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Research Article

IN- VITRO P-SOLUBILIZING ACTIVITY OF SELECTED SOIL MICROORGANISMS ISOLATED FROM DIBRU-SAIKHOWA BIOSPHERE RESERVE FOREST OF ASSAM

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ABSTRACT
An investigation was made to study the *in-vitro* p-solubilization activity of phosphate compounds through some selected microbes *viz. Aspergillus Niger*, *Aspergillus flavus*, *Pseudomonas* sp (PB_1 and PB_2) isolated from the soil of Dibru-Saikhowa Biosphere Reserve (DSBR) Forest of Assam (India). Phosphorous solubilizing microorganisms play an important role in converting insoluble phosphatic compounds such as rock phosphate, basic slag and chemically fixed soil phosphorous into available form. In our study, the above mentioned microorganisms were cultured and screened by applying Pikovskaya's liquid medium with addition of P_2O_5 as insoluble phosphate for microbial phosphate solubilization. *Aspergillus niger,* PB_1 and PB_2 were observed very effective in P-solubilization in presence of P_2O_5. P-solubilizing activity of *A. niger*, PB_1 and PB_2 were increased upto 21 days and decreased immediate after 21 days of incubation. Bacterial isolates PB_1 and PB_2 were observed higher phosphate solubilizing activity (16.54 and 13.37 mg) as compared to fungal species *A. niger* (10.76 mg) and *A. flavus* (9.01 mg) in 28 days incubation.

Key Words: *P- Solubilizing Microorganisms, Insoluble P_2O_5, Solubilization of Phosphate, DSBR*

INTRODUCTION
Study Site
Dibru- Saikhowa Biosphere Reserve (DSBR) forest is our study site. Dibru- Saikhowa Biosphere Reserve forest (National Park) is one of the parts of 19^{th} biodiversity hotspots in the world (Sharma, 1999). It is located between 27^0 35' - 27^0 50' North latitude and $95^0$10' to 95^0 40' East longitude. It is a safe heaven for many extremely rare and endangered species of wild life including over 300 avifauna as well as various species of shrubs, herbs and medicinal plants. Wetlands cover sixty percent of the total area while forest and grasslands covers only 25 percent and 15 percent respectively (Dutta and Phukan, 1997). This biosphere reserve forest is mainly consists of semi wet evergreen forests and tropical moist deciduous forest (Champion and Sheth, 1968).

Microbial solubilization of inorganic compound is of great importance in plant nutrition. Microbial P-solubilization can increase the availability of phosphorus to plants from soil. Phosphate solubilizing microorganisms (PSM) include largely bacteria and fungi, which can grow in media containing tricalcium, iron and aluminium phosphate, bonemeal, rock phosphate and similar insoluble phosphate compounds as the sole phosphate source (Gaur, 1990). It was reported by Yahya and Al-Azawi (1989) that number of soil fungi and bacteria posses mineral phosphates solubilizing activity. They also observed that certain soil fungi *Aspergillus terreus, A. Flavus, A. awamori, A. niger, Penicillum digitatum, P. simplicissimum, Sclerotium rolfsii and species of Fusarium, Rhizoctonia etc.* were also able to solubilize tricalcium phosphates. The solubilization of tricalcium phosphate in soil by inoculation of some species of *Aspergilus* and *Pseudomonas* were better solubilizer to rock phosphates (Ostwal and Bhide, 1972). Chhonkar and Subba Rao (1967) determined the P-solubilizing activity of various kinds of fungi in medium containing soluble KH_2PO_4.

In the earlier worker like Mikanova *et al*., (2002) observed that there were differences not only in the P-solubilizing activity among the tested bacteria, but also in their sensitivity to the presence of soluble

phosphates in medium.. For this reason they decided to test a number of soil bacteria showing a high P-solubilizing activity for its sensitivity to the presence of soluble hydrogen potassium phosphate in medium. This feature is certainly important in the selection of the suitable bacterial strains for practical applications.

