phenolphthalein due to phosphatase enzymes. Then the optical density of this coloured solution was read at 420 nm in a spectrophotometer (Spectronic20D).

b) Culture condition optimization for phosphatase enzymeproduction

i) Effect of pH on phosphatase enzyme production

The phosphate solubilizing bacterial culture that had exhibited maximum phosphatase activity was chosen and subjected to optimization experiments.

Phenolphthalein diphosphate nutrient broth was prepared and pH of the broth medium was adjusted to 5, 6, 7 and 8 using 1 N hydrochloric acid solution (HCl) or 1 N sodium hydroxide solution (NaOH). From each pH group, 10 ml of the broth was transferred into test tubes and sterilized by autoclaving. After sterilization, 100 µl of saline washed cultures were inoculated in all the test tubes aseptically. The tubes were incubated at 37°C for 24 hours and an uninoculated blank was also maintained. A bright pink red colouration was observed. This colouration indicates the liberation of phenolphthalein due to phosphatase enzymes. At six hours interval, phosphatase enzyme activity in its broth culture at different pH was recorded at 420 nm in a spectrophotometer (Spectronic20D).

ii) Effect of temperature on phosphatase enzymeproduction

The phosphate solubilizing bacterial isolates that have exhibited high degree of phosphatase activity was chosen and preceded for optimization experiments.

Phenolphthalein diphosphate nutrient broth was prepared and 10 ml of this broth was transferred into test tubes and sterilized by autoclaving. After sterilization, 100 µl of saline washed cultures were inoculated into the test tubes aseptically. Then, one set of culture inoculated broth tubes were incubated at room temperature for 24 hours. Another set of culture inoculated broth tubes were incubated at 35°C for 24 hours in an incubator. Another set of culture inoculated broth tubes were incubated at 45°C for 24 hours in rotator shaker. In each set blank was maintained. A bright pink red colouration was observed. This colouration

indicates the liberation of phenolphthalein due to phosphatase enzymes. At six hours interval, broth culture was tested for phosphatase enzyme activity of this coloured solution was read at 420 nm in a spectrophotometer (Spectronic 20D).

c) Evaluation of herbicide resistance in phosphate solubilizer by plate assaymethod

To check the herbicide resistance profile of the phosphate solubilizing bacterial isolates, plate assay was carried out.

The nutrient agar medium was prepared and sterilized. The concentration herbicide, one shot (2, 4-D and anilofos) was poured into the sterile nutrient agar medium at different concentrations of 10 ppm and 50 ppm aseptically and mixed well. Then, the mixture of the contents were poured (approximately 15-20 ml) into the sterile Petri dishes and was allowed to solidify. After solidification, the saline washed cultures of phosphate solubilizing bacterial isolates were streaked on one shot nutrient agar plates. Then, the plates were incubated at 37°C for 24-48 hours. The control and replicates were maintained. After incubation, the herbicide resistant bacteria isolates were observed and stored for further analysis.

RESULTS AND DISCUSSION

Total heterotrophic bacterial population in the rhizosphere soil of *Oryza sativa* was enumerated and the results are presented in table - 1. Throughout the sampling period, total heterotrophic bacterial population was found to be in the range of 10 CFU/gm, similar distribution pattern was recorded in phosphate solubilizing bacteria also (table - 2). All the isolation of phosphate solubilizers was recorded to have comparable recovery of phosphate solubilizers. Comparing the data presented in table - 1 and table - 2, it can be understood that the majority of rhizosphere bacterial isolates were comprised of phosphate solubilizers. Since, the root zone represents high metabolic activity one can expect huge populations that have direct or indirect interaction with plant. Phosphate solubilizers are often found to predominate around rhizosphere region where they could utilize the plant exudates and in turn solubilizer phosphates and make it available for the plants.

Table - 1: Total heterotrophic bacterial population in the rhizosphere soil of *Oryza sativa*

S. No.	Sampling Cycle	Bacterial Load (CFU/gm)	Method of Sampling
01	I	36×10^{-3}	
02	II	25×10^{-3}	Pour plate technique
03	III	32×10^{-3}	

Table - 2: Phosphate solubilizing bacterial load in the rhizosphere soil of *Oryza sativa*

S.No.	Sampling Cycle	Bacterial Load (CFU/gm)			Method of Sampling
		Pikovskaya's AgarMedium	HydroxyApatite AgarMedium	Soil Extract AgarMedium	
01	I	50×10^{-2}	30×10^{-2}	20×10^{-2}	Spread
02	II	30×10^{-2}	20×10^{-2}	20×10^{-2}	plate
03	III	30×10^{-2}	30×10^{-2}	10×10^{-2}	technique

On the basis of phosphate solubilization, 25 bacterial isolates were characterized up to generic level and the data was presented in table - 3. From this, it can be noted that the major components are *Micrococcus* sp., *Bacillus* sp. and *Pseudomonas* sp. Among these, both *Micrococcus* sp. and *Bacillus* sp. were equally distributed (48%) and the only other isolate was *Pseudomonas* sp. (4%) presented in figure - 1.

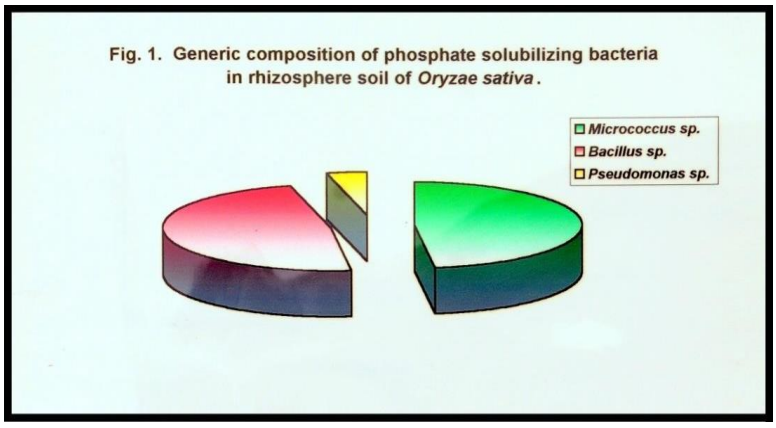

Figure 1: Generic composition of phosphate solubilizing bacteria in rhizosphere soil of *Oryza sativa*

Table – 3: Biochemical characterization of phosphate solubilizing bacterial isolates

