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Organic Manure Amended with Phosphate Solubilizing Bacteria on Soil Phosphorous Availability

B. C. Walpola[1*] and R. H. A. N. Hettiarachchi[1]

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ABSTRACT

Purpose : *Combine use of phosphate solubilizing microorganisms and organic manure has greater agronomic utility as it could improve phosphorous (P) utilization efficiency through conversion of insoluble P to accessible forms. Therefore, the present study was conducted to assess the effect of different organic manures (poultry manure, goat manure and cow dung) with phosphate solubilizing microorganism on phosphorous availability.*

Research Method : *The phosphate solubilizing bacterial (PSB) strain employed in this study was isolated from soil samples collected from agricultural lands in Matara District of Sothern Sri Lanka. The strain was identified as Enterobacter cancerogenous. Eight treatment combinations each replicated three times were used in the study.*

Findings : *P availability of organic manure amended soils was progressively increased irrespectively the inoculation of PSB strain. The bulk of the increment occurred during the first 2 to 3 weeks of the incubation followed by slight reductions at the later stages. P availability of the soil amended with organic manures was significantly ($P \leq 0.05$) higher than that of the control. Furthermore, the specified were much higher when PSB was inoculated to the soil amended with organic manures. PSB inoculants were used with poultry manure showed the highest P availability.*

Originality / Value : *The availability of soil phosphorous is enhanced by combine use of organic manures and PSB inoculants; the practice is suggested to be employed in integrated nutrient management strategies.*

Keywords: *Enterobacter cancerogenous, Organic manure, Phosphate solubilizing bacteria*

INTRODUCTION

Phosphorus (P) nutrition is considered as one of the primary factors limiting crop yields (Zaidi *et al*., 2009).The total P levels of soils usually range between 0.02–0.5%, of which only around 0.1% is available to plants. Therefore, continuous application of phosphotic fertilizers is essential to maximize crop yields. However, more than 80% of the applied phosphotic fertilizers get readily fixed to soil components and become unavailable to plants by forming complexes such as aluminum phosphate and iron phosphate in acidic soils and calcium phosphates in calcareous soils. Therefore, the recovery efficiency of phosphorous is less than 20% from applied and native soil phosphorous (Walpola and Yoon, 2012). Hence, seeking for viable solutions to enhance the recovery efficiency and solubility of applied and native phosphorous gains increased attention.

Conversion of insoluble phosphate complexes to soluble forms by phosphate solubilizing microorganisms through production of organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate and glycolate has been observed in media supplemented with insoluble phosphate sources (Fankem *et al*., 2006; Hu *et al*., 2006). Availability and solubility of phosphorous from applied and native phosphorous could be accelerated by

[1*] *Department of Soil Science, Faculty of Agriculture, University of Ruhuna.*
bcwalpola@soil.ruh.ac.lk
ORCID http://orcid.org/0000-0002-4663-0943

applying different organic amendments such as animal manure, plant residues and green manure (Imran *et al*., 2011; Adesanwo *et al*., 2012;Abbasi *et al*., 2015), composts (Nishanth and Biswas, 2008; Wickramatilake *et al*., 2010). Acidic environment generated by the decomposition process of organic amendments enhances the availability and solubility of applied phosphorous (Nishanth and Biswas, 2008). Therefore, the incorporation of organic manure to soils is considered to be a possible means of increasing recovery efficiency and solubility of applied and native phosphorous. The abundance of soil microorganisms is high in soils amended with different types of organic manure. This may help to solubilize insoluble phosphorous and increase phosphorous availability to the plants (Hu *et al*., 2006).

Combined use of organic manure and phosphate solubilizing bacteria could enhance phosphorous solubilization which in turn would increase the availability of phosphorous to plants. Therefore, the activation of phosphate solubilizing microorganisms and solubilization of insoluble phosphates through the production of organic acids could be expected to occur in soils that are amended with organic manure.