There is a scope to achieve some vigor strains in addition to new strains in a diversified area of a biosphere reserve where soil ecology and floral diversity are quite different so far as the Agricultural land and crop land flora are concerned (Saikia *et al.*, 2004). This paper reveals that the *in-vitro* p-solubilizing activity of some selected soil micro flora isolated from Dibru-Saikhowa Biosphere Reserve Forest of Assam.

MATERIALS AND METHODS

Isolation, Characterization and Identification of Fungi

Potato Dextrose Agar (PDA) was used as basal medium to isolate fungal species. The compositions of PDA medium are: (Infusion form of Potato: 200g, Dextrose: 20g, Agar: 30g, Distilled water: 1000 ml, Final pH: 5.6 ± 0.2). Besides this, Czapek Dox Agar (CDA) (Sodium nitrate: 2g, Dipotassium hydrogen phosphate: 1g, $MgSO_4$: 0.5g, KCl: 0.5g, $FeSO_4$: 0.01g, Sucrose: 30.0g, Agar: 15g, Distilled water: 1000 ml and pH: 7.3 ± 0.2) and Fungal Agar (Papaic digest of soyabean meal: 10g, Dextrose: 10g, Agar: 15g, Distilled water: 1000 ml and pH: 7.0 ± 0.2) media were used to isolate, identify and maintain fungal species (Warcup, 1955 and Gillman, 1995).

Isolation, Characterization and Identification of Bacteria

For isolation of bacteria, Nutrient Agar (Beef extract: 3g, Peptone: 5g, Agar: 15g, Distilled water: 1000 ml, pH: 6.8-7.2 ± 0.2) and phosphate solubilizing bacteria, Pikovskaya's Solid Media (Glucose: 10g, Ca_3PO_4: 5g, Ammonium sulphate, $(NH_4)_2\ SO_4$: 0.5g, NaCl: 0.2g, $MgSO_4$: 0.1g, KCl: 0.2g, Yeast extract: 0.5g, $MnSO_4$: trace, $FeSO_4$: trace, Agar: 15g and pH 7.0 ± 0.2) were used.

Biochemical tests *viz.* Gram stain, Methyl red, Acetyl methyl carbonyl, Catalase test, H_2S production, Glucose test, Dextrose test, Citrate utilization, Urolytic property, Gelatin liquefaction, NO_3 reduction, Starch hydrolysis, Indole production and Congo red test were performed to characterize and inference of the bacterial isolates (Breed *et al*., 1957; Barnett and Hunter, 1972 and Hernot *et al*., 1994).

Screening of Phosphate Solubilizing Microorganisms

Pikovskaya's liquid medium was used for screening of phosphate solubilizing activity of selected soil microorganisms. Microbial solubilizations of insoluble phosphates were measured in Pikovskaya's liquid medium with addition of P_2O_5 as insoluble phosphate. The flask containing the media incubated with *Pseudomonas* sp. (PB_1 and PB_2), *Aspergillus niger* and *A. flavus* were allowed to grow for 0, 7, 14, 21 and 28 days respectively at 30^0 C in an incubator. In case of fungus, the solution is filtered through Whatman Filter Paper No. 42 and in case of bacteria; the culture is centrifuged at 15,000 rpm for 30 minutes. The clear solutions were collected in 100 ml volumetric flask and made upto100 ml with distilled water. 10 ml of chloromolybdic acid was added and with the help of distilled water the volume is made upto about 40 ml. and 5 drops of chlorostannous acid were added. The solution was turned blue. The blue color intensity of the solution was measured at 600 nm and the optical density (OD) was noted. A graph were plotted with concentration of standard KH_2PO_4 (100 ppm) on the X-axis and Y-axis. The changes of pH in media after separating the bacterial cells or fungal biomass were also recorded and data presented in a graph (Figure 1 and 2).

