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Effect of Substrate Concentration and Derivation of Michaelis-Menten Constants of Acid Phosphatase in Alfisols of Andhra Pradesh

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Abstract

Acid phosphatases belong to the group of enzymes, phosphomonoesterases, which play a major role in the mineralization of soil organic P. Substrate concentration of enzyme is one of the factors that affect the enzyme kinetics. To study the role of substrate concentration on soil enzyme activity in alfisols, twenty-five surface soil samples were collected and assayed for the activity of soil acid phosphatase. The acid phosphatase activity as expressed in terms of µg of 4-nitrophenol released g^{-1} soil h^{-1} in these soils varied from 16.9 to 268.4 with a mean value of 58.8 in surface soils. Among them, ten soil samples were selected to study enzyme kinetics with thirteen substrate concentrations under laboratory incubation studies with three replications. Soil acid phosphatase increased with an increase in substrate concentration up to 20mM and almost reached a plateau at a substrate concentration of more than 15mM. The enzyme activity remained almost constant in all the ten alfisols with further increase in substrate concentration, minimal change in enzyme activity was observed. Characteristics of enzyme activities like maximum enzyme reaction velocity (Vmax) and Michaelis constant (Km) were determined using the Michaelis-Menten equation similar to those determined in a homogenous system. The maximum reaction velocity of soil acid phosphatase for soils under study were calculated (µg of 4-nitrophenol g^{-1} soil h^{-1}) and varied from 30.0 to 434.8 and the soils S6, S8, S9 and S10 recorded higher values using Line weaver-Burk plot. The values compared well with Hanes–Wolf transformation 25.5 to 364.7 and the soils S6, S8, S7 and S9 recorded higher values. Under Eadie - Hofstee transformation the values varied from 27.7 to 390.3 and higher values were recorded by S6, S8, S9 and S10. Michaelis constant (Km) of the soil acid phosphatase was calculated using Line weaver-Burk transformation plot, varied from 4.9 to 9.6 mM and the higher values were recorded by S6, S8, S1 and S10. The values compared well with those obtained from Hanes-Wolf 2.6 to 6.8 and soils S6, S8, S4 and S7 recorded higher values. In Eadie-Hofstee plots the values ranged from 3.7 to 7.8 and higher values were found in the soils S6, S8, S10 and S4. It was concluded that the linear transformation of Line-weaver Burk values was superior for estimating Km and Vmax values of enzymes in soil. However significant differences were noticed when Km and Vmax were calculated from the linear transformation of Michaelis plot.

Keywords : Alfisols, Acid phosphatase, Eadie - Hofstee transformation, Hanes - Wolf transformation, Line weaver - Burk transformation, Michaelis-Menten equation, Substrate concentration

Acid phosphatases are the enzymes that belong to the group of phosphomonoesterases that play an important role in the mineralization of soil organic P (Tabatabai and Bremner, 1969 and Eivazi & Tabatabai, 1977). Kinetic parameters Km (Michaelis - Menten) and Vmax (maximum reaction velocity often used to characterize the enzymes, as they are considered to be constant for a specific enzyme under defined experimental conditions (Marx *et al*., 2005), but they may vary independently.

Theories and mathematical analysis of enzyme reactions are based on the concept that an enzyme acts by forming a complex or compound with substrate

presumably the complex of enzyme and substrate is unstable and proceeds through one or more steps or re-arrangement to form the product plus the original enzyme. This theory of enzymes was proposed by Michaelis and Menten and may be expressed by the following equation:

$$S + E \underset{K2}{\overset{K1}{\Leftrightarrow}} ES \xrightarrow{K3} E + P \ldots$$

Where S is the substrate, E is the enzyme, ES is the intermediate enzyme-substrate complex, P is the product of the reaction and K1, K2 and K3 are the respective reaction velocity constants of the three processes.