S. No.	Tests Performed	Phosphate Solubilizing Bacterial Isolates																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
01	Morphology	Small cocci	Rod shaped	Rod shaped	Rod shaped	Small cocci	Small cocci	Small cocci	Small cocci	Small cocci	Small cocci	Rod shaped	*Rod shaped	Rod shaped	Small cocci	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Small cocci	Rod shaped	Rod shaped	Small cocci	Small cocci	Small cocci	Rod shaped
02	Gram staining	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
03	Motility	-	+	+	+	-	-	-	-	-	-	+	+	+	-	+	+	+	+	-	+	+	-	-	-	+
04	Penicillin sensitivity test	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
05	Pigment production test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
06	Oxidation-fermentation test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
07	Luminescence test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08	Kovac's oxidase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Legend: Small cocci indicates Micrococcus sp., rod shaped indicates Bacillus sp., and *rod shaped indicates Pseudomonas sp.*

Growth of this organisms in media supplemented with inorganic phosphate indicates their ability to secrete organic acids that facilitate phosphate solubilization. Most phosphate solubilizing organisms have capabilities of elaborating enzyme namely phosphatase that plays a vital role in the catalysis of organic phosphates. To understand this phenomenon, 5 isolates were selected at random (*Bacillus* sp. PS 11, PS 16, PS 18, PS 25 and *Micrococcus* sp. PS 23) and examined for phosphatase activity with the help of an appropriate indicator. Photometric analysis of this experiment was presented in figure - 2. From this, it can be understood that *Bacillus* sp. PS 11, PS 18 and PS 25 were slightly more efficient in phosphatase production compared to the other two.

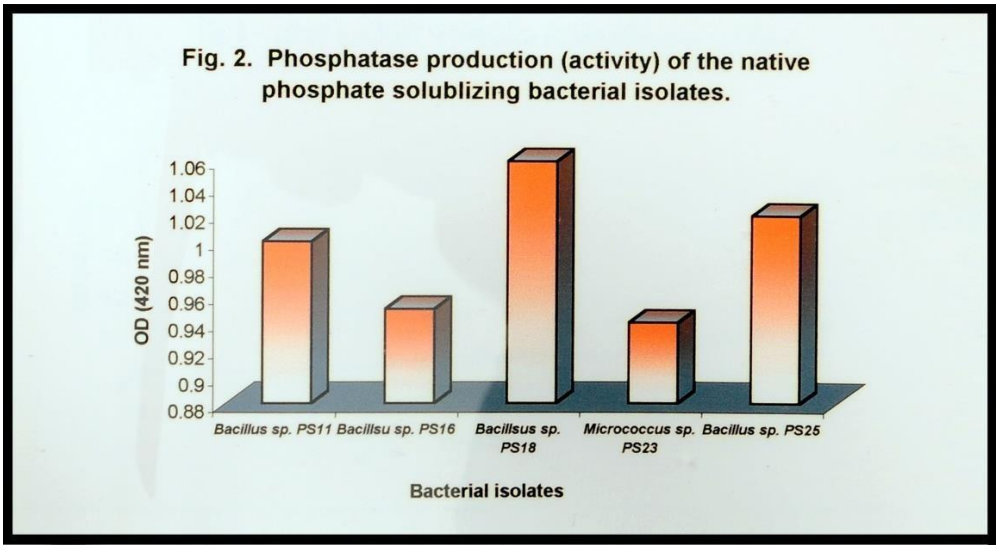

Figure 2: Photometric analysis of phosphatase enzyme production (activity) of the native phosphate solubilizing bacterialisolate

Further all the 5 strains were subjected to culture optimization experiments and the data are presented in figure 3-12. In the case of phosphate solubilizer (PS) 11, optimum pH for enzyme production was recorded to be 7.0 (fig. 3) and the temperature at 45°C (figure -4).

Similar observations can be noted for *Bacillus* sp. PS 16, PS 18 and *Micrococcus* sp. PS 23 (figure - 5-10).

In the case of *Bacillus* sp. PS 25, the optimum pH for phosphatase production was recorded to be pH 8.0 and the temperature of35°C.

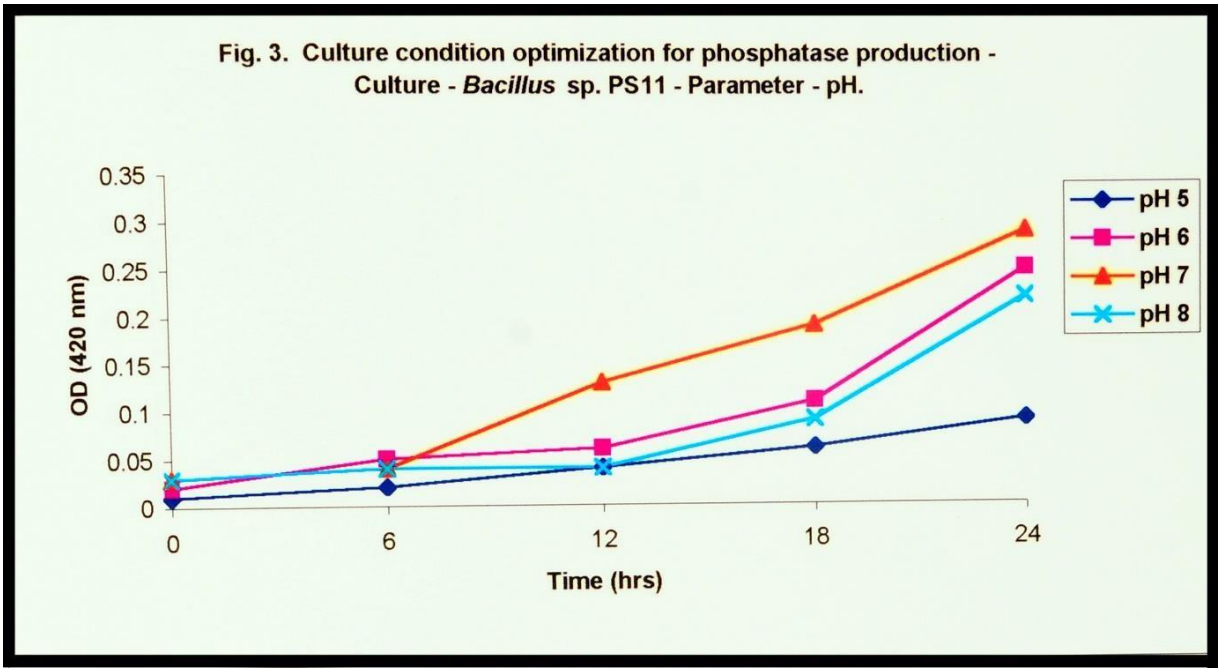

Figure 3: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 11 in the parameter of pH

