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Effect of substrate concentration on soil enzyme acid phosphatase and linear transformation of michaelis menton plot

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Abstract
Substrate concentration of enzyme is one of the factor which effect the enzymes kinetics, to study the role of substrate concentration on soil enzyme Acid phosphatase in selected soils. Forty soil samples were collected and assayed for the activity of soil acid phosphatase and four soil samples, two Alfisols and two Vertisols soils with high activity were selected for further study. The activity of acid phosphatase as expressed in terms of µg of 4-nitrophenol released g^{-1} soil h^{-1} ranged from 18.93 to 51.2 with an average value of 31.79 activities of the surface soils Soil acid phosphatase increased with increase in substrate concentration upto 30mM and almost reached a plateau at a substrate concentration of more than 30mM at higher concentrations the enzyme activity remained almost constant in all the four soils. With further increase in substrate concentration, minimal change in enzyme activity was observed. Characteristics of enzyme activities like maximum enzyme reaction velocity (V_{max}) and Michaelis constant (K_m) were determined using Michaelis – Menten equation similar to those determined in 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 40.0 to 56.6 and followed the sequence VS I >AS II > AS I >VS II using Lineweaver – Burk plot. The values compared well with Hanes – Wolf transformation 46.0 to 56.0 and followed the sequence VS I >VSII > AS II >AS I under and Eadie – Hofstee transformation the values varied from 44.0 to 54.4 and followed the order VS I >VS II >AS II >ASI. Michaelis constant (K_m) of the soil acid phosphatase calculated using Lineweaver – Burk transformation plot varied from 0.44mM to 0.60mM and followed the order is VS I >AS II >AS I >VS II. The values compared well with those obtained from Hanes – Wolf (0.63 to 0.86) and followed the order is VS II >ASI >VS I >AS II. In Eadie – Hofstee plots the values ranged from (0.51 to 0.65) and the order followed is VS I >AS II >AS I >VS II.

Keywords: Alfisols, Eadie - Hofstee Transformation, Hanes - Wolf Transformation, Lineweaver - Burk Transformation, Michaelis–Menten equation, Substrate concentration, Acid phosphatase activity and Vertisols

1. Introduction
The enzymes play a very important role in the mineralization of nitrogen, phosphorus and sulphur. Acid phosphatases is the enzyme which play a very important role in the mineralization of phosphorus. Phosphatases have been used to describe a large group of enzymes that catalyzes the hydrolysis of esters and anhydrides of H_3PO_4 (Tabatabai, 1994) [24]. This enzyme plays a major role in the mineralization of soil organic P. There are five groups of phosphatases phosphoric monoester hydrolases, phosphoric diester hydrolases, triphosphoric monoester hydrolases, phosphoamidase polyphosphatases (Tabatabai and Bremner, 1969 [20]., Eivazi and Tabatabai, 1977 [7], Browman and Tabatabai, 1978) [1]. The phosphomonoesterases are classified as acid and alkaline phosphatases, because they show optimum activities in acid and alkaline ranges, respectively. They play a critical role in P cycle and are correlated to P stress and plant growth. When there is a signal indicating P deficiency in soil, acid phosphatase secretion from the plant root is increased to enhance the solubilisation and remobilization of phosphatidic acid and thus influencing the ability of the plant to cope up with P stressed conditions. Michaelis - Menten constants of soil acid phosphatase have been reported by several workers (Tabatabai and Bremner, 1971 [22], Cervelli *et al.*, 1973 [2] and Thornton and McLaren, 1975). The apparent K_m values of acid phosphatase in soil range from 1.3 to 4.5 mM The K_m values obtained for phosphomonoesterases are affected by shaking the soil substrate mixture using incubation, normally the values are more uniform among soils when shaking than when static incubation technique is employed (Eivazi and Tabatabai, 1977)