Use of phosphate solubilizing microorganisms such as *Pseudomans, Bacillus, Enterobacter, Azospirillum* and *Rhizobium etc.* to solubilize insoluble phosphates has a greater agronomic utility to compensate high cost of phosphatic fertilizer application/production. Such measures could have positive impacts on the environment as well. Consequently, the present study was conducted to assess the effect of poultry manure, goat manure and cow dung amended with phosphate solubilizing microorganisms on phosphorous availability of soil.

MATERIALS AND METHODS

Isolation of phosphate solubilizing bacterial strains

Rhizosphere soil samples collected from agricultural lands in Matara district of Sothern Sri Lanka were employed in isolating phosphate solubilizing bacterial strains. In the laboratory, 10 g of moist soil from each sample were weighed and transferred to 250 ml Erlenmeyerflask containing sterilized 90 ml of 0.85% NaCl solution. The suspension was then shaken for 30 minutes at approximately150 rpm. Immediately after shaking, a series of tenfold dilutions of the suspension was made by pipetting 1 ml aliquot into sterilized 9 ml of 0.85% NaCl solution. Serial dilutions were prepared using dilution plate technique (Wollum II, 1982). Each dilution was plated in the National Botanical Research Institute Phosphorus (NBRIP) agar plates containing 16 g agar, 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water. The pH of the media was adjusted to 7 using HCl. The plates were incubated 4-5 days in an incubator at 30^0C. The colonies with clear halos were considered as phosphate solubilizing colonies (Gyaneshwar *et al*., 1999). They were further purified by re-streaking on the fresh NBRIP agar plates at 30^oC. Bacterial strains which exhibited clear zones on the agar plates were selected as phosphate solubilizing organisms.A total of 15 phosphate solubilizing bacterial strains were isolated and maintained on 30 % glycerol stock until use. The bacterial strain which exhibited the largest halo on NBRIP agar plates was selected for further studies.

Phosphate solubilization under in vitro *conditions*

The selected bacterial strain was allowed to grow in sterilized liquid NBRIP medium (20 ml) at 30^oC for 2 days with continuous shaking at 150 rpm. Aliquots of culture (1 ml) were then transferred to 500 ml flasks (n=3 per strain) containing sterilized liquid NBRIP medium (200 ml) and incubated for 7 days with continuous shaking at 30^oC. Sterilized medium without the bacterial strain served as the control. A sample (10ml) of each cultured and control were taken daily and centrifuged at 10000 rpm for 15 min. The clear supernatant was used in determining the amount of phosphorous released into the medium. The pH of the culture medium was also recorded with the pH meter equipped with glass electrode. The phosphorus availability

was determined using phospho-molybdate blue color method (Murphy and Riley, 1962).

16S rDNA gene sequencing and Phylogenetic analysis of the isolated bacterial strain

The partial sequencing of 16S rRNA for the bacterial strains was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F (5'-AGAGTTTGATCCTGGCT CAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The online program BLAST was used in identifying the related sequences with known taxonomic information available at the data bank of National Centre for Biotechnology Information - NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A Phylogenetic tree was constructed using CLUSTAL Xprogram (Thompson *et al*., 1997), which involved sequence alignment by neighbor joining method (Saitou and Nei,1987) and maximum parsimony using the MEGA4 program (Kumar *et al*., 2001). Grouping of sequences was based on confidence values obtained by bootstrap analysis of1,000 replicates. Gaps were edited in the Bio Edit program and evolutionary distances were calculated using Kimuratwo parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Soil used for the pot experiment

Soil belongs to Red Yellow Podzolic great soil group and classified as Hapludults according to the USDA soil taxonomy (Mapa *et al.*, 1999) were collected randomly from research farm, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka. After removing the surface litter, soil samples were taken from 0 – 15 cm depth using an auger and mixed thoroughly to make composite samples. The soil was then passed through a 2mm sieve to eliminate coarse rock and plant material. A subsample was taken, air dried and used for the determination of physico- chemical characteristics of soil (Table 01).