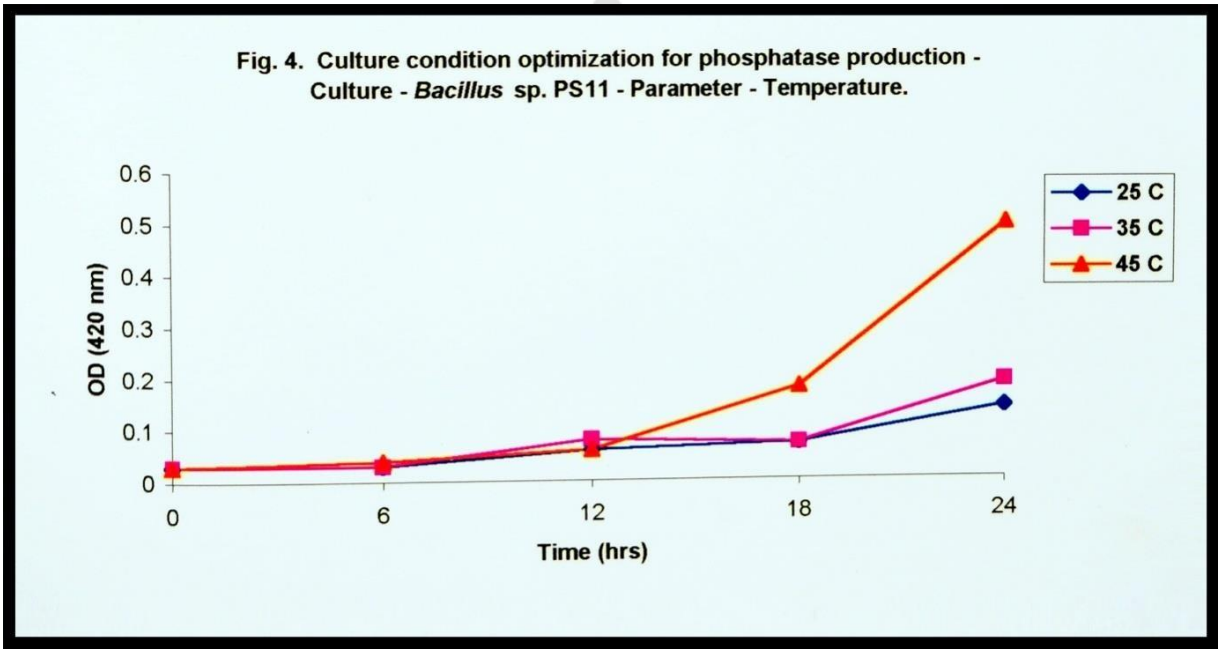

Figure 4: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 11 in the parameter of temperature

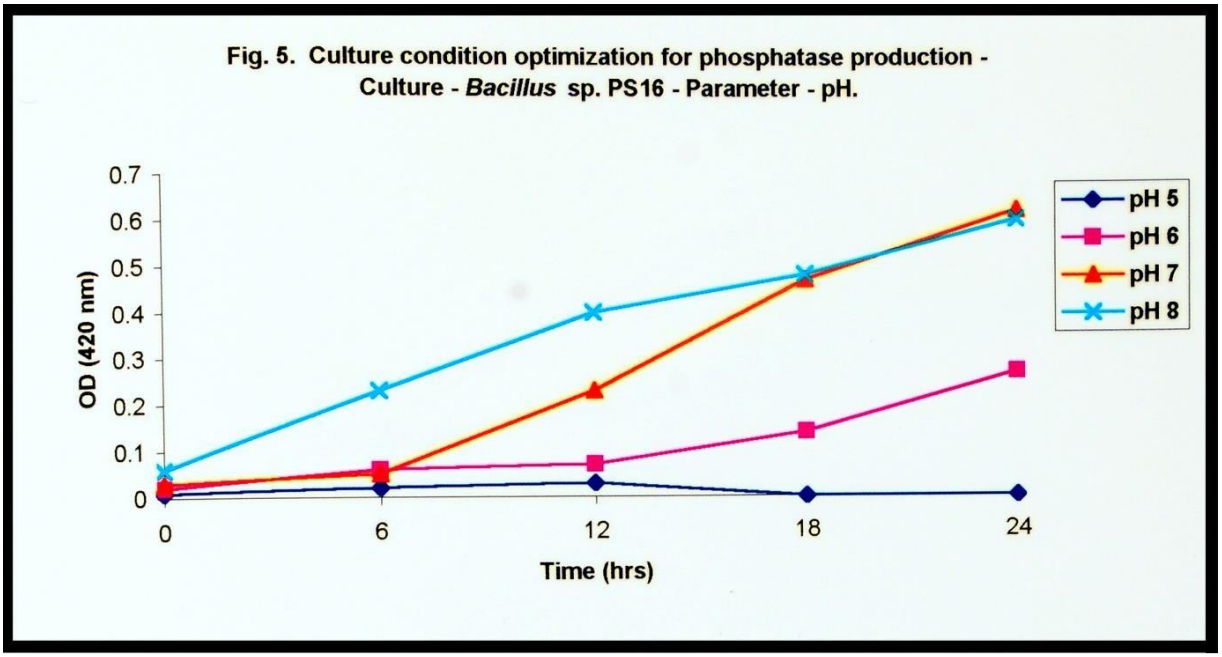

Figure 5: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 16 in the parameter of pH