RESULTS AND DISCUSSION

Microbial Populations

It was observed that 24 Nos. of micro fungi *viz. Aspergillus niger, Aspergillus flavus, Aspergillus terreus, Aspergillus* sp, *Aspergillus fumigatus, Penicillium italicum, Penicillium* sp_1, *Penicillium* sp_2, *Fusarium moniliforme, Fusarium oxysporum, Fusarium* sp, *Trichoderma viride, Trichoderma koningi, Trichoderma* sp, *Curvularia lunata, Curvularia* sp, *Rhizopus nigricans, Rhizopus* sp, *Mucor* sp, *Pythium* sp, *Mycelia*

sterilia, Rhizoctonia solani and *Sclerotium rolfsii* and 20 Nos. of bacterial isolates were very common in the soil of Dibru-Saikhowa Biosphere Reserve Forest of Assam (Table 1 and 2).

Table 1: Characterization (microscopic observations) and identification of isolated Mycoflora from Dibru Saikhowa Biosphere Reserve (DSBR) Forest

Organisms Code/Sites	Microscopic observations	Identification
MF1/8	Fast growing colony with abundant submerged mycelium, reverse usually without color, vegetative mycelium septate, branched, colorless, condiophores smooth, septate, varying length and diameter 200-400 X 7-10 µ, conidial head blackish brown, conidia globose.	*Aspergillus niger*
MF2/12	Colonies on Czapek's agar widely spreading, reverse and agar uncolored or light yellow, conidiophores 400-700 µ long X 5-15 µ in diameter, conidia almost globose, colorless, smooth, 4 x 5 µ in diameter.	*Aspergillus flavus*
MF3/8	Colonies on Czapek's agar from tints of pinkish-cinnamon to deeper brown shades in age, spreading, velvety. Reverse and agar from pale or bright yellow, condiophores 160 µ long, 6 µ in diameter, non- septate, conidia elliptical, 3 µ in diameter, smooth, borne in long, parallel, adherent chains.	*Aspergillus terreus*
MF4/6	Fast growing colony on Czapek's agar with septate branching hyphae, colorless, Condiophores non-septate, enlarging upward, conidia globose, colorless, smooth, 2 µ in long and 3-4 µ in diameter.	*Aspergillus* sp
MF5/8	Colonies on Czapek's agar strictly velvety, varying amounts of tufted aerial mycelium up to felted floccose forms, green to black in colour. Conidiophores short, usually densely crowded, 2-8 µ in diameter. Chains of conidia form soild columns up to 400 x 50 µ. Conidia dark green in mass, globose, 2-3.5 µ.	*Aspergillus fumigatus*
MF6/4	Colonies broadly spreading, bluish green, becoming gray-green when old, reverse brownish; condiophores arise as branches of aerial hyphae, 400- 500 µ long; chains of conidia loosely divergent, long; conidia 3-5 X 2-4 µ, cylindrical.	*Penicillium italicum*
MF7/6	Colonies on Czapek's agar mixed green (gray-green), becoming brownish when old, texture cottony, broadly spreading, reverse yellow; condiophores arising seperately, 300 µ long; conidia elliptical, becoming globose, 3-4 µ, pale green.	*Penicillim* sp_1
MF8/4	Fast growing colonies deeply blue gray, mycelium white in reverse, conidiophores long, uniform in diameter, septate, branched; conidia 3 µ, yellowish green.	*Penicillium* sp_2
MF9/12	Extensive mycelium and cottony in culture with some tinge of pink colour, microconidia in chains and remaining connected, two celled, spindle shaped; macroconidia delicate, crescent shaped, tapering at both ends, 3 septate, chlamydospores lacking.	*Fusarium moniliforme*
MF10/12	Extensive white mycelium, texture cottony, smaller conidia, one or two celled, oval to reniform, are numerous in the aerial mycelium but are lacking in the typical fruiting layers of the macroconidia, 3-septate, 19-40 X 2.5 µ.	*Fusarium oxysporum*