[7]. By changing the buffer system and pH used by Tabatabai and Bremner, (1969) [20] in assay of phosphatase activity in soils and Cervelli *et al*., (1973) [25] showed that the substrate 1-nitrophenyl phosphate is absorbed by soils in the presence of 0.5 M NaOAc buffer pH 4.7. They used adsorption parameters derived from the Freundlich Isotherm to determine concentration of the free substrate and to calculate the K_m values of acid phosphatase. Irving and Cosgrove, (1976) [9] examined the graphical techniques used in calculations of the K_m values of acid phosphatase and concluded that the linear transformation of Eadie – Hofstee is superior. Presumably this graph shows the greatest deviation from the classical Michaelis – Menten equation but recent work shows that the three linear transformations are equally applicable for estimation of the apparent K_m values of enzymes in soils (Dick and Tabatabai, 1978 [4] and Tabatabai and Singh, 1979 [21]). Each transformation gives different weightage to errors in the variables (Dowd and Riggs, 1965) [5] and this is reflected in the variation of estimated K_m and V_{max} values derived for any soil enzyme by using different plots Kinetic parameters (Vmax and Km) are often used to characterize enzymes, they are considered to be constant for a specific enzyme under defined experimental conditions (Marx *et al*., 2005) [11], but they may vary independently. Maximum reaction velocity (Vmax) of an enzyme catalyzed reaction imply splitting velocity or rate of dispersion of enzyme-substrate complex into enzyme and reaction products, which reflects the conjunction affinity between enzyme and substrate. The higher or lower Vmax value can be used as an indicator to speedy or slow enzymatic process. Vmax and Km of an enzyme express the quantity of an enzyme and substrate affinity, respectively (Marx *et al*., 2005 [11]; Davidson *et al*., 2006 [3]) Enzymes catalyzing the same reaction, but derived from different sources of soil have different Km values (Nannipieri *et al*., 1990) [2].

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 enzyme was proposed by Michaelis and Menten and may be expressed by the following equation:

$$S + E \underset{K2}{\overset{K1}{\Leftrightarrows}} ES \overset{K3}{\rightarrow} E + P \ldots (1)$$

Where S is the substrate, E is the enzyme, ES is the intermediate enzyme-substrate complex, P is the product of the reaction and K_1, K_2 and K_3 are the respective reaction velocity constants or rate constant 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:

$$-\frac{ds}{dt} = \frac{dp}{dt} = k_3[ES] = \frac{k_3[E][S]}{K_m + [S]} = \frac{V_{max}[S]}{K_m + [S]}$$

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

$$K_m = \frac{K_2 + K_3}{K_1}$$

$$V_{max} = K_3 E$$

The three linear transformations that commonly used are:

$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]}$ Lineweaver-Burk transformation

$\frac{[S]}{V} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} . [S]$ Hanes-Wolf transformation

$V = V_{max} - K_m \cdot \frac{V}{[S]}$ 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 determination of the constants from a set of experimental data. Once the K_m and V_{max} 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 constant 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 K_m value is useful in estimating the substrate concentration necessary to give a maximum velocity.

Kinetic parameters (V_{max} and K_m) are often used to characterize free enzymes in solution, they are considered to be constant for a specific enzyme under defined experimental conditions (Marx *et al*., 2005) [11], but they may vary independently. Maximum reaction velocity (V_{max}) of an enzyme catalyzed reaction simply splitting velocity or rate of dispersion of enzyme-substrate complex into enzyme and reaction products, which reflects the conjunction affinity between enzyme and substrate. The higher or lower V_{max} value can be used as an indicator to a speedy or slow enzymatic process. V_{max} and K_m of an enzyme express the quantity of an enzyme and substrate affinity, respectively (Marx *et al*., 2005) [11]. However, Michaelis constant (K_m) represents the endurance of an enzyme-substrate complex, which is related with the substrate. The efficiency of the enzymes to decompose substrate at low concentration is directly related to their K_m value (Marx *et al*., 2005) [11]. Higher is the endurance of an enzyme-substrate complex, lower will be the K_m value. Enzymes catalyzing the same reaction, but derived from different sources of soil have different K_m values (Nannipieri *et al*., 1990) [2]. Besides, K_m is independent of enzyme concentration and kinetically reflects the apparent affinity of enzyme for the substrate. In other words, smaller the K_m value, the greater will be the affinity for the substrate (Masciandaro *et al*., 2000) [12]. However, estimating K_m is challenging due to the uncertainty regarding the relative contribution of artificial and naturally occurring substrate under non saturating conditions (Stone *et al*., 2011) [19]. Moreover, enzymes may operate under non-saturating conditions in soil, which supplements K_m an important parameter that merits increased attention (Davidson *et al*., 2006 [3] and German *et al*., 2011) [8]. If substrate concentration is similar to K_m, the measure of affinity for substrate/enzyme can provide information about the adsorption level or enzyme accessibility. Besides, K_m influences enzyme activity at low substrate concentration (Davidson and Janssens, 2006 and Davidson *et al*., 2006) [3]. Many investigations have dealt with