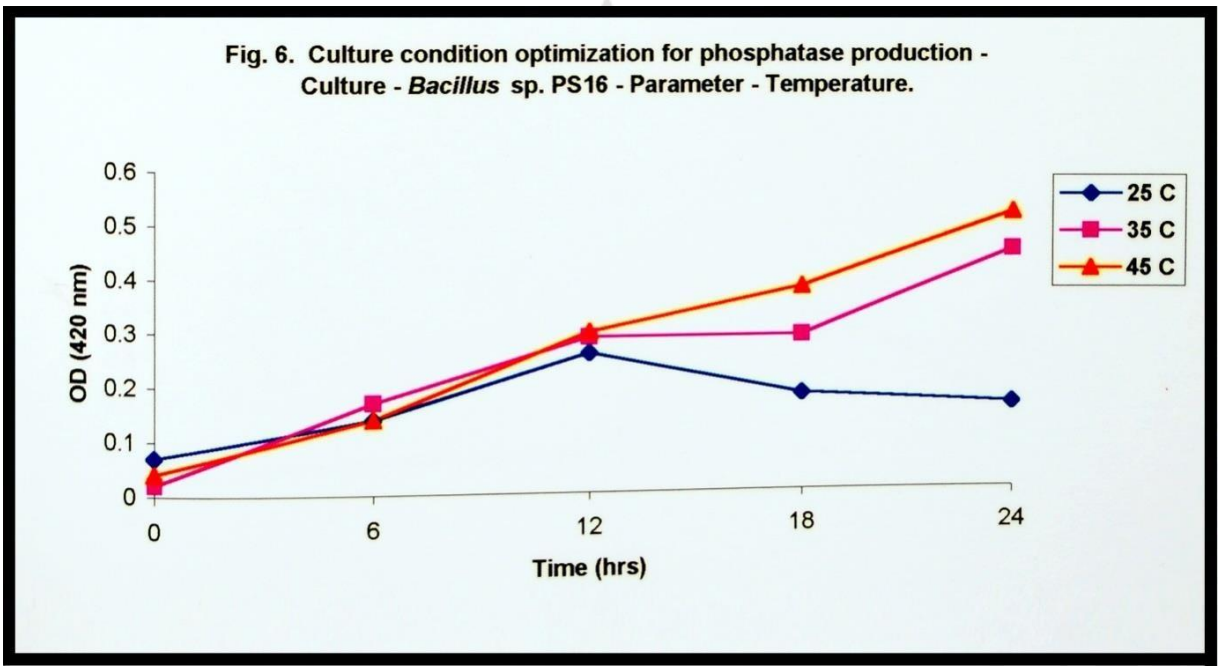

Figure 6: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 16 in the parameter of temperature

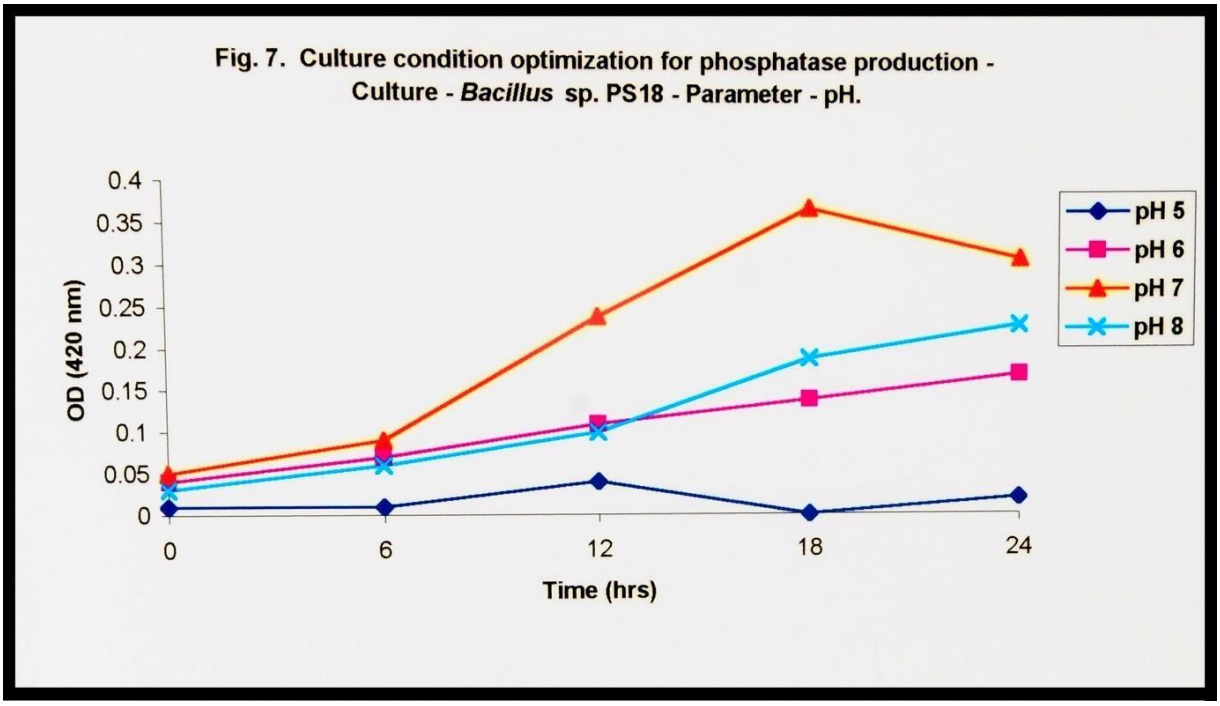

Figure 7: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 18 in the parameter of pH

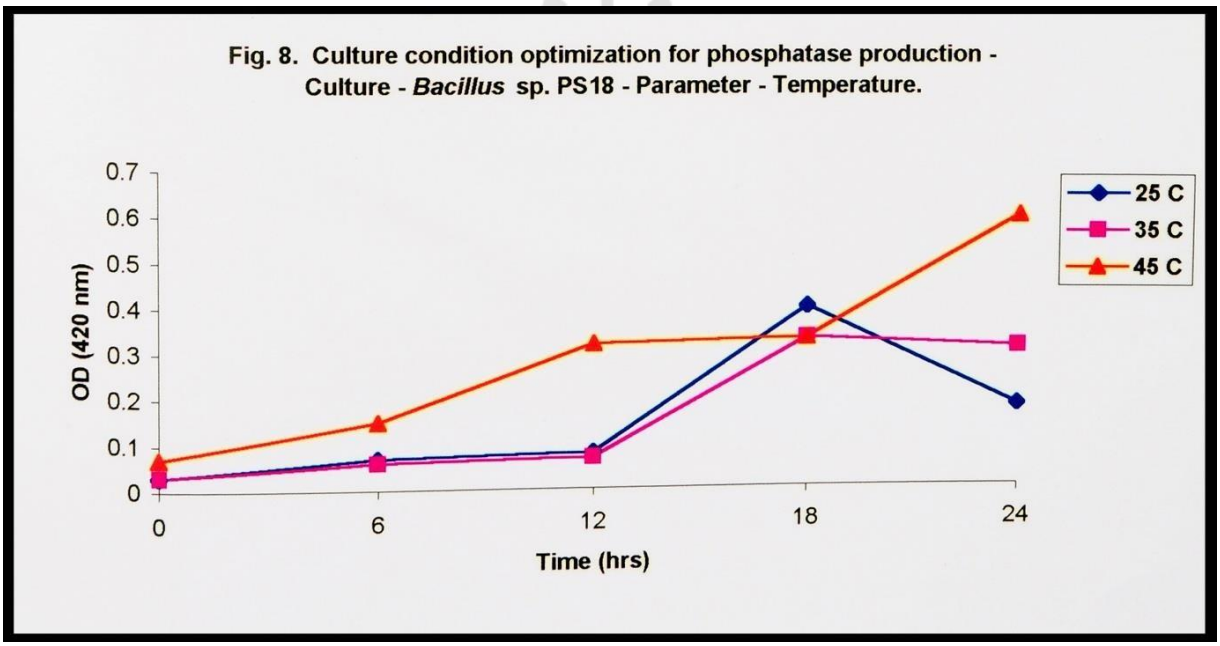

Figure 8: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 18 in the parameter of temperature

