Effect of Substrate Concentration on Soil Enzyme Urease

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A B S T R A C T

To study the effect of substrate concentration on soil enzyme Urease in selected soils. Forty soil samples were assayed to measure the activity of the soil enzyme Urease among them four soil samples two Alfisols and two Vertisolssoils with high activity were selected for further study. Urease activities of the surface soils expressed as µg of NH₄⁺ released g⁻¹ soil h⁻¹ ranged from 5.9 to 16.0 with an average value of 8.74. Soil enzyme Urease activity increased with an increase in substrate concentration in the beginning and almost reached a plateau at a substrate concentration of 30mM for 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 (Vmax) and Michaelis constant (Km) were determined using Michealis – Menten equation similar to those determined in homogenous system. The Km value range from 0.49mM to 0.60mM in Lineweaver - Burk Transformation and 0.50mM to 0.76mM in Hanes - Wolf Transformation and in case of Eadie - Hofstee Transformation the Km value range from 0.62mM to 0.78mM. Vertisols showed more km value than Alfisols. The Vmax value range from 8.1µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 10 µg of NH₄⁺ released g⁻¹ soil h⁻¹ in Lineweaver - Burk Transformation and 8.7(µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 10.3 (µg of NH₄⁺ released g⁻¹ soil h⁻¹ in Hanes - Wolf Transformation and in case of Eadie - Hofstee Transformation the Vmax value range from 9.1(µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 10.5 (µg of NH₄⁺ released g⁻¹ soil h⁻¹ in Alfisols and Alfisols showed more Vmax value than Vertisols

Keywords
Alfisols, Eadie-Hofstee Transformation, Hanes-Wolf Transformation, Lineweaver-Burk Transformation

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Introduction

The enzyme Urease (urea amidohydrolase, EC 3.5.1.5) is the enzyme that catalyzes the hydrolysis of urea to CO₂ and NH₄ (Reithel, F.J. 1971). It is not involved in N mineralization in soils. This enzyme catalyzes the hydrolysis of urea, added to soils as a fertilizer. It breaks the C / N bonds other than peptide bonds in linear amides and releases NH₄ (Ladd and Jackson, 1982; Tabatabai, 1994); thus, belongs to a group of enzymes that include glutaminase and amidase. Urease activity in soil is influenced by many factors.
including crop history, organic matter, heavy metals, soil temperature, pH, soil amendments etc. (Yang et al., 2006).

The two most remarkable properties of enzymes are their specificity and their catalytic efficiency, and it is in these properties that enzymes differ most strikingly from simple catalysts. When it is possible, to compare the enzymatic rates with their own non-enzymatic counterparts, one finds that enzymes enhance the reaction by several orders of magnitude (Segel, 1975).

Soil enzymes are largely immobilized enzymes in soil colloidal particle and hence are different from homogenous systems. Nevertheless, with small substrates, the rate of reactions is not expected to be very much reduced as most of the diffusion mobility resides with the substrate.

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:

\[
S + E \rightleftharpoons ES \rightarrow E + P \quad \text{(1)}
\]

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.

\[
K_m = \frac{K_2 + K_3}{K_1}
\]

\[
V_{max} = K_3E
\]

\( K_m \) is equal to substrate concentration (expressed in moles per liter) at \( V = V_{max} / 2 \). When \( K_2 \) is greater than \( K_3 \), \( K_m \) may be set equal to dissociation constant \((K_2/K_1)\) of enzyme-substrate complex and \( 1/K_m \) then becomes the affinity constant. Although these equations are basic, it must be kept in mind that pH, ionic strength, temperature and many other factors influence the values of \( K_1, K_2 \) and \( K_3 \) (Irving and Cosgrove, 1976). For the experimental determination of \( V_{max} \) and \( K_m \) linear form of the Michaelis-Menten equation are generally used.

The three linear transformations that commonly used are:

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

2. \[
\frac{[S]}{V} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \cdot [S]
\]
   Hanes-Wolf transformation

3. \[
V = V_{max} - K_m \cdot \frac{V}{[S]}
\]
   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 $V_{\text{max}}$ 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 ($V_{\text{max}}$ 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), but they may vary independently. Maximum reaction
velocity ($V_{\text{max}}$) 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 $V_{\text{max}}$ value can be used as
an indicator to a speedy or slow enzymatic
process. $V_{\text{max}}$ and $K_m$ of an enzyme express
the quantity of an enzyme and substrate
affinity, respectively (Marx et al., 2005).