the kinetic properties of enzymes (Masciandaro *et al*., 2000, Zhang *et al*., 2009 and 2010, Juan *et al.*, 2010) [12, 28, 29, 10].

Although, the literature on soil enzyme is on the increase, reports on kinetic constants like Michaelis constant and V_{max} and their correlations with soil properties are limited. Values for both K_m and V_{max} vary with the type of soil and also its physical fractions. Then, values are also influenced by assay conditions like choice of substrate and buffer, use of shaken or unshaken soil suspensions.

When the Michaelis–Menten model is applied to ecological systems, V_{max} and K_m no longer reflect the biochemical attributes defined in its original context. In such cases, these parameters are more accurately described as apparent V_{max} ($AppV_{max}$) and apparent K_m ($AppK_m$) with $AppV_{max}$, a relative measure of enzyme abundance, and $AppK_m$, a relative measure of substrate (Wallenstein *et al*., 2011) [27].

K_m values may also fluctuate, depending on whether it is in the free or in an absorbed state (McLaren and Packer, 1970) [13]. While investigating the enzyme splitting of urea in the presence of bentonite, (Durand, 1966) [6], obtained higher K_m values for adsorbed than for free enzyme. K_m values also varied with pH of assay, being lowest at the pH optimum. In general K_m for soil enzymes are greater than that for the corresponding pure enzymes. Paulson and Kurtz, (1970) [15], indicating a much lower apparent affinity of the adsorbed enzyme for the substrate compared to that of the native enzyme. Shaking of soil suspension during assay decreased K_m values and increased V_{max} values for soil urease (Tabatabai, 1973) [23].

Materials and Methods

The procedure of Tabatabai and Bremner (1969) [20] and Eivazi and Tabatabai (1977) [7] were adopted for the assay of acid and alkaline phosphatases respectively. Modified Universal Buffer (MUB) Stock: The stock of MUB was prepared by mixing 12.1 g of Tris (hydroxymethyl) aminomethane (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 6.5 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-nitrophenyl phosphate solution (0.025M): This was prepared by dissolving 0.420 g of disodium salt of p-nitrophenyl phosphate in 40ml of MUB pH 6.5 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 to 1 litre with water. Standard p-nitrophenol solution: Primary stock solution of 1000 µg ml^{-1} of p-nitrophenol was prepared by dissolving 1 g of p-nitrophenol in distilled water and made up to 1 litre. From this, secondary stock of 100 µg ml^{-1} and 20 µg ml^{-1} solutions were prepared. Working standards of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg ml^{-1} were prepared from 20 µg ml^{-1} stock and the absorbance of these standards were recorded at 420nm in spectrophotometer. This was used for the standard curve. To 1 g of soil sample taken in glass tubes, 4 ml of modified universal buffer pH 6.5 was added followed by addition of 1 ml of 4-nitrophenyl phosphate solution. Different concentrations *viz*. 2, 4, 6, 8, 10, 20, 40, 60, 80 and 100 mM of substrate solution i.e. 4 – nitrophenyl phosphate were prepared separately and 1 ml solution was added to each glass tube in triplicates. The final concentrations of substrate in the incubation mixtures were 1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 mM. After 2 hours of incubation at 37ºC, the glass tubes were removed and 1 ml 0.5 M $CaCl_2$ 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 Whatman No. 42 filter paper. The absorbance of yellow color of 4-nitrophenol liberated due to hydrolysis of the substrate by phosphomonoesterases 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 1 ml of 0.5M $CaCl_2$ and 4 ml of 0.5M NaOH. Corrections were made for control / blank values.