It can be shown that with the soluble substrate in excess, the rate of reaction, that is, the decrease in concentration of the substrate with time or the increase in concentration of the product is given by:

$$\frac{-ds}{dt} = \frac{\phi}{dt} = K3[ES] = \frac{k3\ [E]\ [S]}{Km + [S]} = \frac{Vmax\ [S]}{Km + [S]}$$

Where S and ES are the concentration of substrate and enzyme-substrate complex respectively, Km is Michael is constant.

$$Km = \frac{K2 + K3}{K1}$$

$$Vmax = K3\ E$$

The three linear transformations that commonly used are:

$$\frac{1}{V} = \frac{1}{Vmax} + \frac{Km}{Vmax} \cdot \frac{1}{[S]} \quad \textit{Lineweaver - Burk transformation}$$

$$\frac{[S]}{V} = \frac{Km}{Vmax} + \frac{1}{Vmax}\ [S] \quad \textit{Hanes - Wolf transformation}$$

$$V = Vmax - Km \cdot \frac{V}{[S]} \quad \textit{Eadie - Hofstee transformation}$$

Plots of the variables of such relationships normally give straight lines. The value of the slope and intercept are commonly used for the determination of the constants from a set of experimental data. Once the Km and Vmax are known for a particular enzymatic reaction under a given set of conditions, the reaction velocity, V can be calculated for any substrate concentration. The Michaelis constant is by far the most fundamental in enzyme chemistry. It has the dimensions of concentration (that is, moles per liter) and it is a constant for the enzyme only under rigidly specified conditions. The Km value is useful in estimating the substrate concentration necessary to give a maximum velocity. Although the literature on soil enzymes is on the rise reports on kinetic constants like Michaelis constant and Vmax and their correlations with soil properties are inadequate values for both Km and Vmax as they vary with the type of soil and also its physical fractions. The, values are also influenced by assay conditions like choice of substrate and buffer, use of shaken or unshaken soil suspensions.

Material and Methods

To study the effect of substrate concentration on soil enzyme acid phosphatase, twenty-five surface soil samples belonging to Alfisols were collected and assayed for their activity. The acid phosphatase activity as expressed in terms of µg of 4-nitrophenol released g^{-1} soil h^{-1} in these soils varied from 16.9 to 268.4 with a mean value of 58.8 in surface soils. Among them, ten (10) were selected to study enzyme kinetics with thirteen (13) substrate concentrations under laboratory incubation studies with three replications. The procedure of Tabatabai and Bremner (1969) was adopted for the assay of acid phosphatase.

Modified Universal Buffer (MUB) Stock : The stock of MUB was prepared by mixing 12.1g of Tris (hydroxy methyl) amino methane (THAM), 11.6 g of maleic acid, 14 g of citric acid and 6.3 g of boric acid in 488 ml of 1N sodium hydroxide and the solution was diluted to 1 litre with distilled water.

Modified Universal Buffer (pH 6.5) : 200 ml of MUB stock was transferred to 1 litre beaker and kept on a magnetic stirrer and the pH of the solution was

adjusted to pH 11.0 with 0.1N HCl and volume was made up to 1 litre with distilled water. The MUB buffer was wrapped with carbon paper and stored in a refrigerator.

P-nitro Phenyl Phosphate Solution (0.025M) : This was prepared by dissolving 0.420g of disodium salt of p-nitrophenyl phosphate in 40ml of MUB pH 6.5 (for assay of acid phosphatase) and the solution was diluted to 50 ml with MUB of the same pH. The solution was wrapped with carbon paper and stored in a refrigerator.

Calcium Chloride (0.5M) : This was prepared by dissolving 73.5g of $CaCl_2 2H_2O$ in distilled water and made up to 1 litre.

Sodium Hydroxide (0.5M) : 20 g of sodium hydroxide was dissolved in 700 ml of distilled water and diluted to1litre with water.