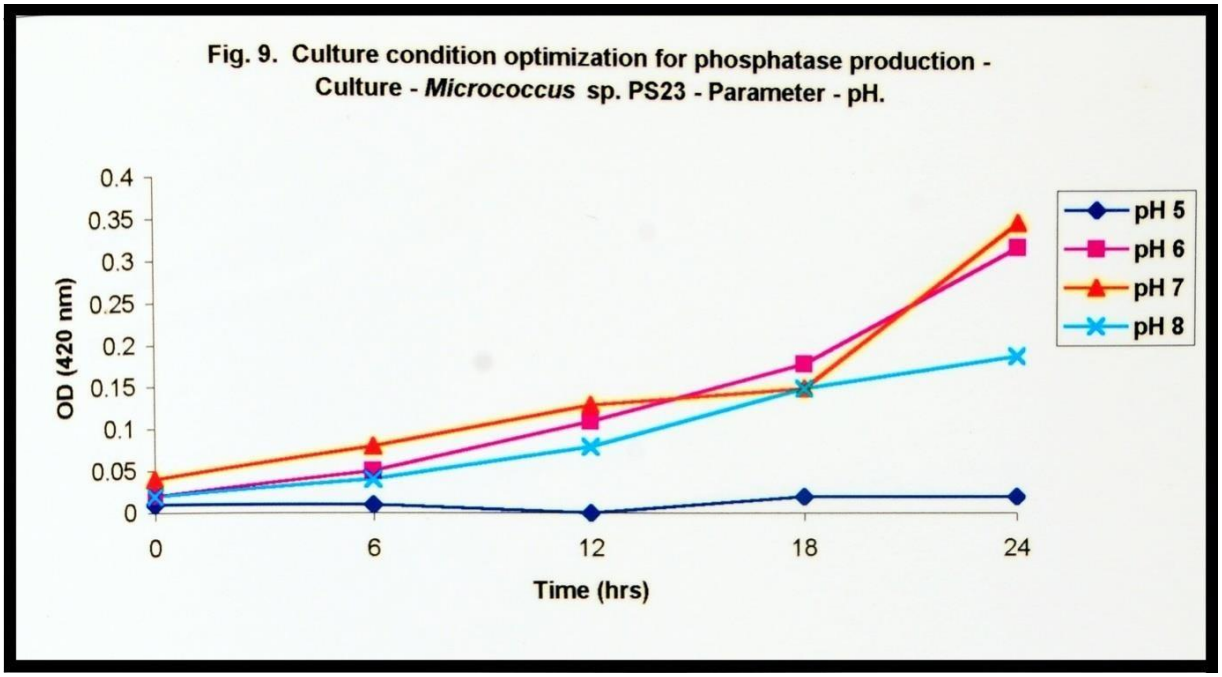

Figure 9: Culture condition optimization for phosphatase production of *Micrococcus* sp. PS 23 in the parameter of pH

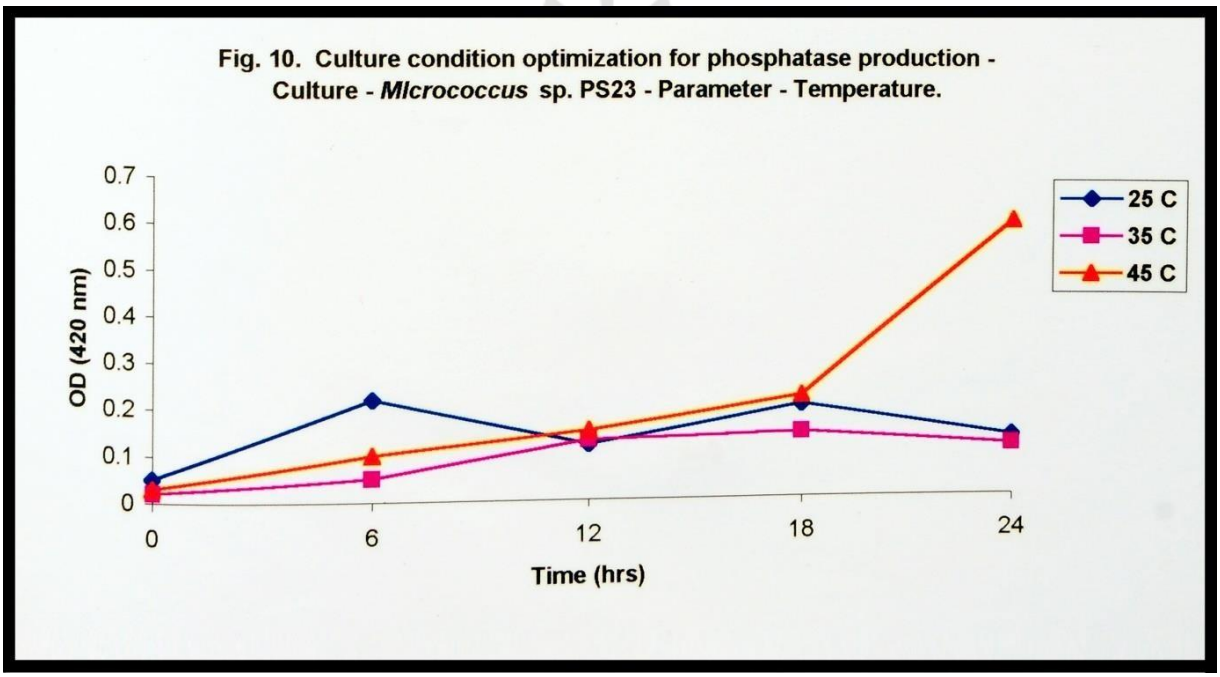

Figure 10: Culture condition optimization for phosphatase production of *Micrococcus* sp. PS 23 in the parameter of temperature