Results and Discussions

The effect of substrate concentration on acid phosphatase activity is presented in (Table 1) and is depicted in (Figure 1). Increase in substrate concentration increased soil acid phosphatase activity upto 30mM in all the soils and almost reached a plateau at a substrate concentration of more than 30mM at higher concentrations the enzyme activity remained almost constant. Eivazi and Tabatabai, (1977) [7]., Tabatabai and Bremner, (1971) [22]., Srinivas and Raman, (2008) [18] and Vandana, (2012) [26] also obtained similar results for phosphomonoesterases. Plots of three linear transformations of Michaelis-Menten's equation for acid phosphatase are shown in (Figure. 2, 3 and 4).

Table 1: Effect of substrate concentration on soil acid phosphatase activity

Substrate concentration (mM)	Acid phosphatase activity (µg of 4-nitrophenol released g^{-1} soil h^{-1})			
	VS1	VS2	AS1	AS2
1.0	25.6	27.6	29.6	32.5
2.0	28.2	30.1	32.7	35.0
3.0	30.4	32.3	35.5	38.2
4.0	32.9	33.7	37.9	40.1
5.0	34.6	35.1	41.5	43.9
10.0	36.1	37.3	44.9	47.9
20.0	38.0	39.5	47.2	50.1
30.0	38.7	40.3	48.0	52.4
40.0	38.9	40.5	48.2	53.6
50.0	38.9	40.5	48.2	53.6