Standard P-nitro Phenol Solution : Primary stock solution of 1000 $\mu g ml^{-1}$ of p-nitrophenol was prepared by dissolving 1g of p-nitrophenol in distilled water and made up to 1 litre. From this, secondary stock of 100 $\mu g\ ml^{-1}$ and 20 $\mu g\ ml^{-1}$ solutions were prepared. Working standards of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 $\mu g\ ml^{-1}$ were prepared from 20 $\mu g\ ml^{-1}$ stock and the absorbance of these standards were recorded at 420 nm in spectrophotometer. This was used for the standard curve.

Procedure : To 1 g of soil sample taken in glass tubes, 4 ml of modified universal buffer pH 6.5 was added followed by the addition of 1ml of 4-nitrophenyl phosphate solution. Different concentrations *viz*., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 100 mM of substrate solution *i.e.,* 4-nitrophenyl phosphate were prepared separately and 1ml solution was added to each glass tube in triplicates. The final concentrations of substrate in the incubation mixtures were 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15 and 20mM. After 2 hours of incubation at 37°C, the glass tubes were removed and 1 ml 0.5M $CaCl_2$ and 4 ml 0.5 /M NaOH was added to each tube. The acid phosphatase activity was determined by estimating the 4-nitrophenol released. The glass tubes were swirled and the soil suspension was filtered through What man No. 42 filter paper. The absorbance of yellow color of 4-nitro phenol liberated due to hydrolysis of the substrate by phosphomonoesterases (acid phosphatase) was measured at 420 nm. Controls were run simultaneously following the same procedure except adding 1 ml of 4-nitrophenyl phosphate after the addition of 1ml of 0.5M $CaCl_2$ and 4 ml of 0.5M NaOH. Corrections were made for control/ blank values.

Results and Discussion

The effect of substrate concentration on acid phosphatase activity is depicted in Fig. 1. Increase in substrate concentration increased soil acid phosphatase activity upto 20 mM in all the soils and almost reached a plateau at a substrate concentration of more than 15 mM and at higher concentrations the enzyme activity remained almost constant. Tabatabai and Bremner (1971), Srinivas & Raman (2008) and Vandana (2012) also obtained similar results for phosphomonoesterases. Plots of three linear transformations of Michaelis-Menten' sequation

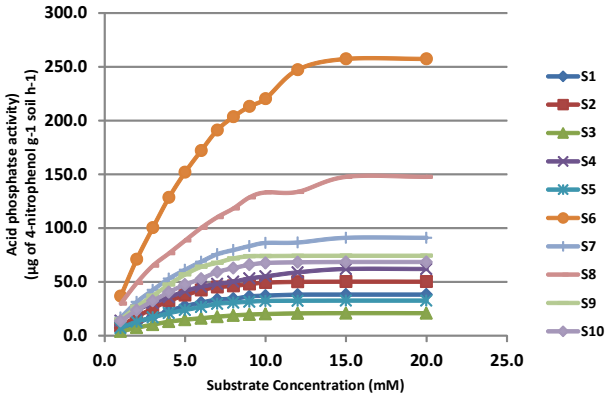

Fig. 1 : Effect of substrate concentration on acid phophatase activity in alfisols

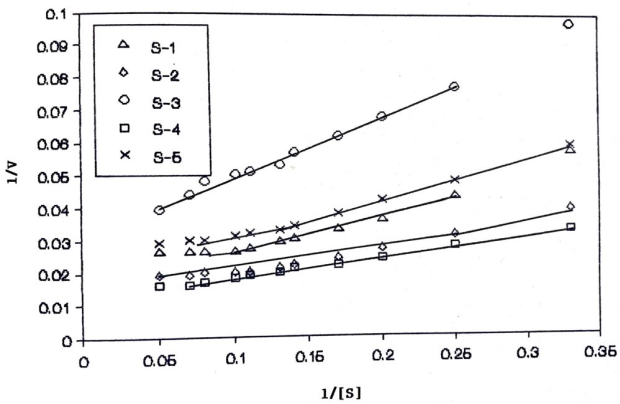

Fig. 2 : Line weaver burk plot of soil acid phosphatase activity in alfiols (1 to 5)