MF11/5	Fast growing colonies on Czapek's agar medium, extensive white mycelium with cottony texture, conidiophores slender and simple, terminal, branched, spindle shaped, many celled with distinct cross-walls, conidia 3-septate.	*Fusarium* sp
MF12/8	Colonies on Czapek's agar spreading, floccose, white at first, becoming light green in four to five days but never becomes deep green, reverse colorless; conidiophores hyaline, much branched, conidia (phialospores) hyaline, 1-celled, ovoid, borne in small terminal clusters.	*Trichoderma viride*
MF13/8	Colonies on Czapek's agar spreading, floccose, white at first, becoming light green in four to five days but never becomes deep green, reverse colorless; vegetative hyphae septate, hyalin; condiophores arise as branches of aerial mycelium, alternate, 25 µ in height X 3.0 µ in diameter, dichotomously branched; conidia oblong, smooth, hyalin, 3.2 –4.8 µ long 1.8 – 3.0 µ wide.	*Trichoderma koningi*
MF14/9	Colonies on Czapek's agar broadly spreading, hyalin, fruiting areas appear as tufts, white at first, and becoming various deep green shades with age, reverse colorless; condiophores arise as aerial mycelium, septate, dichotomously branched; condia globose, smooth.	*Trichoderma* sp
MF15/7	Colony spreading, dark olive gray, reverse bluish black, hyphae septate and much branched, 3-3.6 µ in diameter, unbranched. Spores borne more or less in a whorl at tip of conidiophore, three septate, curved, brown.	*Curvularia lunata*
MF16/12	Mycellium branched, septate, subhyalin or brown; conidiophores brown, thread like, unbranched, septate. Conidia acrogenous cylindrical, three or four septate.	*Curvularia* sp
MF17/5	Stolons creeping, recurving to the substrate in the form of arachnoid hyphae, rhizoids well developed. Sporangiophores united in groups of three to five or more. Spores unequal, irregular round or oval, angular, grey blue, 7.5-8 µ in diameter; zygospores round or oval, 160-220 µ in diameter. No chlalmydospore formation.	*Rhizopus nigricans*
MF18/3	Mycelia of two kinds, one submerged in the substratum and the other aerial, constituting filaments. The sporangiophores arise from the nodes where rhizoids present.	*Rhizopus* sp
MF19/10	Mycelium widespread in and on the substratum, rhizoids and stolons are absent, richly branched, hair fine, colorless; terminal sporangia in clusters. Spores spherical or ellipsoid with thin smooth membrane.	*Mucor* sp
MF20/1	Mycelium well developed rather corse on PDA plates. Hyphae large, branching irregular and free, septate in old cultures. Sporangia spherical or oval, terminal and intercalary. Conidia usually numerous, intra and extrametrical, 15-25 µ in diameter, round, oval.	*Pythium* sp
MF21/11	Sclerotiawithout definite form, often grown together, horny-fleshy with thinner undifferentiated edges, frequently embedded in the mycelium and bound together by mycelial strands.	*Mycelia sterilia*
MF22/12	Stolons creeping, the hyphae are more or less branched, rhizoids well developed, sporangiophores rarely single, united in groups of three to five or more, 0.5-4 mm in height X 24-42 µ in diameter, sporangia hemispheric, 100-350 µ.	*Rhizopus nigricans*

MF23/8	Sclerotia without definite form, often grown together, horny-fleshy, with thinner undifferentiated edges, frequently imbedded in the mycelium and bound together by mycelial strands.	*Rhizoctonia solani*
MF24/12	Mycelium densely floccose, not ropy and bearing numerous pinkish-buff to olive-brown to clove-brown globose sclerotia, 0.8-2.5 mm in diameter.	*Sclerotium rolfsii*

MF *= Mycoflora*
Sampling Sites: 1- Tarali area 2- Kundakhat area 3- Colomee area 4- Tongkong area 5- Sobha area 6- Pulibari area 7- Uriambari area 8- Kalia area 9- Miripathar area 10- Bordubi area 11- Holokhbari area 12- Surkey area

Table 2: Number of colonies (fungi and bacteria) and their percent contribution of each isolates obtained from soils of Dibru-Saikhowa Biosphere Reserve (DSBR) Forest.

