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Phosphatases of Soils and Microbes of Tea Gardens of Dibrugarh District, Assam.

KEYWORDS

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ABSTRACT *Soil fertility can be related to the phosphatase activity of soil. Thus the soil health of the tea gardens of Dibrugarh District was determined by the phosphatase activity of the soil. The acid phosphatase activity was found at the range of 273.29 ± 4.67 - 451.62 ± 11.72 units/g of soil and the alkaline phosphatase activity was found at the range of 295.79 ± 3.86 - 539.88 ± 20.92 units/g of soil.*

Rhizospheric soil from the shade trees was further screened and microbes with phosphate solubilising capacity were isolated on Pikovskaya agar medium. Seventeen microbes were obtained with phosphate solubilising capacity. On the basis of solubilisation zone and efficacy of viability during enrichment culture four active isolates were selected for study. From the active isolates obtained the phosphatase activities were determined. The acid phosphatase specific activity was obtained at a range of 9,397.56 ± 61.33 - 10,021.22 ± 91.78 and the alkaline phosphatase specific activity was obtained at a range of 10,411.67 ± 73.47 - 13,854.33 ± 64.02.

Introduction

Phosphorous is an important element required by the living beings. It is highly reactive and is therefore found as either inorganic phosphate rock or organic phosphate. Phosphate occurs mostly in insoluble form; only small part is soluble in nature. In order to obtain phosphorous the phosphate must be converted into soluble form.

Phosphate solubilising microbes aid in converting the insoluble form of phosphate to soluble form (U. Chakraborty, B.N. Chakraborty & A.P. Chakraborty, 2012; Sharma, Subba & Saha, 2012). Phosphate solubilising microbes consist of members of both bacteria and fungi. They help in the release of phosphorous from inorganic and organic phosphate by solubilization and mineralization. Soil phosphorous dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes. Principal mechanism in soil for mineral phosphate solubilization is lowering of soil pH by microbial production of organic acids that dissolve phosphatic minerals and/or chelate cationic partners of the phosphate ions, directly thus, releasing phosphorous into soil. And the release of phosphorous from organic phosphate is by mineralization of organic phosphate with phosphatases (He, Bian & Jhu, 2002; Khan et al. 2009).

Phosphatases are group of enzymes secreted as soluble periplasmic proteins or retained as membrane bound lipoproteins. Phosphatases are usually able to dephosphorylate a broad array of structurally unrelated organic phosphoesters (nucleotides, sugar phosphates, phytic acid etc.) to acquire inorganic phosphate and organic by-products (Gandhi & Chandra, 2012). Activities of soil phosphatases and phosphodiesterases are among the major sources of fresh organic phosphate inputs to soil (Cosgrove. 1967).

Phosphatase enzyme significantly accelerates the release of inorganic phosphate from organically bound phosphate and returns it to the soil (Phukan, Samanta & Barthakur, 2011). Phosphatases are usually classified as neutral (EC 3.1.3.-), alkaline [EC 3.1.3.1] and acid [EC 3.1.3.2] based on pH optima (Hoffmann, 1968; Akanji and Adesokan, 2005; Raghav et al. 2011; Banerjee, Sanyal & Sen, 2012).

In India, phosphorous content in an average soil is 0.05%. Only 0.1% from the total phosphorous forms is available to plant, rest of the phosphorous forms become insoluble salt (Bhattacharya and Jain. 1996; Nisha et al. 2014). Large amount of phosphorous applied as fertilizer in form of superphosphate, NPK enters into the immobile pools through precipitation reaction with highly reactive aluminium (Al+) and iron (Fe3+) in acidic, and calcium (Ca2+) in calcareous or normal soils (Gyaneshwar et al. 2002; Hao et al. 2002; Devi et al. 2012).

The soil of Assam is acidic in nature which favours the growth of tea plants. Like other soil, tea garden soils also harboured microbial population. To meet the needs of consumers, tea industry largely rely on use of chemicals in the form of fertilizers and pesticides for better production. The phosphorous applied as fertilisers usually get converted to insoluble form. The Phosphate solubilising microbes in the soil helps in solubilising phosphate from the insoluble pool, which in turn, is available for the plants. Dibrugarh located along the river Brahmaputra is known as Tea City of India. There are 167 tea estates in Dibrugarh. It is the largest tea exporting town in India. Therefore, the study is designed to determine the phosphatases activity of both the soil and the phosphate solubilising microbes of the rhizosphere soil of shade trees of Dibrugarh.