Organic manures

Poultry manure - PM, goat manure - GM and cow dung - CD) used in this study were collected from the Faculty farm, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka. Composite samples of well dried manure was taken, crushed into smaller particles by hand pressing, homogenized and passed through a 1mm sieve before use. Total nitrogen in manure samples were determined by the Kjeldahl method and the total phosphorous content was determined by vanadomolybdate yellow color using spectrophotometer (Table 02).

Table 01: Some important physico-chemical properties of soil used in incubation study

Soil properties	Value
Sand (%)	84±1.25
Silt (%)	12±0.48
Clay (%)	4±0.85
Soil texture	Loamy sand
Bulk density (g/cm^{3-})	1.28±0.23
pH	6.43±0.54
Organic carbon (%)	0.85±0.18
Total N (%)	0.15±0.02
NH_4^+ - N (mg/kg soil)	82±2.12
NO_3^- - N (mg/kg soil)	31±1.63
Available P (mg/kg soil)	48±2.15
Available K (mg/kg soil)	118±6.55
CEC (cmol $^{(+)}$/kg soil	12.1±0.25

Table 02: Chemical composition of organic manure used for the experiment

Organic manure	Organic C (%)	N (%)	P (%)
Poultry manure	18.53±0.31	2.21±0.13	2.95±0.12
Goat manure	16.24±0.22	1.85±0.09	0.68±0.07
Cow dung	15.64±023	1.42±0.11	0.72±0.04

Inoculum preparation for incubation study

A Single colony of isolated bacterial strain was transferred to 500 ml flask containing nutrient broth. The Colony was then grown aerobically in flask on a rotating shaker (150 rpm) for48 h at 30°C. The bacterial suspension was then diluted in sterile distilled water to a final concentration of10^8CFU ml^{-1} and resulting suspension was used as inoculums for the pot experiment.

Incubation study

Plastic pots (25 cm in diameter,35 cm in height) were filled with the soil mentioned above and moisture content of soil was adjusted to 60% of water holding capacity (WHC) by adding water. They were then kept in dark for one week prior to addition of organic manure for pre-incubation. After pre-incubation period, addition of organic manure was done on the base of the recommended mulch application rate of 5 tons dry matter per hectare, assuming that top 15 cm of 1ha land contains 2.31×10^6 kg of soil (bulk density of the soil 1.542 gcm^{-3}). Accordingly, 10 ml of bacterial suspension was inoculated into the middle part of the pots. The control pots received 10 ml of diluted LB broth with no bacteria. Following the addition of all amendments, the soil was thoroughly mixed and the weight of each pot was recorded. Pots were covered with black polythene which was perforated with a needle to ensure natural gas exchange.

The pots were then arranged in a completely randomized block design with three replications per treatment. The experimental plan was based on eight treatments as follows; (1) soil without PSB or organic manure-control; (2) soil + poultry manure; (3) soil + goat manure; (4) soil + cow dung; (5) soil + poultry manure + PSB; (6) soil + goat manure + PSB; (7) soil + cow dung + PSB; (8) soil + PSB.All the amended pots along with the controls that were incubated in the dark at room temperature (30 ± 1°C). Constant moisture content of the soil was maintained by daily monitoring and adding distilled water when necessary.

Soil analysis

Samples of all treated and controls incubated for different time intervals were analyzed for changes in soil-available P and pH. Triplicate samples from each treatment including control were taken at 7, 14, 21, 28, 35, 42 and 56 days of incubation and analyzed for available P. The soil-available P was measured by ammoniummolybdate blue color method (Murphy and Riley, 1962) using a spectrophotometer. At each sampling time, 10 g soil from each pot was taken and used for measuring the changes in pH using a glass electrode in a 1 : 2.5 (v=v) soil–water suspension.

Statistical analysis

All data from the incubation experiment were subjected to analysis of variance (ANOVA) using SAS package (SAS, 1999). The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at $P \leq 0.05$.Values were given as means ± SD for triplicate samples.