S. No.	Fungal/Bacterial isolates	Dibru Range		Saikhowa Range	
		No. of Colonies (gm^{-1} of soil)*	Percent Contri-bution	No. of Colonies ($gm^{-1}$of soil)*	Percent Contri-bution
1	*Aspergillus niger* (MF1/8)	77.66±2.04	8.40 %	81.00±3.06	7.42 %
2	*Aspergillus flavus* (MF2/12)	75.33±2.07	8.15 %	42.33±0.67	3.88 %
3	*Aspergillus terreus* ((MF3/8)	68.66±1.92	7.42 %	74.65±0.81	6.84 %
4	*Aspergillus* sp (MF4/6)	27.33±1.03	2.96 %	27.34±0.50	2.51 %
5	*Aspergillus fumigatus* (MF5/8)	18.33±0.27	1.98 %	69.32±0.45	6.35%
6	*Penicillium italicum* (MF6/4)	79.00±2.27	8.55 %	51.30±0.42	4.70 %
7	*Penicillim* sp (MF7/6)	65.66±0.99	7.10 %	78.00±1.70	7.15 %
8	*Penicillim* sp (MF8/4)	35.66±0.20	3.86 %	73.00±1.56	6.69 %
9	*Fusarium moniliforme* (MF9/12)	46.66±1.13	5.05 %	59.16±1.39	5.42 %
10	*Fusarium oxysporum* (MF10/12)	78.00±1.49	8.44 %	34.20±0.32	3.13 %
11	*Fusarium* sp (MF11/5)	12.00±0.19	1.30 %	63.13±1.22	5.78 %
12	*Trichoderma viride* (MF12/8)	43.33±1.13	4.69 %	12.00±0.08	1.10 %
13	*Trichoderma koningi* (MF13/8)	10.66±0.08	1.15 %	43.53±0.74	3.99 %
14	*Trichoderma* sp (MF14/9)	8.66±0.02	0.94 %	10.66±0.35	0.98 %
15	*Curvularia lunata* ((MF15/7)	23.30±0.11	2.52 %	66.00±0.51	6.05 %
16	*Curvularia* sp (MF16/12)	9.33±0.39	1.01 %	27.33±0.54	2.50 %
17	*Rhizopus nigricans* (MF17/5)	11.00±0.68	1.19 %	13.10±0.45	1.20 %
18	*Rhizopus* sp (MF18/3)	60.33±0.69	6.53 %	38.10±1.57	3.49 %
19	*Mucor* sp (MF19/10)	42.66±0.78	4.62 %	60.38±1.91	5.53 %
20	*Phythium* sp (MF20/1)	33.33±1.46	3.61 %	42.65±0.50	3.91 %
21	*Mycelia sterilia* (MF21/11)	8.45±0.92	0.91 %	6.23±0.16	0.57 %
22	*Rhizoctonia solani* (MF23/8)	5.66±0.31	0.61 %	23.43±1.33	2.15 %
23	*Sclerotium rolfsii* (MF24/12)	3.33±0.30	0.36 %	11.36±0.41	1.04 %
24	*Bacillus* sp (MDB_{18})	33.13 ± 1.46	3.58 %	23.65±0.25	2.17 %
25	*Pseudomonas* sp (MDB_2)	29.00 ± 1.26	3.14 %	39.42±0.74	3.61 %
26	*Rhizobium* sp (MDB_1)	17.86 ± 0.48	1.93 %	20.11±0.72	1.84 %
	Total	**924.32**		**1091.27**	

** = Mean of 3 replicates and SE*

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Selected microorganisms were further applied in phosphate solubilizing activity on the basis of literature (Gaur, 1990; Mikanova and Novakova, 2002). Table 2 revealed that the highest (79.00 gm^{-1}) and lowest (3.33 gm^{-1}) number of populations were observed in *Pencillium italicum* and *Sclerotium rolfsii* respectively in the soil of Dibru range while the highest (81.00 gm^{-1}) and lowest *(*10.66 gm^{-1}) number of populations were recorded in *Aspergillus niger* and *Trichoderma* sp. respectively in Saikhowa range. Similar observation was reported by Yasmeen (2002) in Binsar Wildlife Sanctuary, Almora.

During the investigation period it had been observed that the microbial population of the soil was very less in the soil environment of the reserve forest. The qualitative and quantitative composition of the soil mycoflora depends upon the nature of soil and its chemical constituents (Kamal and Bhargava, 1973). The numbers of *Ascomycotina* and *Bacillus*, *Pseudomonas* and *Rhizobium* were observed dominating fungi and bacteria in both the ranges respectively. The higher number of the species of this fungus can be attributed to its ability to grow in diverse conditions (Saikia *et al.*, 2004). Soil fungi make a very important part of the ecosystem along with other microbes in turnover of the biomass (James and Hyde, 1998).