Materials and methods

Study site

Soil samples were collected from five selected tea gardens of Dibrugarh district. (Fig. 1, Table 1)

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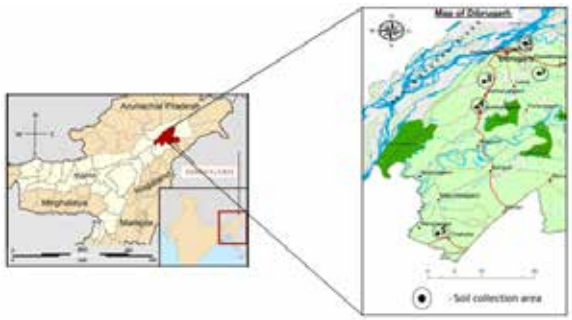

Fig .1 Geographic map of Dibrugarh District, Assam showing sampling area.

The lower aerobic zone of soil was selected for collection due to the abundant microbial activity that can be observed here. The soil samples were collected from the rhizosphere of the shade trees at a depth of (7-10) inches and packed in sterile zipper polybags. Cylindrical auger and spade were used for digging the soil. In order to make the samples random, each tea garden was arbitrarily divided into four plots. From each plot further ten different sampling spots were chosen. The soil collected from the ten sampling spots was put together as one sample. Thus four samples, each comprising 10 sites were prepared from each garden.

Table 1. Soil sampling - Tea gardens.

Soil Sampling Sites		
Tea Gardens	Geographic coordinates	
Moran Tea Garden	27˚09′ N,	94˚53′ E
Durgapur Tea Garden	27˚23′ N,	94˚52′ E
Borborooah Tea Garden	27˚24′ N,	94˚53′ E
Bokel Tea Garden	27˚27′ N,	94˚59′ E
Jalannagar Tea Garden	27˚29′ N,	94˚56′ E

Media

a. Nutrient agar medium

Peptone- 10g

Lemco- 5g

Sodium chloride- 1g

Distilled water- 1000 ml

Agar- 20g

pH- 7.2

Sterilised in autoclave at 15 lb pressure, 121 ˚C for 20 mins.

b. Pikovskaya agar medium

Dextrose- 10g

Calcium phosphate- 5g

Ammonium sulphate- 0.5g

Magnesium sulphate- 0.1g

Potassium chloride- 0.2g

Yeast extract- 0.5g

Trace amount of Manganese sulphate and Ferrous sulphate

Distilled water- 1000ml

Agar- 15g

Sterilised in autoclave at 15 lb pressure, 121˚C for 20 mins.

The microbial population was determined by Serial Dilution technique (Waksman. 1922) using Nutrient Agar and Pikovskaya agar media (Phukan. 2012). Dilution plating was done under sterile conditions for determining the microbial population of the collected soil samples. Soil dilution was done by adding 1g soil in 100 ml sterilised distilled water. The microbial population was estimated by culturing 1 ml of the soil dilution in petri plates containing Nutrient agar medium, in triplicate. The phosphate solubilising microbes were estimated by culturing 1 ml of the soil dilution in petri plates containing Pikovskaya medium, in triplicate.

Isolation of Phosphate solubilising microbes (PSM)

The PSM were isolated and maintained at Pikovskaya medium. 10 g soil was added in 100 ml sterilised Pikovskaya media and incubated in the incubator at 28˚C. The inoculated was transferred to another flask containing the same media after seven days. The process was repeated till the fourth transfer. After the fourth transfer dilution plating was done using Pikovskaya agar medium. The PSM showed distinct transparent halo zone in Pikovskaya medium. (Fig. 2)

Seventeen colonies were picked up as phosphate solubilising microbes from the five tea gardens. The isolates were subcultured in Pikovskaya medium using enrichment culture technique. Four active isolates were selected on the basis of phosphate solubilisation on Pikovskaya agar medium and efficacy of viability. The isolates were numbered for further studies.

Phosphatase activities

Phosphatase activities, mainly Alkaline phosphatase [orthophosphoric monoester phosphohydrolase (EC 3.1.3.1)] and Acid phosphatase [orthophosphoric monoester phosphohydrolase (EC 3.1.3.2)] was carried out. It was determined by p-nitrophenol released after incubation with p-nitrophenyl phosphate. The Phosphatase activity is expressed in terms of unit enzyme. One unit of enzyme is expressed as the amount of enzyme required to liberate one µMole nitrophenol per hour. Specific activity of enzyme is expressed as µ mole p-nitrophenol liberated per hour per mg protein. The method used is modification of the original method by Tabatabai and Bremmer (1969), Choudhery (2005) and Phukan (2012).