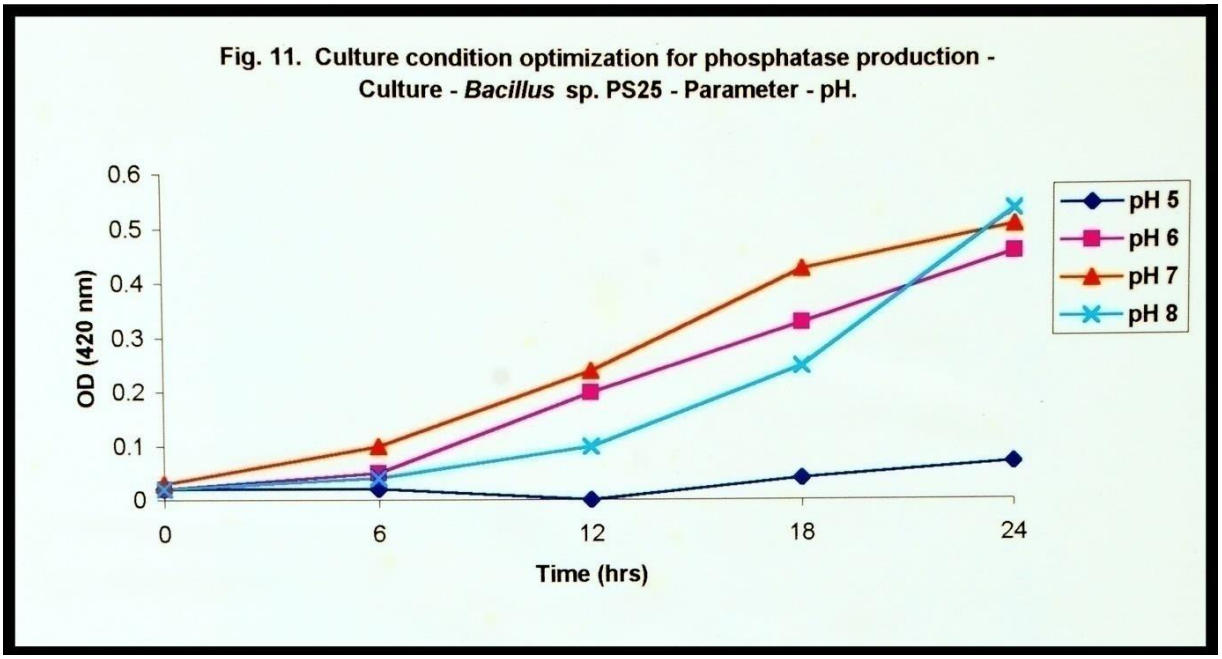

Figure 11: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 25 in the parameter of pH

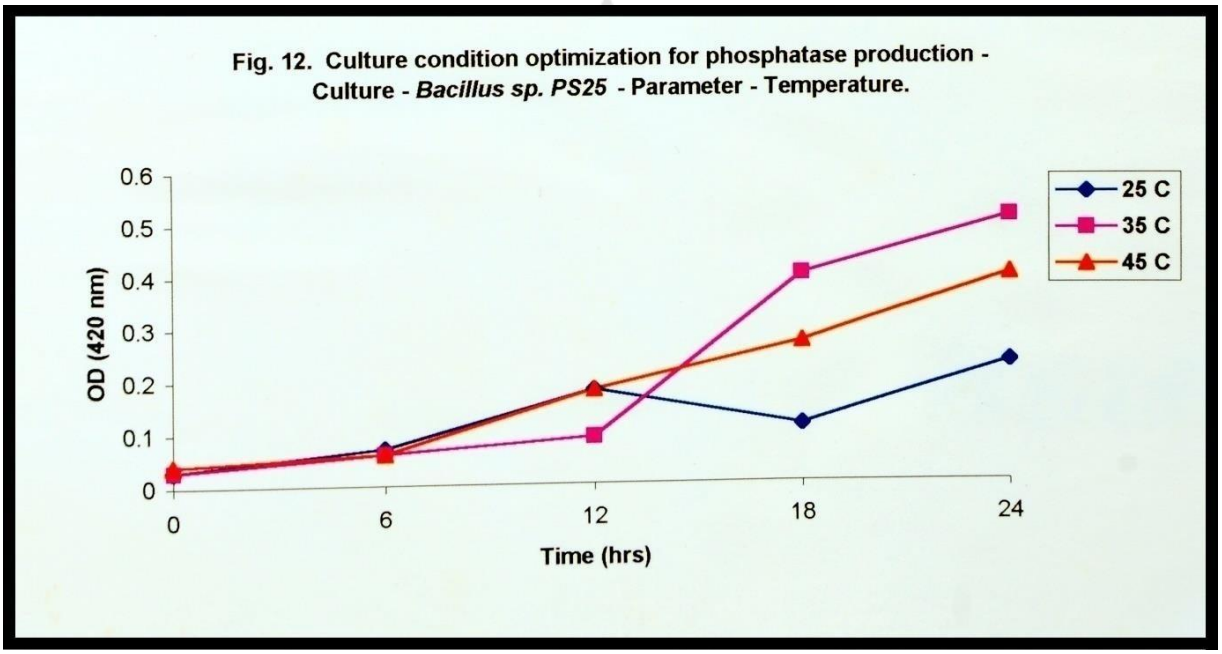

Figure 12: Culture condition optimization for phosphatase production of *Bacillus* sp. PS 25 in the parameter of temperature

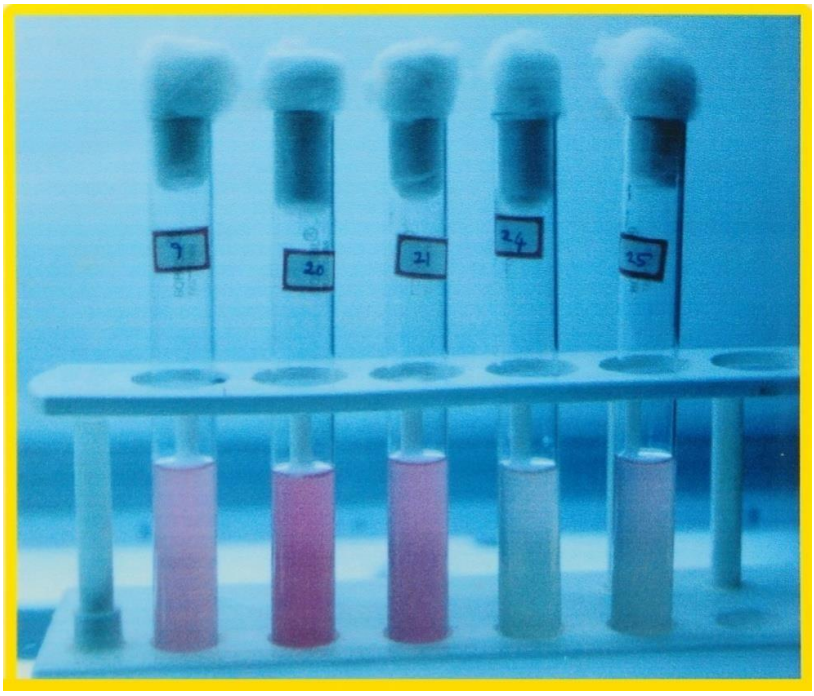

Figure 13: Phosphatase enzyme assay (before adding 1 N NaOH solution)

