International Journal of Technical Innovation in Modern Engineering & Science (IJTIMES)
Impact Factor: 5.22 (SJIF-2017), e-ISSN: 2455-2585
Volume 5, Issue 03, March-2019

ISOLATION AND IDENTIFICATION OF PHOSPHATE SOLUBILIZING MICROORGANISMS FROM SOIL OF KOSAMBA REGION

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Abstract
One of the macronutrient which is highly needed for plant growth, uptake and development is Phosphorus, apart from Nitrogen and Pottasium. Comparison to other elements it is the least mobile element in plant as well as in soil and so its needed that the Phosphorus present in the soil in the anonymous form should reach to the plants in the soluble and usable form. Phosphorous solubilizing organisms play key role in phosphorous nutrition by enhancing and increasing its avalibilty to the plants through the release from various niches by solubilisation and mineralization. Soils from various parts of garden were taken and a laboratory study was carried out for isolation and identification of Phosphorus solubilizing Microorganisms (PSM). PSM isolated from various soil samples indicated the mechanism behind the Phosphorous solubilisation is pH reduction by production of various organic acids. The efficacy of PSM to solubilize phosphorous enhances transpiration, photosynthesis, respiration, flower formation, fruit production. The present study indicates that the presence of diverse plants in the garden associated with Phosphorus solubilizing micro organisms act as potential and efficient bio fertilizers which can be a new tred in present scenario.

Key words: Phosphorous, Phosphorous solubilizing microorganisms, mineralization, pH reduction, biofertilizers, bacteria, fungi.

1. Introduction

Phosphorus isthe secondmost important element and mineral , next to nitrogen most commonly limiting the growth of plant. Phosphorus is an essential macro element. It is an important component of biomolecules like DNA, RNA, ATP, phospholipids and plays a key role in energy transfer reactions, respiration, photosynthesis, stimulates root and shoot development, flower formation, fruit production, seed formation, stalk and stem strength, maturity, crop development, quality, and overall for plant development and growth making up about 0.2% of plant dry weightand resistance to plant diseases.Plants acquire phosphorus from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation withions such as Ca +2,Mg +2 , Fe +3 and Al +3, depending on the particular properties of soil. Phosphate being in this configuration is highly unsolvable and unapproachable to plants. So the asccesible amount is in very diminutive which isacquitanted to plant. [1].
Many soil fungi and bacteria jointly known as phosphorous solubilizing microorganisms (PSM) are known to solubilize inorganic phosphate to soluble form by the process of acidification, chelation, exchange reactions and production of organic acids. These are known as efficient P solubilizers. PSM play imperative role in soil phosphorous crescendos (6, 7).

The heart of any reaction is its mechanism and the main gadget used for solubilisation of mineral phosphate is organic acids production and the paramount role is of acid phosphatases for mineralization of organic phosphorus in soil. The crucial mechanism of mineral phosphate amalgamation is by action of coalescence of organic acids by soil microorganism.Organic acid generation ensue in acidification of the microbial cell and its surroundings (2,3).

Ouahmane et al. stated that there are several advantages of microbial mediated phosphorous over orthodox chemical fertilizers for agricultural and land purposes.Thesepluses are as follows: (1) microbial merchandises are considered safer than many of the chemical fertilizers. (2) neither toxic substances nor microbes themselves will be amassed in the food chain; and (3) self-replication of microbes circumvents the need for repeated application. Thus, inoculation with PSM along with rock phosphate could be another strategy to improve the physicochemical and biological properties of soil and help in improving crop and land production (4,5).
Fungi are the most important components of soil flora, than bacteria and actinomycetes depending on soil depth, texture and nutrient condition, soil characters and physic chemical characteristics of soil. Fungi have been reported to have greater ability to solubilize the insoluble phosphate than bacteria. A wide variety of bacteria and fungi such as *Bacillus sp, Pseudomonas, Rhizobium , Azotobacter, sp, Aspergillus, Penicillium, filamentous fungi*, are reported to solubilize phosphate and are receiving greater attention.(8, 9,17)

Plant-microbes interactions in the rhizosphere are gratifying determinants of plant health and soil fertility [13]. The important genera of P-solubilizing bacteria include *Rhizobium, Bacillus, and Pseudomonas* amid the rhizosphere microbe[14, 15]. The ruling P-solubilizing filamentous fungi found in rhizosphere *is Penicillium and Aspergillus* spp.[16]. RP solubilization critically needs filamentous fungi as they are producers of organic acids. As they are of foremost in solubilization of Phosphate and other biotechnological importance such as biocontrol, biodegradation, and phosphate mobilization, trials have been conducted for *Aspergillusniger* and some *Penicillium* species [10, 17, 18].