Acid phosphatase activity

Acid phosphatase activities were studied using the substrate p--Nitrophenyl phosphate in Sodium citrate buffer (pH 4.5). The Acid phosphatase activity of the soil samples was determined by incubating 1g soil with 5mM p-nitrophenyl phosphate solution (pH 4.5).

The Acid phosphatase activity of the isolates was determined using 1 ml of the isolate and 5mM p-nitrophenyl

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phosphate solution (pH 4.5).

A control was prepared by stopping the reaction at zero hour. The reaction was terminated by addition of 2ml of 0.1 N NaOH at the end of the set time. The reading was taken in UV-VIS- spectrophotometer at 425 nm. The reading obtained was compared to standard graph prepared from p-nitrophenol solution made in the same buffer (pH 4.5). The experiments were carried out in triplicate.

Preliminary results indicate that the soil samples showed optimum reading after an incubation period of four hours. Therefore, acid phosphatase of soil was tested after four hours of incubation.

Preliminary results showed that the activity of the isolates was maximums at twenty fours of growth period. Therefore, all the experiments were carried out using twenty four hour growth of the culture.

Alkaline phosphatase activity

The assay of Alkaline phosphatase activity is similar to that of Acid phosphatase activity except the substrate 5 mM p-nitrophenyl phosphate solution was prepared in Tris buffer (pH 9).

Protein estimation

The protein content of the active isolates was estimated after growth period of twenty-four hours. Protein was estimated using Lowry method (1951) (Phukan. 2012) and with appropriate modifications in it. The isolate was washed in distilled water and centrifuged. In the cell suspension 2ml of 10% Sodium dodecyl sulphate was added. The sample was further subjected to cell disruption using sonicator. Different concentrations were prepared from the samples in tubes with distilled water. Sodium dodecyl sulphate and Sodium cholate (0.1 ml of each) were added in the tubes. The tubes were warmed for 30 minutes in the water bath at 60˚C. After cooling 2.5 ml of protein reagent (100ml of 4% Na_2CO_3 prepared in 0.1 N NaOH + 1 ml $CuSO_4$ + 1 ml Na–K tartarate) was mixed to it. The sample was then allowed to stand for 15 minutes. Folin ciocalteau reagent was added in the tubes at the end of the set time and the reading was taken in spectrophotometer at 660 nm. The reading obtained was compared to standard graph prepared using Bovine serum albumin.

Table 2. Acid Phosphatase and Alkaline Phosphatase activity of the soil samples.

TEA GARDENS	Alkaline phosphatase (Units/g of soil)	Acid phosphatase (Units/g of soil)
	Mean ±SD	Mean ±SD
Jalannagar Tea Garden	410.07 ± 12.35	273.29 ± 4.67
Bokel Tea Garden	539.88 ± 20.92	451.62 ± 11.72
Durgapur Tea Garden	465.62 ± 10.41	360.68 ± 7.00
Borborooah Tea Garden	295.79 ± 3.86	411.82 ± 21.53
Moran Tea Garden	308.68 ± 9.17	278.77 ± 11.35

Table 3. The active isolates from the different gardens.

TEA GARDENS	Active Isolates
Bokel Tea Garden	LS-T-F-3
Moran Tea Garden	LS-T-F-5
Jalannagar Tea Garden	LS-T-B-1
Durgapur Tea Garden	LS-T-B-6

Table 4. Protein content of the active isolates.

ACTIVE ISOLATES	PROTEIN CONTENT (µg/ml) Mean ± SD
LS-T-F-3	36.85 ± 0.26
LS-T-F-5	34.05 ± 0.81
LS-T-B-1	37.70 ± 1.19
LS-T-B-6	33.87 ± 0.34

Table 5. Acid phosphatase activity of the active isolates.

ACTIVE ISOLATES	Acid phosphatase	
	Units/ml Mean ±SD	Specific Activity Mean ±SD
LS-T-F-3	346.3 ± 2.26	9397.56 ± 61.33
LS-T-F-5	323.76 ± 4.85	9508.37 ± 142.44
LS-T-B-1	377.8 ± 3.46	10021.22 ± 91.78
LS-T-B-6	337.54 ± 9.54	9965.75 ± 281.67

Table 6. Alkaline phosphatase activity of the active isolates.