RESULTS AND DISCUSSION

Isolation and identification of phosphate solubilizing bacterial strains

A total of 15 bacterial isolates that exhibited clear zones on the agar plates were selected as phosphate solubilizing bacterial isolates (Figure

01). The most efficient phosphate solubilizing bacteria (PSB-1) was selected for further studies. The selected strain had a marked insoluble phosphate solubilizing ability as visualized by the clear zone development around the colonies after incubation. According to 16S rRNA sequence analysis, the strain was identified as *Enterobacter cancerogenous*(PSB-1). Comparison of the16S rRNA sequence among available strains of *Enterobacter* species closely associated *Enterobacter cancerogenus LMG 2693*with 99.2% identity. The neighbor-joining method was employed to construct the phylogenetic tree which illustrates the relationships of 16S rRNA strain sequence and other *Enterobacter* species (Figure 02). A sequence of the *Enterobacter cancerogenus LMG 2693* was deposited in the GenBank nucleotide sequence data library under KX815170 accession number.

Phosphate solubilization under in vitro conditions

An inoculant is a complex biological formulation that combines two elements: cultured microorganisms and compounds secreted into their growth medium under controlled conditions. Changes in pH of NBRIP medium and soluble P content, released from inorganic P, due to the addition of bacterial inoculants are shown in Figure 03. A significant ($P \leq 0.05$) increment in soluble P content was observed with PSB inoculation. A significant reduction in the pH of the PSB-inoculated medium was also observed as compared with the control where it remained constant (pH 7). A strong negative correlation ($r = -0.934 \pm 0.1$, $P \leq 0.05$) could be observed between phosphate solubilization and pH.

Phosphate solubilization under invivo conditions

The effect of different organic amendments with and without phosphate solubilizing bacterial strain on changes in soil pH over 8 weeks of incubation is presented in Table 03 and the overall effect of different organic amendments on soil pH change is presented in Figure 04. Soil pH was significantly decreased ($P \leq 0.05$) in all treatments throughout the incubation period except control. The reduction of pH was rapid during the initial stages of incubation (first 2 to 3 weeks of incubation) followed by slower decrease. However, some fluctuations in pH were observed from some treatments. A significant decrease ($P \leq 0.05$) in soil pH was recorded from PSB inoculated soils than the un-inoculated soils. The pH of the initial soil was 6.43, which subsequently decreased down to 5.85, 5.95 and 5.96 at the end of the incubation period respectively for the poultry manure, goat manure and cow dung treated soil and 5.56, 5.81 and 5.84 respectively for the soil treated with poultry manure, goat manure and cow dung with PSB strain. Among the different treatments, soil treated with poultry manure + PSB strain showed the lowest pH at the end of the incubation period (Figure 04).

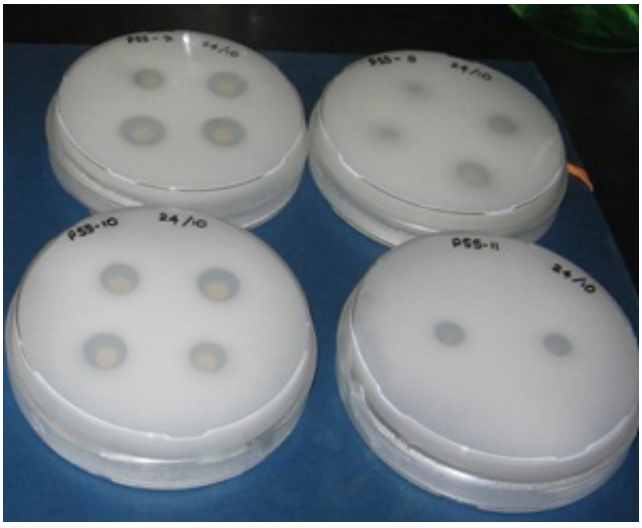

Figure 01: Isolated phosphate solubilizing bacterial strains showing clear halos produced in NBRIP solid medium