2. Materials and Methods

2.1 Soil Sample Collection

The soil samples were collected from differentfield sites of the Kosamba region having crop field such as site 1, Site 2, Site 3, Site 4, Site 5, Site 6 rhizospheric soil of field plants. The collected soil sample were brought into laboratory and used for further studies.

2.2 Isolation of Phosphate Solubilizing Microorganisms (PSM)

Soil samples collected were further used for isolation and identification of PSM on Pikovskaya's (PKV) agar medium, containing the following (g/L): 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.02 g NaCl, 0.02 g KCl, 0.003 g $FeSO_4{\cdot}7H_2O$, 0.003 g $MnSO_4{\cdot}H_2O$, 5 g $Ca_3\,(PO_4)_2$, 10.0 g glucose, 0.5 g yeast extract, 15.0 g agar, and 1000 mL distilled water [22]. The medium was autoclaved at 121°C for 15 minutes; about 20 mL of the sterilized molten agar medium was poured into each petri dish and allowed to solidify before inoculation.
1gm of various soil samples were added in 10 ml sterile d/w and further serial dilutions of the same were performed from 10-1, 10-2, 10-3, 10-4, 10-5, 10-6 for isolated growth. From the dilutions 0.1ml of inoculum were added on PVK plate and spread plate technique was also performed. (2,3)
Also soil samples were randomly sprinkled on the PVK plate (Jadeja) so that one can get difference between serial dilutions and direct soil application method which helps in isolation of PSM as well as in getting different characteristic PSM.

The incubated plates were at at 37°C for 3-5 days. Colonies showing clear zones around after incubation were further purified by transferring into Pikovskaya's agar medium and observed for colony characteristics, morphological characteristics and microscopic view The pure cultures were preserved on PVK Slant, Potato Dextrose Agar (PDA) slant and N agar slant at 4°C for further investigation.

2.3 Identification and Characterization of Phosphate Solubilizing Microorganisms (PSM)

After incubation, each individual bacterial colony which showed zone of solubilisation was picked with help of nichrome wire and fungal colony which showed zone of solubilisation was picked from the edge with a sterile fine tipped inoculation needle and transferred on to the slide. [10]. The microorganisms were identified based on thecolony characteristics, morphological characteristics and microscopicmorphological, conidial (fruiting bodies) and culture characters. The classifications of the fungi were carried out following standard procedures. All the isolated fungi were named and maintained in test tubes and Petri plates containing PDA media and used for further studies (4).

2.4 Budding Phosphate Solubilizers

The budding phosphate solubilisers were inoculated on the PVK agar plate, incubated for 3 to 5 days at 37 c temperature and isolated on the base of zone of solubilisation or halo found around the growth of organisms. The zones diameter were further measured for showing clear zone of soulbilization. The budding phosphate solubilizers were then selected for primary screening technique. The isolates were then transferred on the slant for preservation and were transferred to broth for further studies.

3. Result and Discussion

3.1 Isoaltionof Phosphate Solubilising Microorganisms

6 different soils samples were collected from different croplandsites of Kosamba region.

Total of 74 Phosphate Solubilising Microorganisms were isolated from which 43 were fungal isolates and 31 were bacterial isolates.

A total of 43 fungal species were isolated from different sites from which 6 fungal isolates were from Site 1, 7 were isolated form site 2 which is the main garden area indicationg high fertility and presence of PSM, 6 isolates from site 3, 4 isolates from site 4, 6 from site 5 and 13 isolates from site 6 which is field soil or agricultural land. All the fungal isolates were labeled as AF1 to AF 43.

Table 1 showing the total number of fungal and bacterial species from various sites

Sr No	Fungal Species	Bacterial Species	Total Number
Site 1	6	3	9
Site 2	7	3	10
Site 3	6	4	10
Site 4	4	7	11
Site 5	7	5	12
Site 6	13	9	22
Total Number	43	31	74

Total 43 fungal isolates belonging to genera *Aspergillus, Penicillium, Fusarium, Trichoderma, Neurospora, Alternaria, Curvularia*were isaloted and identified based on the colony characterisitcs, morphological characteristics and Microscopic view.

Table 2 showing the total number of fungal species belonging to various Genera from various sites

Sr No	*Aspergillus*	*Penicillium*	*Fusarium*	*Trichoderma*	*Neurospora*	*Alternaria*	*Curvularia*	ToTal
Site 1	2	1	1	1	-	1	-	6
Site 2	3	2	-	-	-	-	2	7
Site 3	-	1	1	1	2	1	-	6
Site 4	-	-	-	1	1	1	1	4
Site 5	2	1	1	1	-	2	-	7
Site 6	3	3	2	1	1	2	1	13
Total	10	8	5	5	4	7	4	43

A total of 31 bacterial species were isolated from different sites from which 3 bacterial species from site 1, 8 bacterial species from site 2, 4 from site 3, 7 from site 4, 3 from site 5 and 9 form site 6.