ACTIVE ISOLATES	Alkaline phosphatase	
	Units/ml Mean ±SD	Specific Activity Mean ±SD
LS-T-F-3	442.8 ± 12.06	12016.28 ± 327.27
LS-T-F-5	471.74 ± 2.18	13854.33 ± 64.02
LS-T-B-1	392.52 ± 2.77	10411.67 ± 73.47
LS-T-B-6	427.2 ± 9.37	12612.93 ± 276.65

Results and Discussions

Soil health

The soil of the tea gardens in Dibrugarh District is generally acidic in nature. Five different tea gardens were chosen to study the phosphatase activity of soil (Table 2). The Bokel Tea Garden showed the highest alkaline phosphatase activity at 539.88 ± 20.92 units/g of soil while the Borborooah Tea Garden showed the lowest alkaline phosphatase activity at 295.79 ± 3.86 units/g of soil. In case of acid phosphatase activity, the highest activity at 451.62 ± 11.72 units/g of soil were seen in the Bokel Tea Garden whereas the lowest acidic phosphatase activity was recorded in the Jalannagar Tea Garden at 273.29 ± 4.67 units/g of soil. The soil health was studied using phosphatase activity and the result showed that both the acid and the alkaline phosphatase activities of the Bokel Tea Garden were very high.

The soil fertility can be related with the phosphatase activity of the soil. Grierson and Adams (2000) observed that acid phosphatase activity of Jarrah (Eucalyptus marginata Donn ex Sm) forest soils ranged from 30 to 40 µ mol/g/

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hr. In soil with pH range 3.8 – 11 the phosphatase activity was estimated to be in the range of 0.05 – 14 µ mol/g/hr (Nannipieri et al. 2011). It was seen that some agroforestry species (Tithonia diversifolia, Tephrosia vogelii and Crotalaria grahmiana) stimulated acid phosphatase activity of rhizosphere soil, whereas maize stimulated alkaline phosphatase activity of rhizosphere soil (George et al. 2002). Banerjee et al (2012) reported that the maximum unit of activity was 6.393 units (where one unit of enzyme activity was described as the degradation of one mM substrate in the standard assay conditions).

Under fertiliser treated tea garden soil in China, Lin (2013) reported the acidic phosphatase activity to be in the range of 0.79 ± 0.04 to 1.36 ± 0.08 µmol/g/h. The alkaline activity of the tea garden soil of Dibrugarh was estimated to be 16.20 ± 0.031 µg/ml/hr (Nath & Samanta, 2012). Our observations are in accordance with the earlier observations made by Venkatesan and Senthurpandian (2006) where they found that the tea garden soils in South India had phosphatase activity to be 523-823 µg/g/hr and alkaline phosphatase activity to be 403-505 µg/g/hr.

In the studies conducted by Venkatesan and Senthurpandian, the acid phosphatase activity was found to be higher than the alkaline phosphatase activity. However, in the current study, alkaline phosphatase activity was found to be higher than acid phosphatase activity. The phosphatase activity obtained in the present study was higher compared to the works of other researchers.

The Phosphate Solubilising Microbes (active isolates)
Though several bacteria and fungi showed clear zone around the colonies, only seventeen colonies were picked up as phosphate solubilising microbes on the basis of clearing zone. The Isolates were labelled as LS-T-B-1, LS-T-B-2, LS-T-F-1, LS-T-F-2, LS-T-B-3, (from Jalannagar Tea Garden) LS-T-F-3, LS-T-B-4, LS-T-B-5, (from Bokel Tea Garden), LS-T-F-4,LS-T-B-6,LS-T-B-7,LS-T-B-8 (from Durgapur Tea Garden), LS-T-B-9, LS-T-B-10, LS-T-F-5 (from Moran Tea Garden) and LS-T-F-6, LS-T-B-11 (from Borborooah Tea Garden).

The isolates were subcultured in Pikovskaya medium using enrichment culture technique. Two active fungal culture (namely LS-T-F-3, LS-T-F-5) and two active bacterial culture (namely LS-T-B-1, LS-T-B-6) were taken for further study on the basis of solubilisation zone and efficacy of viability during enrichment culture (Table 2). The active isolates were repurified in Pikovskaya agar medium. The cultures were subcultured in both liquid and agar Pikovskaya medium for further studies.

The growth of four active isolates as studied by the increase in protein content showed range 33.87 ± 0.34 - 37.70 ± 1.19 µg/ml after twenty four hours (Table 4). The same cultures were used to study both acid and alkaline phosphatase activities.