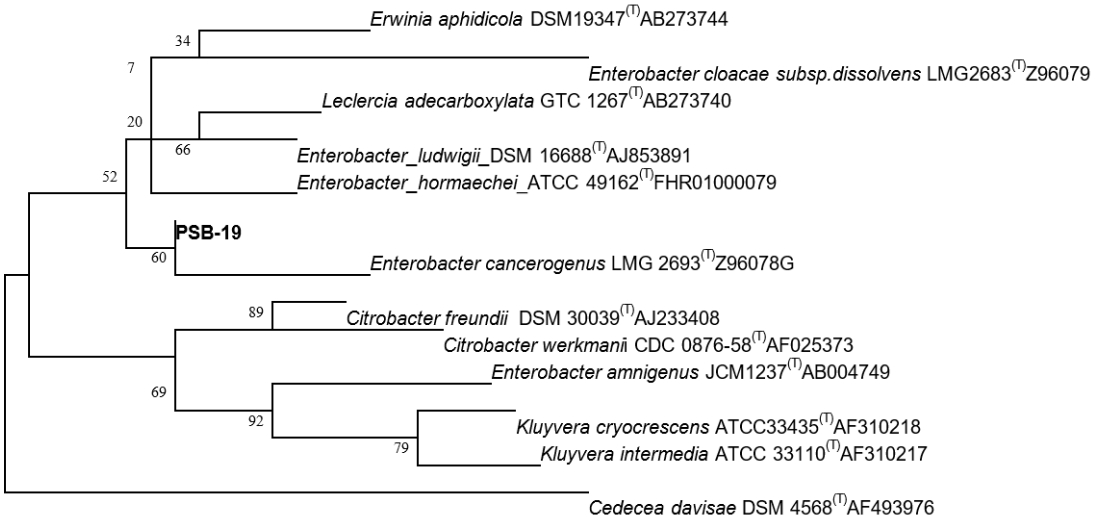

Figure 02: **Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Enterobacter cancerogenus* (PSB-19) strain with respect to related species. The scale bar indicates 0.002 substitutions per nucleotide position and accession numbers are given in parenthesis.**

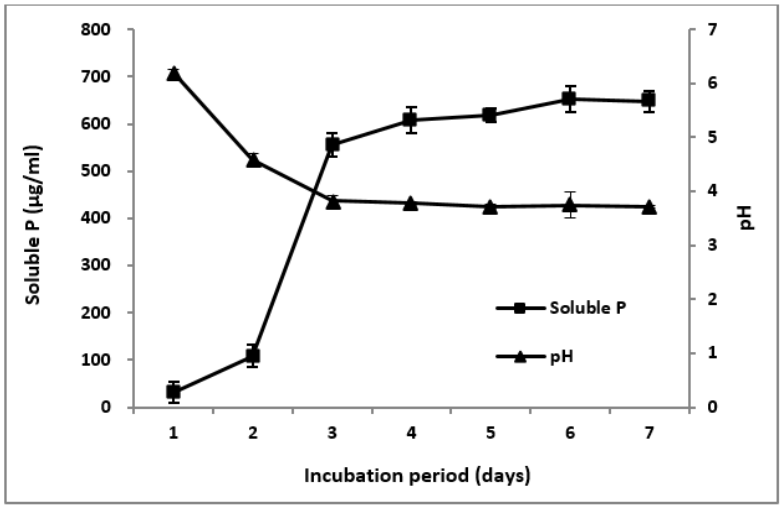

Figure 03: **Insoluble Phosphate solubilization and changes of pH in NBRIP culture medium containing *Enterobacter cancerogenus*. Values given here are the means (n = 3) ± standard deviation.**

Table 03: **Changes of the pH in the soil after application of organic amendments with and without PSB**