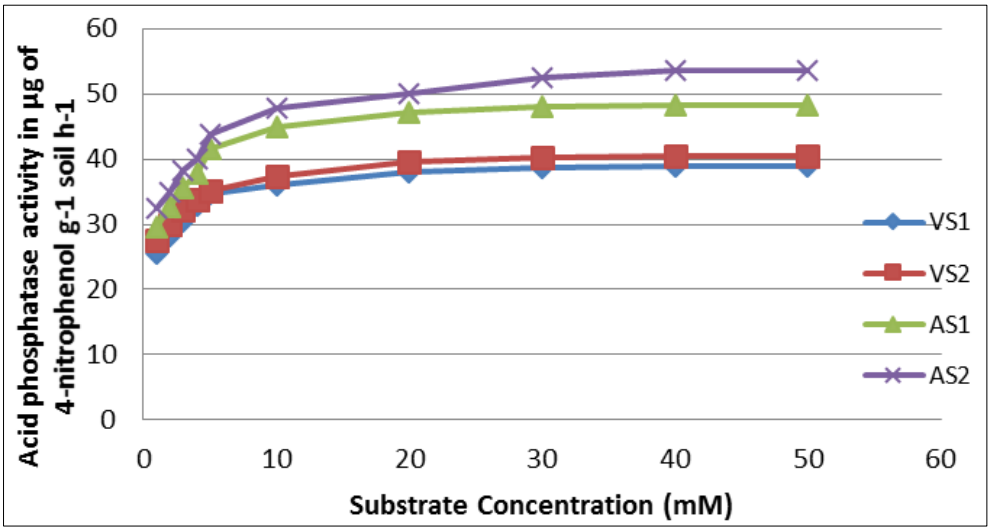

Fig 1: Effect of substrate concentration on soil acid phosphatase activity

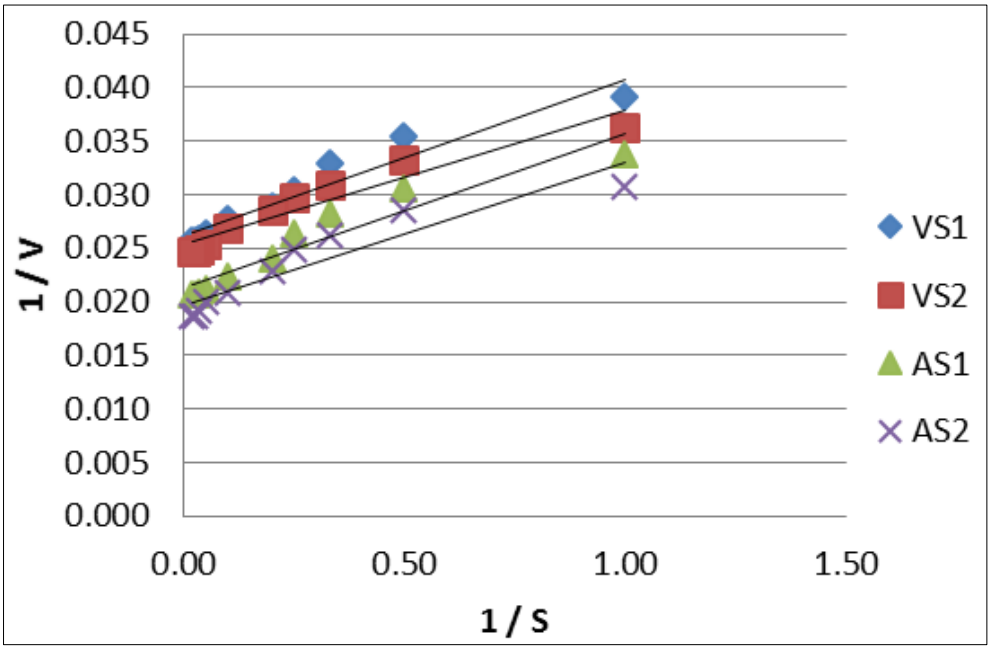

Fig 2: Lineweaver - Burk plot of soil acid phosphatase activity

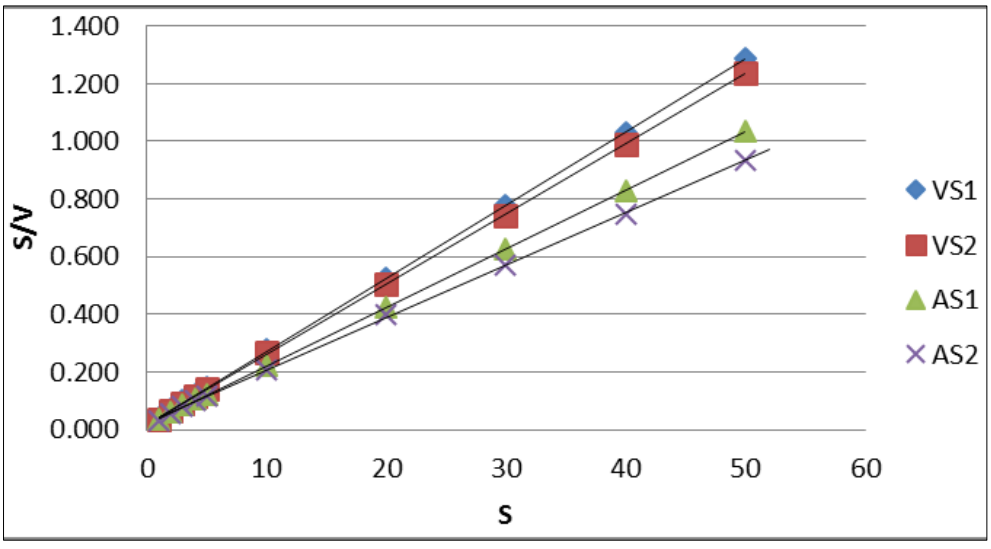

Fig 3: Hanes - Wolf plot of soil acid phosphatase activity

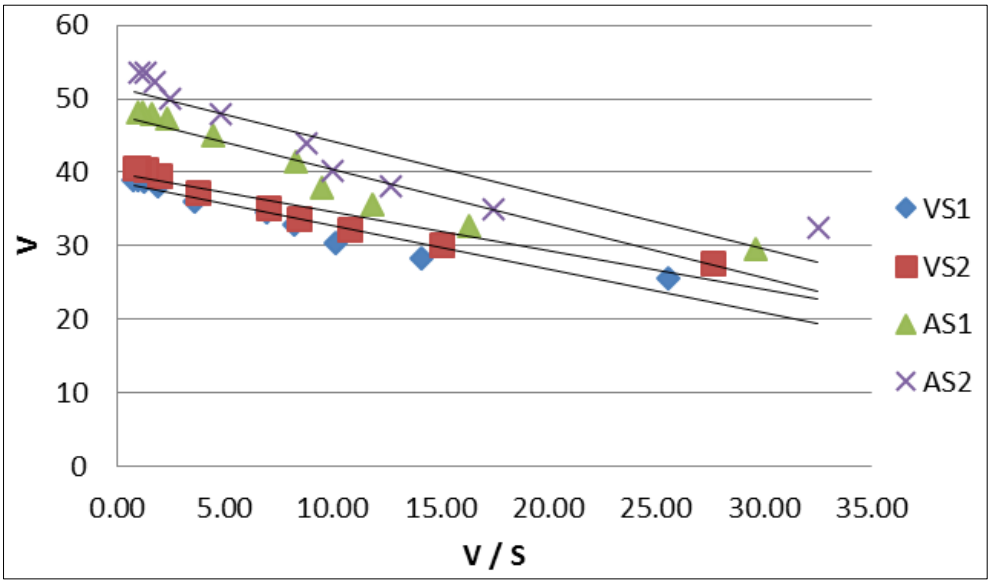

Fig 4: Eadie - Hofstee plot of soil acid phosphatase activity

The linearity of these plots is indicated that the values obtained for acid phosphatase activity fitted the three linear transformations of Michaelis-Menten's equation. The values of V_{max} and K_m obtained by least square analysis of these plots are presented in (Table. 2).From the graph, it is observed that with all the soils, reasonably linear plots were obtained in all the cases. The values of V_{max} and K_m obtained from the least square analysis of these plots are presented in (2) and (Figures. 2, 3 and 4).

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 40.0 to 56.6 and followed the sequence VS I >AS II > AS I >VS II using Lineweaver – Burk plot. The values compared well with Hanes – Wolf transformation 46.0 to 56.0 and followed the sequence VS I >VSII > AS II >AS I under and Eadie – Hofstee transformation the values varied from 44.0 to 54.4 and followed the order VS I >VS II>AS II >ASI.

Table 2: Maximum enzyme reaction velocity (V_{max}) and Michaelis Constant (K_m) values of soil acid phosphatase activity