Tea gardens harboured wide range of microbes. Species of Trichoderma, Aspergillus, Penecillium and Mucor were reported to be found in the rhizosphere of the tea bushes of different regions of the Indian Himalayas (Singh et al.2007). Members of Rhizobium, Burkholderia and Enterobacter were isolated from the tea gardens of Silchar (Huidrom et al. 2011). Rhizospheric soil from tea bushes of Darjeeling hills was screened for the presence of phosphate solubilising bacterial populations on Pikovskaya agar. One of the potent strains was identified as Kurthia sp (Sharma et al.2012). Three rhizobacteria Bacillus amyloliquefaciens, Serratia marcescens and Bacillus pumilus were isolated from the rhizospheric soil of tea gardens that showed phosphate solubilisation in Pikovskaya Media (Chakraborty et al.2013). After screening of fourteen isolates MM PSM 10 (Aspergillus niger Code 1228.07NCFT) was found to have phosphate solubilizing activity as evidenced by measuring the P -solubilization zone in solid Pikovskaya's medium (Phukan et al. 2011).

From the works carried out by different researchers it can be seen members of both bacteria and fungi showed phosphate solubilisation.

Phosphatase activity of the active isolates.
The four active isolates namely LS-T-B-1, LS-T-B-6, LS-T-F-3 and LS-T-F-5 were used to study their phosphatase activity under cultural conditions. Both acid and alkaline phosphatase activities were measured. All the four microbes showed both alkaline and acidic phosphatase activity. In case of acid phosphatase, (Table 5) showed that the isolate LS-T-B-1 showed the highest specific activity 10,021.22 ± 91.78. Isolate LS-T-F-5 showed lowest specific activity 9,397.56 ± 61.33 of acid phosphatase. In case of alkaline phosphatase, (Table 6) showed the isolate LS-T-F-5 showed the highest specific activity 13,854.33 ± 64.02. While isolate LS-T-B-1 showed lowest specific activity 10,411.67 ± 73.47 of alkaline phosphatase.

Specific activity expressed in terms of mmole/hr/mg protein the acid phosphatase activity was at the range of 9.398 ± 0.061- 10.021 ± 0.092 and the alkaline activity was at the range of 10.412 ± 0.073 - 13.854 ± 0.064.

The acidic phosphatase activity was higher in bacterial cultures compared to the fungal cultures. It was noticed that alkaline phosphatase activity was higher in the fungal cultures compared to the bacterial cultures.

Amount of available phosphate was determined as 40.62 ± 1.1 mg/l in Kurthia sp (Sharma et al.2012). The different strains of PSB were screened and phosphatase activity was obtained at a range of 18.33-28.50 mmol/ml/hr (Balamurugan et al. 2010). The acid phosphatase specific activity of MM PSM 10 (Aspergillus niger Code 1228.07NCFT) was estimated to be 3.21 ± 0.001 mM/hr/mg while the alkaline phosphatase specific activity was estimated to be 5.24 ± 0.055 mM/hr/mg (Phukan et al.2011). In the present study the specific activity of alkaline phosphatase is 10.412 ± 0.073 - 13.854 ± 0.064 which is higher than the results obtained in other studies.

It was reported that the phosphatase activity was higher in the bacteria compared to the fungi. In the phosphatase activity carried out with the active isolates, the fungi showed higher alkaline phosphatase activity and the bacteria showed higher acid phosphatase activity.

This shows that the shade trees harbour PSM with high phosphatase activities and these microbes have the potential to be used as bio fertilizers.

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Fig.2 PSM in Pikovskaya media.

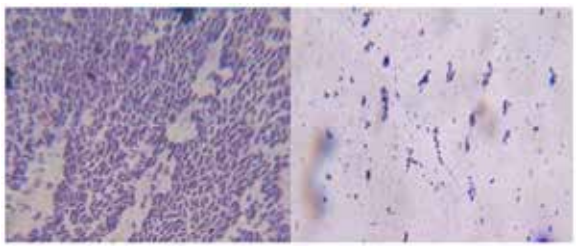

Fig 2.1 Gram positive rods

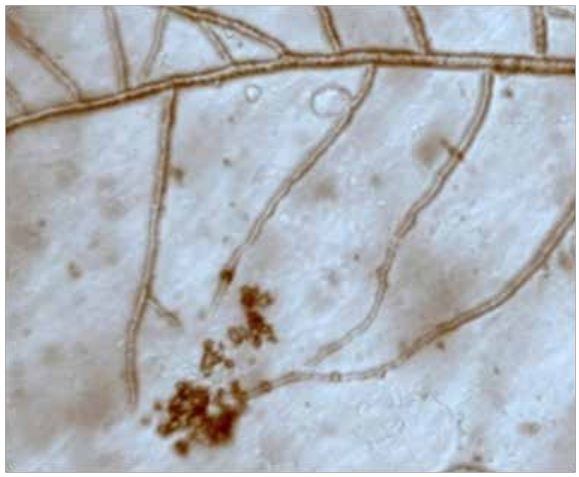

Fig 2.2 Fungal hyphae with spores

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