Legend: Light pink colour indicates the presence of phosphatase enzyme

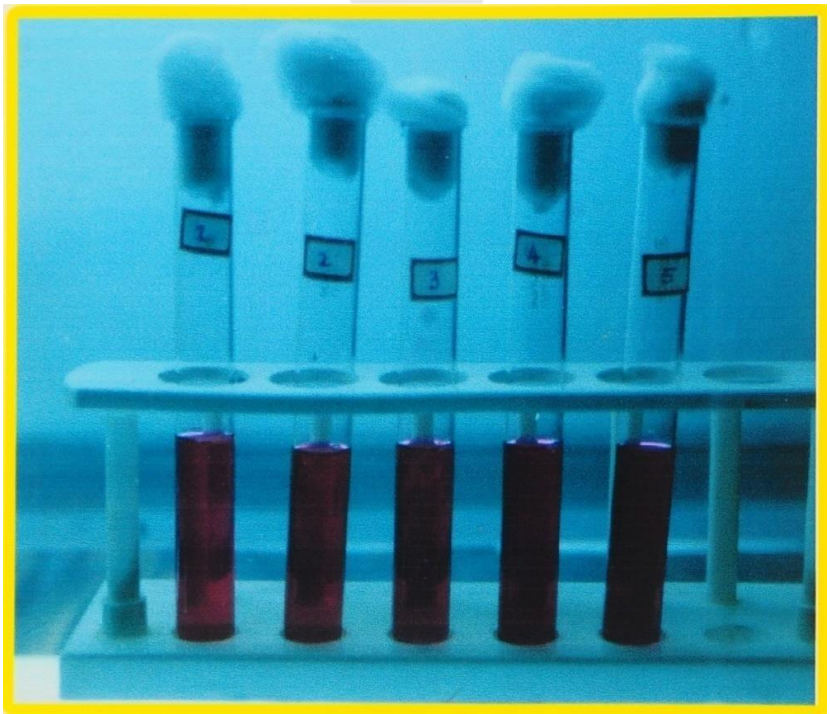

Figure 14: Phosphatase enzyme assay (after adding 1 N NaOH solution)

Legend: Bright pink colour indicates the presence of phosphatase enzyme

Phosphatase production is an important property that enables the bacteria to flourish especially in the rhizosphere region. As plants are continuously amended with organic fertilizers, these organisms would generate phosphate by more than one mechanism. Furthermore, phosphatase producers would be of special value especially when organophosphate pesticides through phosphatase enzyme thereby ensure the faster disappearance in soil. Even though these organisms are expected to resist organophosphonates, resistance to other pesticides would be an added advantage as these organisms can be used both as biofertilizers and bioremedial agents. In this background, one isolate from each genera was exposed to a commonly used herbicide, 'one shot' (2, 4-D and anilofos) at two different concentrations (10 ppm and 50 ppm) and checked for their tolerance level, while *Micrococcus* sp. and *Bacillus* sp. were unable to tolerate this herbicide even at low concentration (10 ppm). *Pseudomonas* sp. was able to tolerate this farm chemical at lower concentration (table -4).

Table - 4: Herbicide (one shot) resistance pattern of phosphate solubilizing bacterial isolates

S. No.	Culture Tested	Concentration of Herbicide	
		10ppm	**50 ppm**
01	*Micrococcus* sp.	-	-
02	*Bacillus* sp.	-	-
03	*Pseudomonas* sp.	+	-

Legend: + sign indicates the appearance of bacterial growth and - sign indicates the absence of bacterial growth.

CONCLUSION

This study indicates, the abundance of phosphate solubilizing bacteria in the rhizosphere region of *Oryza sativa*. Furthermore, their phosphatase activity would prove beneficial especially in inactivating residual organophosphate pesticides that would prevent the problem of pesticide pollution due to this chemical. Further research on the nature and production of phosphatase in these strains would be useful in their commercial applications.

ACKNOWLEDGEMENTS

First and foremost of all, I thank the almighty for blessing me to complete my Master of Science course in Microbiology and the project work successfully. I am grateful to express my deep sense of gratitude to my profound research guide Dr. GowrishankarRajam M.Sc., Ph.D. I owe him personnel in debtness for giving valuable suggestion for problem, his guidance, wise council, unstinted encouragement both psychologically and academically. His keen interest and effort helped me in completing this project. I feel pleasure, immense pride and as most blessed person in presenting the work under his guidance.

REFERENCES

1. Goldstein AH. Bacterial phosphate solubilization: Historical perspective and future prospects. Am J Alt Agric. 1986; 1: 57-65.
2. Gyaneshwar P, Naresh Kumar G, Parekh LJ. Effect of buffering on the phosphate solubilizing ability of microorganisms. World J Microbiol Biotech. 2002; 14:669-673.
3. Kennedy AC. Rhizosphere, In: Principles and applications of soil microbiology, Sylvia DM, Fuhrmann JJ, Hartel PG, Zuberer DA (eds.), 2nd ed., Pearson, Prentice Hall, New Jersey; 2005, pp.242-262.
4. Hiltner L. Uber neueerfahrungen und problem auf demgebiet der bodenbakteriologieunterbesondererberuksichtigung der grundungung und brache. Arb DtschLanwirtGes. 1904; 98:59-78.
5. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil. 2003; 255: 571-586.
6. Antoun H, Kloepper JW. Plant growth promoting rhizobacteria. In: Brenner S, Miller JF (eds.). Encyclopedia of Genetics, Academic Press: 2001, pp.1477-1480.
7. Goldstein AH, Rogers RD, Mead G. Mining by microbe. Bio Technol. 1993; 11: 1250- 1254.
8. Fasim F, Ahmed N, Parsons R, Gadd GM. Solubilization of zinc oxide and zinc phosphate by a bacterium isolated from air environment of a tannery. FEMS Microbiol Lett. 2002; 213(1):1-6.
9. Khan MS, Zaidi A, Wani PA. Role of phosphate solubilizing microorganisms in sustainable agriculture - a review. Agronomy for Sustainable Development. 2006; 26: 1- 15.
10. Glick BR. The enhancement of plant growth by free-living bacteria. Can J Microbiol. 1995; 41:109-117.
11. Iguala J, Valverdea M, Cervantesa E, Velazquezb E. Phosphate solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. 2001; 21: 561-568.
12. Wu SC, Cao ZH, Li ZG, Cheung KC, Wong MH. Effects of biofertilizers containing N- fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma. 2005; 125(1/2):155-166.
13. Artidave K, Patel HH. Inorganic phosphate solubilizing soil Pseudomonads. Indian J Microbiol. 1999; 39:161-164.
14. Neelima Garg M, Mishra M, Garg KL. Microbial solubilization of insoluble phosphates. In: Mukerji KG, Singh VP and Garg KL (eds.). Frontiers in Applied Microbiology, New Delhi; Rastogi and Company:1989.
15. Sen A, Paul NB. Solubilization of phosphates by some common soil bacteria. Curr Sci. 1957; 26:222-223.
16. Skujins J. Enzymes in soil. In: Melaren AD and Peterson GH (eds.). Soil Biochemistry, New York; Marces Dekker: 1967, pp.311-414.
17. Swaby RJ, Sperber II. Phosphate dissolving microorganisms in the rhizosphere of legumes in nutrition of legumes. New York; Academic Press: 1959, pp.289-294.
18. Sperber JI. Solution of mineral phosphates by soil bacteria. Nature. 1957; 180(4593): 994-995.