Soils	Maximum enzyme reaction velocity (V_{max}) (µg of 4-nitrophenol g^{-1} soil h^{-1})			Michaelis constant (K_m)(mM)		
	Lineweaver - Burk Transformation	Hanes - Wolf Transformation	Eadie - Hofstee Transformation	Lineweaver - Burk Transformation	Hanes - Wolf Transformation	Eadie - Hofstee Transformation
VS I	52.6	56.0	54.4	0.60	0.67	0.65
VS II	40.0	54.0	44.0	0.46	0.86	0.58
AS I	44.0	46.0	45.0	0.44	0.78	0.51
AS II	46.0	48.0	47.0	0.52	0.63	0.61

Michaelis constant (K_m) of the soil acid phosphatase calculated using Lineweaver – Burk transformation plot varied from 0.44mM to 0.60mM and followed the order is VS I>AS II >AS I>VS II. The values compared well with those obtained from Hanes – Wolf (0.63 to 0.86) and followed the order is VS II >ASI >VS I >AS II. In Eadie – Hofstee plots the values ranged from (0.51 to 0.65) and the order followed is VS I>AS II>AS I>VS II. Irving and Cosgrove, (1976) [9] concluded that the linear transformation of Eadie – Hofstee values were found to be superior but the work shown by others (Dick and Tabatabai, 1978 [4].,Tabatabai and Singh, 1979 [21]., Rao, 1989 [16]., Srinivas, 1993 [17] and Vandana, 2012 [26]) shows that the three linear transformations are equally applicable for estimation of K_m and V_{max} values of enzymes in soil. significant difference was not noticed when Km and Vmax were calculated from the linear transformation of Michaelis plot

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