*Total 31 bacterial isolates belonging to genera Bacillus, Rhizobium, Azotobacter, Pseudomonas, Enterobacter, Pantoeasp*have been isolated and identified.

Table 3 showing total number of bacterial species belonging to various genera from various sites

Sr No	*Bacillus subtilis*	*Bacillus megaterium*	*Rhizobium*	*Azotobacter*	*Pseudomonas*	*Enterobacter*	*Pantoea*	ToTal
Site 1	1	-	2	-	-	-	-	3
Site 2	2	2	2	-	1	-	-	7
Site 3	-	-	-	3	1	-	-	4
Site 4	-	-	2	-	1	2	-	5
Site 5	1	-	2	-	-	-	-	3
Site 6	2	4	-	-	-	-	3	9
Total	6	6	8	3	3	2	3	31

The clearing zones of isolates were quantified on the 5th day of incubation using the equation

Phosphate solubilizing index = colony diameter+ clearing zone / colony diameter * 100

3.2 Identification of Phosphate Solubilizing Microorganisms

Morphological Characterization
Morphological characteristics of isolates viz. shape, size, elevation, surface form, margins and surface texture, color were observed for their characterization.

Gram's staining
The isolate was characterize for its gram staining uniqueness as per the following standard Procedure: Take the smear on the glass slide with the help of inoculate loop let it be air dry. After this with the help of flame fix it with the heat. Add crystal Voilet for 3o seconds. Wash it with the distilled water, let it be dry. After that add Gram's iodine for 60 seconds. Wash it with 95% Ethyl alcohol, Add saffranin for 30 seconds after this wash it with the distilled water. Air dry it with the help of blotting paper. Observe in the microscope. The pink colonies will show the gram negative bacteria and the purple colonies will show the gram positive bacteria.

Lactophenol Blue Staining

The fungal isolates were stained with lactophenolblueas per standard procedure : Take the fungi with some agar from the plate, fix it on slide, tap it, spread it and cover it with coverslip. After this add Lactophenol blue and mount it with the stain.

Figures Showing Phosphate solubilising Zone of Bacteria and Fungi as well as Lactophenol blue Mounting and Gram's Staining.

Fig 1: Showing Phosphate Solubilising zone of bacteria.

Fig 2: Showing Phosphate Solubilising zone of bacteria.

Fig 3: Showing Phosphate Solubilising zone of Fungi.

Fig 4 :Showing Spores of *Fusarium*

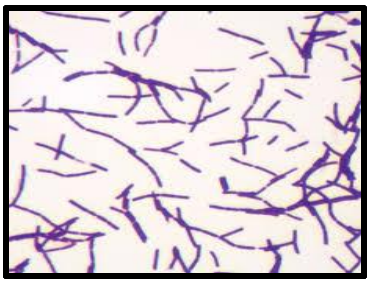

Fig 5: Showing Gram Positive *Bacillus*

Fig 6: Showing Gram Negative *Rhizobium*

Fig 7: Showing spores of *Curvularia*

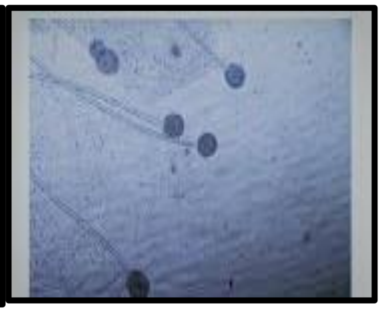

Fig 8: Showing structure of *Aspergillus*

3.3 Discussion

In this study we have screened for phosphate solubilizing microorganusms (PSM) collected and isolated form various soil samples from varois sites of Kosamba region.

Total 74 PSM were isolated from which 43 were fungal isolates belonging to various genera as showed in Table 2, 31 were bacterial isolates from which 12 were gram positive bacteria and 19 were gram negative bacteria belonging to various genera as shown in Table 3.
PSM strains were isolated using Pikovsakyas agar medium based on zone of solubilisation and halo formation around the growth of colonies.(18,19)

The PSM were identified based on colony characteristics, morphological characteristics, spores pattern, microscopic view, grams staining and biochemical tests and further growth on specific differential medium that confirms the organism.

The present study reveals that the population of PSM and their diversity vary in soil from various sites due to abiotic factors of soil, climate, soil and cropping history, organic carbon content, phosphorus content, physical and chemical properties of soil, as well as surrounding atmosphere and water as main source for plantation.
Screening for PSM clearly indicated that there must be change in pH of medium which may be acidic due to production of various organic acids that facilitates the solubilisation of phosphate.