Treatments	Days after treatment application						
	7	14	21	28	35	42	56
Control	6.43±0.22	6.42±0.14	6.41±0.12	6.42±0.12	6.4±0.17	6.42±0.22	6.44±0.21
Soil + PM	6.33±0.18	6.13±0.16	6.11±0.17	6.05±0.17	6.02±0.15	5.95±0.14	5.85±0.17
Soil + GM	6.34±0.15	6.32±0.14	6.21±0.15	6.16±0.12	6.04±0.12	6.02±0.17	5.96±0.12
Soil + CD	6.22±0.15	6.23±0.18	6.19±0.26	6.28±0.32	6.26±0.11	6.01±0.10	5.95±0.12
Soil + PM + PSB	6.31±0.24	6.13±0.22	6.03±0.16	5.94±0.12	5.84±0.12	5.78±0.25	5.56±0.23
Soil + GM + PSB	6.26±0.11	6.21±0.12	6.02±0.23	6.01±0.22	5.87±0.25	5.82±0.26	5.81±0.20
Soil + CD + PSB	6.24±0.27	6.21±0.19	6.07±0.25	5.86±0.21	5.86±0.17	5.85±0.15	5.84±0.18
Soil + PSB	6.25±0.15	6.25±0.23	6.24±0.23	6.31±0.26	6.27±0.14	6.23±0.16	6.22±0.17

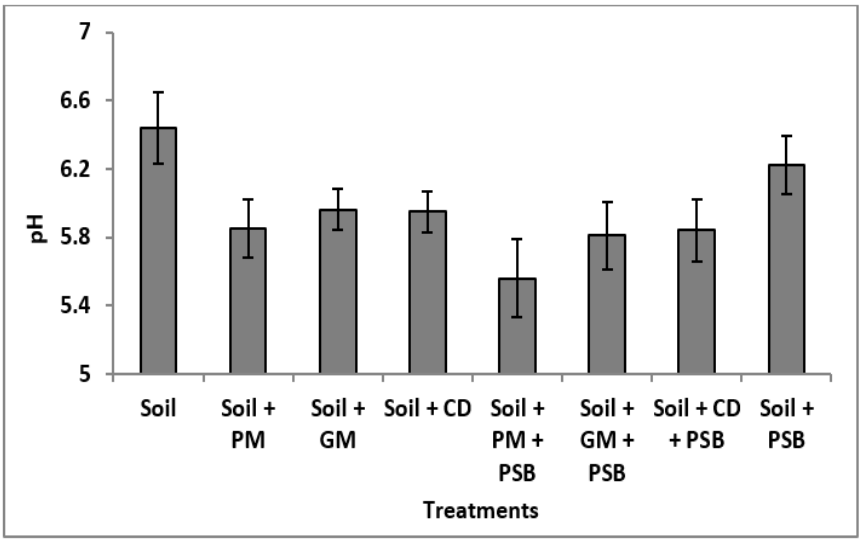

Figure 04: **The final effect of different organic amendment on pH change**

Table 04: **The available soil phosphorous content in soil with and without PSB strain**

Treatments	Days after treatment application (mg/kg soil)						
	7	14	21	28	35	42	56
Control	12.42±1.22	16.58±1.52	32.03±2.45	29.35±2.73	33.98±1.92	34.54±2.12	37.28±1.23
Soil + PM	22.26±1.58	32.55±2.51	46.38±3.74	51.44±3.73	51.09±3.51	50.32±3.25	50.21±4.51
Soil + GM	19.16±1.65	22.02±1.64	42.45±2.69	42.13±2.51	45.57±2.81	43.14±2.86	42.37±3.54
Soil + CD	23.65±2.14	27.6±2.84	40.86±3.82	48.52±2.68	48.63±2.68	48.45±2.84	47.66±5.31
Soil + PM + PSB	36.19±2.67	40.63±3.24	54.43±3.62	58.68±3.51	63.66±3.54	63.61±3.54	62.24±2.94
Soil + GM + PSB	23.95±1.87	27.34±1.82	45.82±3.95	53.85±3.62	52.63±3.21	52.32±2.94	51.56±3.68
Soil + CD + PSB	14.21±1.93	24.64±1.74	45.73±2.57	54.39±3.71	55.05±2.62	53.65±3.47	53.34±3.54
Soil + PSB	10.53±1.57	16.28±1.33	34.41±2.63	31.32±1.62	36.72±2.46	38.46±3.12	39.61±2.52