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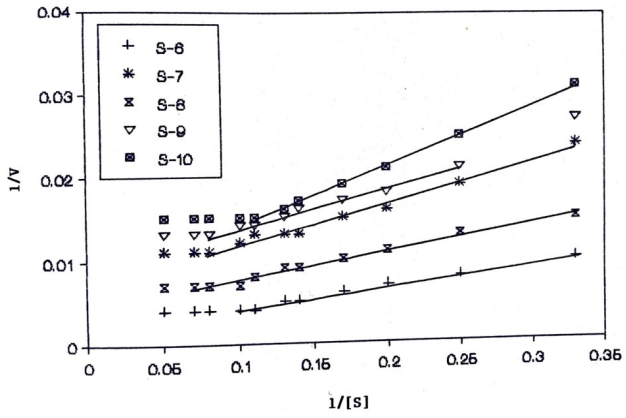

Fig. 3: Line Weaver-Burk plot of soil acid phosphatase activity in alfiosls (6 to 10)

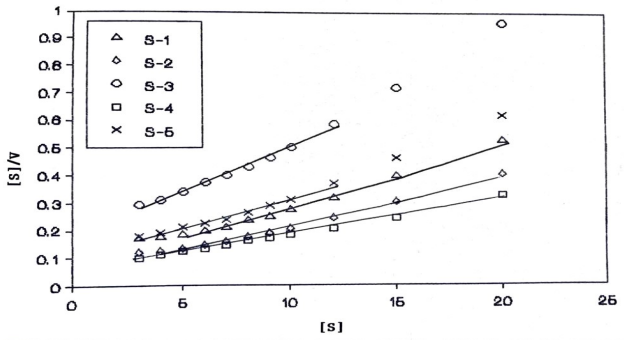

Fig. 4: HANES-WOLF of plot of soil acid phosphatase activity in alfisols (1 to 5)

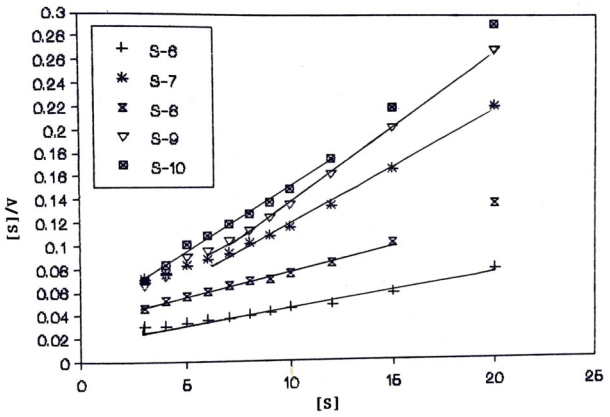

Fig. 5: HANES-WOLF of plot of soil acid phosphatase activity in alfiosols (6 to 10)

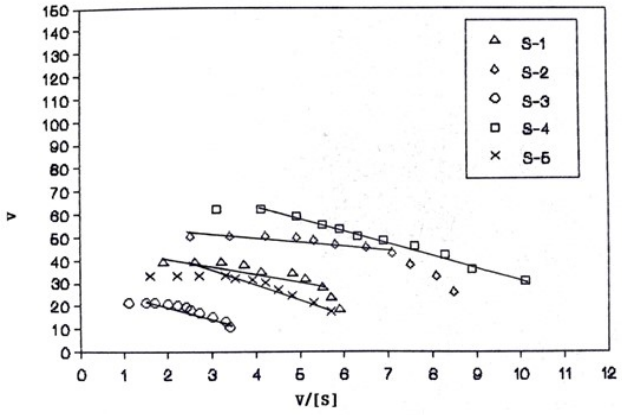

Fig. 6: EADIE-HOFTSEE of plot of soil acid phosphatase activity in alfiosls (1 to 5)