Another correlation may be due to the presence of phosphatase enzyme in the PSM.PSB Strains were identified by studying the cultural, morphological, physiological, microscopic and growth on differential and specific media as well as various biochemical tests using Bergeys Manual of Dterminative Bacteriology (27). PSF species were identified by studying the cultural, morphological, physiological, microscopic view, spore structure (23,24,25).

Phosphorous deficiencies are nowdays spreading in world wide soil and specially restraining the crop productivity. NPK fertilizers are the major cost for agricultural production and their use directly effects the soil fertility, soil microbe miscellany, crop productivity and has long term effect on environment in terms of Eutrophication, restoration of fertility, carbon footprint. Many bacteria, fungi, Actinmoyctes are potential P solubilisers in soil playing a major and imp role for making it available to plants. In this regards PSM have been seen best eco- friendly means for P nutrition for crops (14).

In the present study, a total of 74 PSM were isolated from various field sites of various cropland.

A total 43 fungal isolates were isolated from rhizosphere soil and screened for their ability to solubilize Phosphate in PVK medium. A total of 31 bacterial species were isolated from rhizosphere soil and screened for their ability to solubilize Phosphate in PVK medium. There are reports on both bacterial and fungal stains exhibiting P solubilizing activity by the formation of clear halo zone or zone of solubilisation. These results support the findings of Mendes et al where fungi specially*Aspergillus and Penicillium*solubilse all forms of Phosphate.

It has been also noticed in the present study that the fungal diversity is overall more as compared to bacteria. (16).

It has also been seen that sites where fungal diversty is more has lesser bacterial species and soil where there is higher bacterial diversity has less fungal species.

The reason for this may be due to productionof antifungal agents, antibacterial agents, antibiotics, primary or secondary metabolites or toxic metabolites which may have antagonistic effect on other species or genus. It has been also observed that *Aspergillus* species were in the highest number as compared to other fungal isoaltes proving the fact that *Aspergillus* is the robust organism, having high soil diversity and it is producumg maximum zone of solubilisation confirming the fact that its most efficient in phosphate solubilisation (17, 22).
P solubilisation may be the work of either phosphatase or phytase or may be combined effect.

Curvularia and Neurospora strains are less in number and *Fusarium ,Alterneria and Penicillium* species are ascetically present.

It has been delineated by Sudisha et al. [23] that, tomato seeds treated with phosphate solubilizing fungi like*Trichoderma, Penicillium*, etc., enhanced seed germination and seedling. , solubilization of tri-calcium phosphate (TCP) and improvement in the growth of soyabean in TCP amended soil by *A. nigerand P. italicum*[24]. Seeds treated with *T. harzianum* in sunflower [25] and *Penicilliumchrysogenum and P. oxalicum* in pearl millet [13, 26] showed enhancedseed germination and seedling vigor over the control. Finding the lead of coffins, that phosphate solubilization, IAA production and other related compounds by the fungus interact with plants as part of its colonization, leading to growth promotion, induced resistance, and modification of basal plant defense mechanisms [19-22]. Ca, Fe and Al metal cationsprecipitate the Phosphorous fertilizer in soil upto70-90% making insoluble form which are not efficiently taken up plants. Phosphate solubilizing microorganisms inoculation in soil has been shown to be in good termsof insoluble phosphate resulting in higher crop performances. PSM isolated were hyper efficient phosphate solubilizer (22).

4. Conclusion

The study reveals that the isolates isolated from Kosamba region, have phosphate solubilizing efficacy and can be used for further studies on phosphate solubilisation and crop field experiments which could prove the fertility of soil and help in enriching the soil diversity.

The study also reveals that there are major fungal isolates found in the soil as compared to bacteria.Fungi are important components of the soil microbes as compared to bacteria as they have more biomass , can live in robust conditions, also in soil depth, less nutrient conditions, and have greater ability to solubilize phosphate then bacteria. *Aspergillus* and *Penicillium* are the most common species but *Fusarium*, *Curvularia* and *Alterneria*have also been found efficient of Phosphate solubilisation. Now days, PSM technology largely depends on good quality inoculants to farming communitites .For identification and characterization of more PSM, there is a need for extensive and consistent research efforts with greater efficiency for their ultimate application under field conditions. Hence the results of the present study may contribute to the findings of other researchers, where we were able to notice the efficacy of bacteria as well as fungi to solubilize phosphate which can be helpful as biofertilizers.

The lack of fertilizer industries in developing countries,the rise in the cost of chemical fertilizers and the growing environmental issue and biodiversity loss using chemical fertilizers, biofertilizers are clinching momentousness as alternative for increased productivity and yield.

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