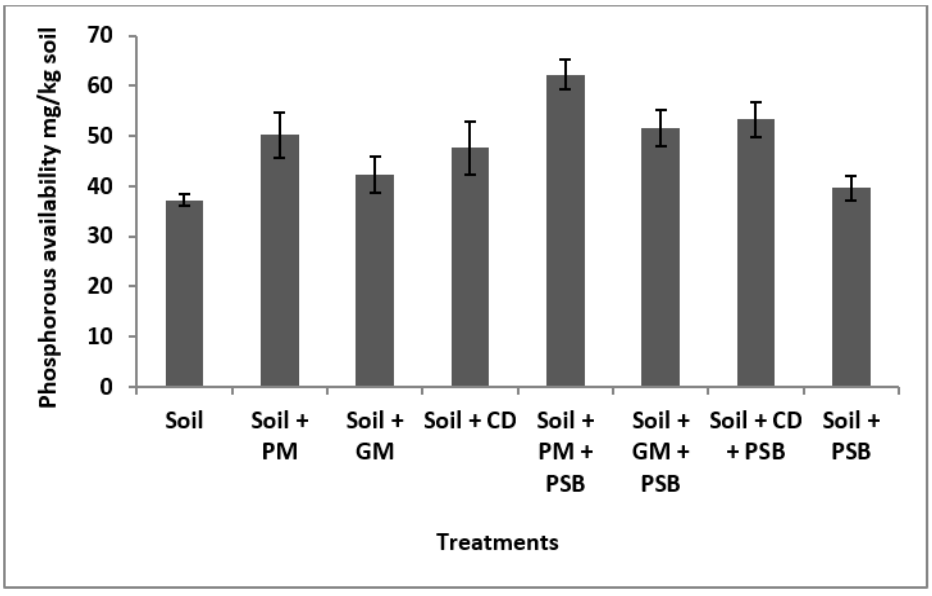

Figure 05: **The final effect of different organic amendment on phosphorous availability**

The effect of different organic amendments with and without PSB strain on soil phosphorous availability over 8 weeks of incubation is presented in Table 04 and the overall effect of different amendments on soil phosphorous availability is presented in Figure 05. The phosphorous availability of organic manure amended soil (with and without PSB strain) was progressively increased and the bulk of the increment occurred during the first 2 to 3 weeks of the incubation. However, subsequently a slight reduction was observed during the later stage of the incubation. Phosphorous availability of the soil amended with the organic manure was significantly ($P \leq 0.05$) higher than that of the control. However, the figures were lower than that of the soil amended with organic manure + PSB strain. The application of PSB strain without organic amendments did not show any remarkable effect on phosphorous availability. Soil amended with PM + PSB strain showed the highest phosphorous availability throughout the incubation period.

The application and incorporation of organic amendment can result in an increase organic matter levels in soil. Several reports are available on the effects of organic matter in enhancing the availability of soil phosphorous (Kouno *et al*., 2002;Wu *et al*., 2007). Organic matter can have a synergistic effect on mineral phosphate availability in phosphorous fixing soils (Agbenin and Igbokwe, 2006; Gichangi and Mnkeni, 2009). The decomposition of organic amendment generate acidic environment by releasing organic anions. These organic anions can compete with phosphorous sorption sites and they enhance the chelation of soluble Al and reduce the precipitation and of Al-P. The density and diversity of soil microorganisms are increased when soils amended with organic amendments due to the fact that readily available substrates are released into the soil (Nishio and Kusano, 1980). Therefore, application of organic amendments to soil increase microorganism's density and diversity, organic anion production and help to increase phosphorous availability to plants by solubilizing insoluble phosphorous (Fankem *et al*., 2006; Hu *et al*., 2006).These findings are in agreement with the present results of increased soluble phosphorous content and decreased pH in organic manure amended soil during the incubation period (Table 01and 02). Reduction in soil pH after manure application has been reported by Abebe (2001) also. However, the range of pH reduction is found to be slightly varying (from 6.43 to 5.85) which might be due to high buffering capacity of the experimental soil as stated by Abu-Zahraand and Tahboub (2008).