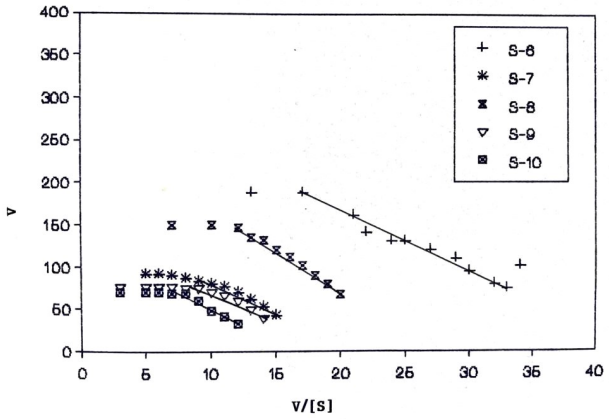

Fig. 7: EADIE-HOFTSEE of plot of soil acid phosphatase activity in alfiols (6 to 10)

drawn for acid phosphatase are shown in Fig. 2 to 7 in vertisols. The linearity of these plots indicated that there was a good fit to the Michaelis-Menton equation for acid phosphatase in vertisols.

The maximum reaction velocity of soil acid phosphatase (Table 1) for soils under study was calculated (µg of 4-nitrophenol g^{-1} soil h^{-1}) and varied from 30.0 to 43.4.8 and the soils S6, S8, S9 and S10 recorded higher values using Line Weaver Burk plot. The values compared well with Hanes-Wolf transformation 25.5 to 364.7 and the soils S6, S8, S7 and S9 recorded higher values. Under Eadie - Hofstee transformation the values varied from 27.7 to 390.3 and higher values were recorded by S6, S8, S9 and S10. Similar results were also reported by Rao (1989) and Tabatabai & Bremner (1971).

TABLE 1

Maximum enzyme reaction velocity (Vmax) and Michaelis Constant (Km) values of soil acid phosphatase activity

Soils	Maximum enzyme reaction velocity (Vmax) (µg of 4-nitrophenol g^{-1} soil h^{-1})			Michaelis constant (Km) (mM)		
	Lineweaver - Burk Transformation	Hanes - Wolf Trans-formation	Eadie - Hofstee Transformation	Line Weaver Burk Transfor-mation	Hanes - Wolf Transformation	Eadie - Hofstee Transfor-mation
S1	60.0	47.4	51.6	5.0	2.7	3.7
S2	72.1	59.6	64.8	5.4	3.4	4.4
S3	30.0	25.5	27.7	5.1	4.4	4.9
S4	82.7	76.0	81.0	4.9	2.8	3.8
S5	46.7	38.0	42.6	9.6	6.8	7.8
S6	434.8	364.7	390.3	6.3	4.0	5.1
S7	13.7	114.8	124.8	7.4	5.8	6.8
S8	224.7	201.2	215.1	5.4	2.6	3.6
S9	111.9	88.2	96.7	6.5	3.7	4.9
S10	106.9	86.1	94.7	6.2	4.0	5.0
Mean	118.3	110.2	118.9	5.0	2.7	3.7

Michaelis constant (Km) of the soil acid phosphatase (Table 1) was calculated using Line Weaver Burk transformation plot, varied from 4.9 to 9.6 mM and the higher values were recorded by S6, S8, S1 and S10. The values compared well with those obtained from Hanes-Wolf (2.6 to 6.8) and soils S6, S8, S4 and S7 recorded higher values. In Eadie-Hofstee plots the values ranged from (3.7 to 7.8) and higher values were found in the soils S6, S8, S10 and S4. Irving and Cosgrove (1976) concluded that the linear transformation of Eadie-Hofstee values were found to be superior but the work shown by others (Dick & Tabatabai, 1978; Tabatabai & Singh, 1979 and Rao, 1989 & Vandana, 2012) shows that the three linear transformations are equally applicable for estimation of Km and Vmax values of enzymes in soil. Further, the kinetic parameters, beyond simple measurements of enzyme activity, is important for understanding enzyme mechanisms, enzyme roles in specific soil conditions, enzyme responses to changes in metabolite concentration and also enzyme control mechanisms (Marinari *et al.,* 2008 and Henry, 2012).

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