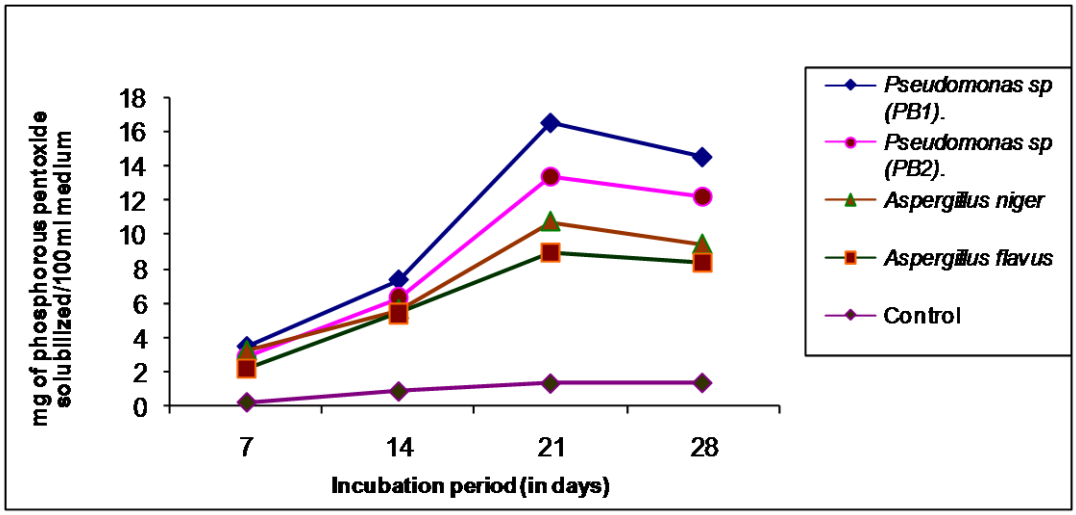

Figure 1: Phosphate solubilizing activity of selected microorganisms with respect to Incubation period

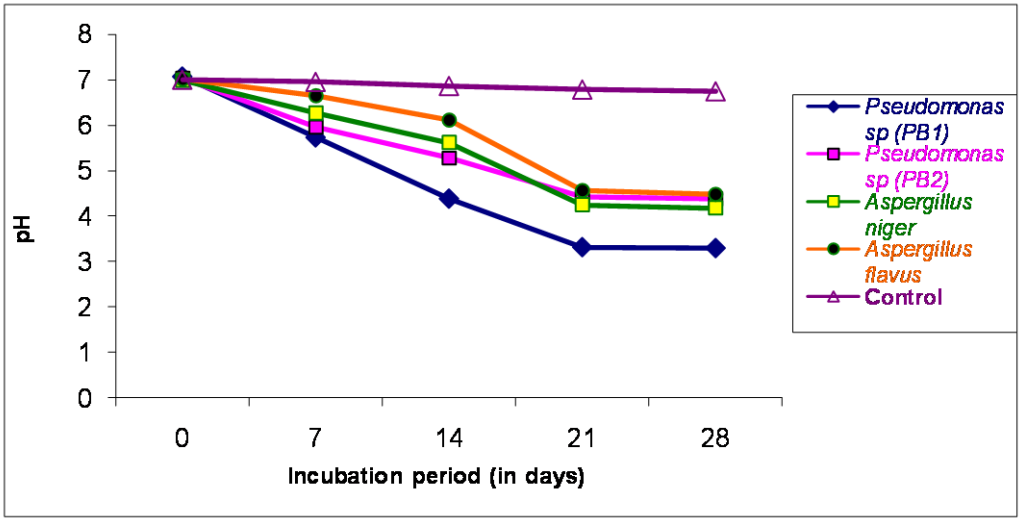

Figure 2: Phosphate solubiliizing activity with changes of ph in media against the Incubation period (in days)