Soil microorganisms are responsible for determining the status of organic matter decomposition, nutrient cycling, soil degradation and bioremediation of soil pollutants. They play a key role in making available soil phosphorous to plants by solubilizing and mineralizing organic and inorganic phosphorous sources (Xiao *et al.,* 2013). Recently, emphasis has been paid to use of phosphate solubilizing bacteria in increasing the phosphorous efficiency thereby improving the growth and yield of crops. The major mechanisms involved in the phosphate solubilization by phosphate solubilizing microorganisms are acidification, chelation, ion exchange reactions and production of low molecular weight organic acids such as gluconic, oxalic and citric acids (Chaiharn and Lumyong, 2009; Ekin, 2010). Therefore, organic acid production contributes significantly to the process of acidification and pH reduction through which facilitates the phosphate solubilization. Present results of higher pH reduction and phosphate solubilization in soil amended with organic manure + PSB strain are therefore, in agreement with the early reports of Aria *et al*.,(2010) and Khanand Sharif, (2012), who conducted similar experiments with PSB inoculations. The effectiveness of different *Enterobacter* species in solubilizing phosphates have been identified previously also (Mirza *et al*.,2001; Deepa *et al*.,2010).

According to the results, the phosphorous availability of organic manure amended soil (with and without PSB strain) was found to be increased progressively and the bulk of the increment occurred during the first 2 to 3 weeks of the incubation. Simultaneously a rapid reduction in pH was observed during the period indicating that the acidification of the medium may have contributed to the enhanced

solubilization of phosphates. Subsequent slight reduction of phosphorous availability observed during the later stages of the incubation might be due to the inhibitory effect caused by available phosphorous, formation of organo-phosphate compounds or depletion of nutrients especially carbon source for the microbial activity and production of organic acids as suggested by other researchers (Varsha-Narsian *et al*., 1994; Illmer and Schinner, 1995; Chaiharn and Lumyong, 2009). Phosphate solubilizing microorganisms are capable of utilizing a variety of carbon sources to meet their energy requirement. Therefore, the growth and phosphate solubilization of phosphate solubilizing microorganisms may vary with the carbon source. Carbon source is considered to be an important factor for active proliferation and organic acid production by the organisms. The nature of available carbon sources is thus decisive in determining the type and concentration of organic acid produced by the phosphate solubilizing microorganisms which in turn controls the degree of phosphate solubilization (Patel *et al*., 2008).

The overall effect of different organic amendments on pH and phosphate solubilization is shown in Figure 01 and 02. Poultry manure showed the highest pH reduction and phosphate solubilization compared to other treatments. In accordance with our results, Azeez and Van Averbeke (2012) also observed lowest pH values for poultry manure amended soil. The high phosphorous content in poultry manure could also contribute towards the high phosphorous concentration in soil after 56 days of incubation.

Similar to present findings, positive impacts of organic manure and PSB on phosphorous availability have been reported previously by Begum *et al*., (2004); Toor, (2009) and Abbasi *et al*., (2013). Production of organic acids from these organic manures and PSB strains in the root rhizosphere could reduce phosphorous fixation, induce greater P availability and form phosphor–humic complexes that are easily assimilated by plants (Toor, 2009). These mechanisms could ultimately result in converting greater amounts of applied P to available P in root rhizospere.

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CONCLUSIONS

Availability of soil phosphorous is enhanced by amendment with organic manure such as poultry manure, cow dung and goat manure with and without PSB. When applied with isolated PSB strain, greater phosphorous availability is resulted in poultry manure amended soils compared to other sources. However, further studies under field conditions are needed to confirm the results and recommend commercial applications. Economic feasibility of this application should also be quantified through a series of field trials.

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