19. Illmer P, Schinner F. Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Soil BiolBiochem. 1992; 24: 389-95.
20. Tewari SK, Das B, Mehrotra S. Cultivation of medicinal plants - tool for rural development. J Rural Tech. 2004; 3: 147-50.
21. Chabot R, Antoun H, Cescas MP. Stimulation of growth of maize and lettuce by inorganic phosphorus solubilizing microorganisms. Canadian J Microbiol. 1993; 39: 941- 47.
22. Donahue RL, Miller RW, Shickluna JC. Soils: An introduction to soils and plant growth. New Delhi - 110 001; Prentice Hall of India Private Limited: 1990, pp.222-4.
23. Khalil S. Direct application of phosphate rock and appropriate technology fertilizers in Pakistan. In: direct application of phosphate rock and appropriate technology fertilizers in Asia - what hinders acceptance and growth, Proc. Int. Workshop, (eds.): Dahanayake K, VankauWenbergh SJ and Helluums DT. Feb: 20-25. Int. Fertilizer Devel. Centre, Kandy, Srilanka; 1995, pp.231-36.
24. Edwards CA, Bater JE. An evaluation of laboratory and field studies for assessment of the environmental effects of pesticides. Proceedings of the Brighton Crop Production Conference on Pests and Diseases. 1990;963-968.
25. Khan M, Sculion J. Effect of soil on microbial responses to metal contamination. Environ Pollut. 2000; 110: 115-125.
26. Miligi L, Costantini AS, Veraldi A, Benvenuti A, Vineis P. Cancer and pesticides: an overview and some results of the Italian multicenter case-control study on hematolymphopoietic malignancies. Ann N Y Acad Sci. 2006; 1076:366-377.
27. Vyas Sc. Soil microorganisms and their activities. Nontarget effects of agricultural fungicides, FL, Boca Raton; CRC Press: 1988, pp.258-275.
28. Ahemad M, Khan MS, Zaidi A, Wani PA. Remediation of herbicides contaminated soil using microbes. In: Khan MS, Zaidi A and Musarrat J (eds.). Microbes in Sustainable Agriculture, New York; Nova Publishers:2009.
29. Barriuso JS, Marin S, Mellado. Effect of the herbicide glyphosate on glyphosate tolerant maize rhizobacterial communities: comparison with pre-emergency applied herbicide consisting of a combination of acctochlor and terbuthylazine. Environ Microbiol. 2010; 12: 1021-30.
30. Pereira JL, Picanco MC, Silva AA, Santos EA, Tome HVV, Olarte JB. Effects of glyphosate and endosulfan on soil microorganisms in soybean crop. Planta Daninha. 2008; 26:825-30.
31. Srinivas T, Sridevi M, Mallaiah KV, India G, Nagar N. Effect of pesticides on *Rhizobium* and nodulation of green gram *Vigna radiate* (L.) Wilczek. ICFAI J Life Sci. 2008; 2: 37- 40.
32. Ahemad M, Khan MS. Phosphate solubilizing and plant growth promoting *Pseudomonas aeruginosa* PS1 improves green gram performance in quizalafop-p-ethyl and clodinafop amended soil. Arch Environ ContamToxicol. 2010; 58: 361-372.
33. Bellinaso ML, Greer CW, Peralba MC, Henriques JA, Gaylarde CC. Biodegradation of the herbicide trifluralin by bacteria isolated form soil. FEMS Microbiol Ecol. 2003; 43: 191-194.
34. Wani PA, Zaidi A, Khan AA, Khan MS. Effect of phorate on phosphate solubilization and indole acetic acid releasing potentials of rhizospheric microorganisms. Ann Plant Prot Sci. 2005; 13:139-144.
35. Ahemad M, Khan MS. Effect of insecticide-tolerant and plant growth promoting Mesorhizobium on the performance of chickpea grown in insecticide stressed alluvialsoils. J Crop SciBiotechnol. 2009; 12:213-222.
36. Oves M, Zaidi A, Khan MS, Ahemad M. Variation in plant growth promoting activities of phosphate solubilizing microbes and factors affecting their colonization and solubilizing efficiency in different agro-ecosystems. In: Khan MS, Zaidi A (eds.). Phosphate Solubilizing Microbes for Crop Improvement, New York; Nova Science: 2009, pp.247-263.
37. Aiso K, and Simidu V. Occurrence and distribution of heterotrophic bacteria in seawater from the komagawa bay. Bull Jap SocSci Fish. 1962; 28:1133-1141.
38. Sundara Rao WVB, and Sinha MK. Phosphate dissolving organisms in the soil and rhizosphere. Ind J Agric Sci. 1963; 33:272-278.
39. Ayyakkannu K, and Chandramohan D. On the occurrence and distribution of phosphobacteria in the marine environment at Porto Novo. Curr Sci. 1970a; 39: p. 398- 399.

40. Katznelson H, and Bose B. Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere and non-rhizosphere soil. Can J Microbiol. 1959; 5:79-85.
41. Bergey's Manual of Systematic Bacteriology. Baltimore, Hd.: Williiams and Wilins, 1984-1989.
42. Bauer AW, Kirby WMM, Sherris JC, and Turc M. Antibiotic susceptibility testing by a standardized single disc method. Am J ClinPathol. 1966; 36:493-496.
43. Hugh R, and Leifson E. J Bacteriol. 1953; 66:24.
44. King EO, Ward MK, and Raney DE. J Lab and Clin Med. 1954; 44: 301-307.
45. Kovacs N. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature (London). 1956; 178:703.