Table 3: Morphology and bio-chemical tests for bacterial isolates and their inference

Organ-ism	Shape & Gram Stain	Methyl Red Test	Acetyl Methyl Carbonyl Test	Catalase Test	Triple test	Sugar	Iron Agar	Citrate utilization Test	Urolytic property Test	Gelatin Test	NO_3 Redu-ction	Starch Hydro-lysis Test	Indole Produ-ction Test	Congo Red Test	Inference
					H_2S	Glucose	Glucose + Dextrose								
MDB_1	Rod/-ve	+	-	-	-	-	+	-	+	-	+	-	-	+	*Rhizobium* sp
MDB_2	Rod/-ve	-	-	+	-	+	-	-	-	+	-	-	-	-	*Pseudomonas* sp (PB_1)
MDB_3	Rod/+ve	-	+	-	+	-	-	-	-	-	-	+	-	+	Unidentified
MDB_4	Cocci/-ve	-	-	-	-	+	-	-	+	-	-	-	-	-	Unidentified
MDB_5	Rod/-ve	+	-	+	-	+	-	-	-	+	-	-	-	-	*Pseudomonas* sp (PB_2)
MDB_6	Cocci/+ve	+	+	-	-	-	-	-	+	+	-	-	-	-	Unidentified
MDB_7	Rod/-ve	-	+	-	-	-	-	-	+	-	+	-	+	+	*Rhizobium* sp
MDB_8	Cocci/-ve	-	-	-	-	-	-	-	+	-	-	-	-	-	Unidentified
MDB_9	Rod/-ve	+	+	-	-	-	+	-	+	+	+	-	+	+	*Rhizobium* sp
MDB_{10}	Rod/-ve	-	+	-	+	-	-	-	+	-	+	-	+	+	*Rhizobium* sp
MDB_{11}	Rod/-ve	-	+	-	+	-	-	-	+	+	+	-	+	+	*Rhizobium* sp
MDB_{12}	Rod/-ve	+	+	-	-	+	-	-	-	+	+	-	+	+	*Rhizobium* sp
MDB_{13}	Cocci/-ve	+	+	-	-	-	+	-	-	-	-	-	+	-	Unidentified
MDB_{14}	Cocci/-ve	+	-	-	+	-	-	-	-	+	+	+	+	-	Unidentified
MDB_{15}	Rod/-ve	-	+	-	-	-	-	-	+	-	+	-	+	+	*Rhizobium* sp
MDB_{16}	Rod/+ve	-	-	-	-	-	-	-	+	-	-	-	-	-	Unidentified
MDB_{17}	Cocci/-ve	-	-	-	-	-	-	-	+	-	-	-	-	-	Unidentified
MDB_{18}	Rod/+ve	-	-	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* sp
MDB_{19}	Cocci/-ve	+	+	-	-	+	-	-	+	+	-	-	+	+	Unidentified
MDB_{20}	Rod/-ve	-	-	+	-	+	-	-	-	+	-	-	-	-	*Pseudomonas* sp (PB_1)

MDB= Microbial Diversity of Bacteria (B_1 - B_{20}) '+' = Positive, '-' = Negative

Phosphate Solubilizing Activity of Selected Microorganisms

Phosphate solubilizing activity of a few selected isolates *viz. Aspergillus niger, A. flavus* and isolates of *Pseudomonas* sp. (PB_1 and PB_2) were increased upto 21 days incubation and its activity decreased immediately after 21 days of incubation against insoluble P_2O_5. *Pseudomonas* sp. (PB_1 and PB_2) were observed higher phosphate solubilizing activity (16.54 and 13.37 mg) as compared to *Aspergillus niger* and *A. flavus* (10.76 and 9.01 mg) (Figure 1). The pH of media gradually decreased with the increase of incubation period due to production of organic acids by efficient phosphate solubilizing bacteria or fungi (Figure 1). Due to production of organic acid and the pH level of the medium becomes acidic and the phosphate solubilizing activity of the organism decreased gradually (Gaur, 1990 and Mikanova and Novakova, 2002).

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