However, Michaelis constant ($K_m$) represents
the endurance of an enzyme-substrate
complex, which is related with the substrate.
The efficiency of the enzyme to decompose
substrate at low concentration is directly
related to their $K_m$ value (Marx et al., 2005).
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).
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). However,
estimating $K_m$ is challenging due to the
uncertainty regarding the relative contribution
of artificial and naturally occurring substrate
under nonsaturating conditions (Stone et al.,
2011).

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 and German et al., 2011). 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).
Many investigations have dealt with the
kinetic properties of enzymes (Masciandaro et
al., 2000, Zhang et al., 2009 and 2010, Juan et
al., 2010).

Although, the literature on soil enzyme is on
the increase, reports on kinetic constants like
Michaelis constant and $V_{\text{max}}$ and their
correlations with soil properties are limited.
Values for both $K_m$ and $V_{\text{max}}$ 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, $V_{\text{max}}$ 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 $V_{\text{max}}$ (App$V_{\text{max}}$) and apparent $K_m$
(App$K_m$) with App$V_{\text{max}}$, a relative measure of
enzyme abundance, and App$K_m$, a relative
measure of substrate (Wallenstein et al., 2011).

\( K_m \) and \( V_{max} \) values for ureases of different particle size fractions of soils differed from each other and from those of unfractionated soils (Tabatabai, 1973). Generally the \( K_m \) values of fractions were greater than those of unfractionated soils but no relationship of these values with particle size could be so established. \( V_{max} \) values of all fractions were considerably less than those of the unfractionated soils, indicative of urease destruction, perhaps during the sonic vibrating the fractions.

\( K_m \) values may also fluctuate, depending on whether it is in the free or in an absorbed state (McLaren and Packer, 1970). While investigating the enzyme splitting of urea in the presence of bentonite, Durand, (1966), 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), 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).

Materials and Methods

Urease activity was assayed by quantifying the rate of release of \( NH_4^+ \) from the hydrolysis of Urea as described by Tabatabai and Bremner (1972), but with some modifications as suggested by Dorich and Nelson (1983) and Rao (1989). Urea solution (0.2 M): This was obtained by dissolving 1.2 g of Urea in 80 ml distilled water and volume was made up to 100 ml. Potassium chloride (2 M) - Silver Sulphate (100 ppm) KCl-Ag2SO4 solution: 100 mg of Ag2SO4 was dissolved in 700 ml of distilled water to which 300 ml of water containing 149 g of KCl was added. MgO: Magnesium oxide was heated in an electrical furnace at 500°C for an hour and the powder was collected in dessicator and stored in a tightly stoppered bottle.

4% Boric acid: 40 g of Boric acid was dissolved in a beaker containing hot distilled water about 800 ml. Then 5 ml bromocresol green and 15 ml of methyl red were added and the volume was made up to 1 litre with hot distilled water. 0.005 N \( H_2SO_4 \): This solution was prepared by taking 5 ml of 1N \( H_2SO_4 \) is taken in a 1 litre volumetric flask and make up to the mark by the addition of distilled water. Soil samples (5 g) were taken in 50 ml capacity glass tubes to which 9 ml distilled water was added.

Substrate i.e. urea solution of mM strength were added to different glass tubes in triplicates so as to obtain 1, 2, 3, 4, 5, 10, 20, 30, 40 and mM urea in the glass tubes. These tubes were made air tight and were incubated for 2 hours at 37°C. Thereaction was terminated by the addition of KCl- Ag2SO4. The contents were agitated on mechanical shaker for one hour to release all \( NH_4^+ \) formed and the suspension was allowed to settle. Thirty ml of the supernatant with KCl-Ag2SO4 extract was taken and transferred to Kjeldahl flask.

To this a pinch of MgO was added which was kept at one end of the distillation unit. During steam distillation for 4 min, the solution containing MgO was heated. The ammonia was released into boric acid containing mixed indicator through a tube dipped in the solution. The ammonia released would change the color of the solution from pink to pale green at the end of the distillation. This was titrated against standardized 0.005N \( H_2SO_4 \) and the amount released was
calculated and expressed as μg of NH4+ released g-1 soil h-1.

**Results and Discussion**

Soil urease activity increased with an increase in substrate concentration in the beginning and almost reached a plateau at a substrate concentration of 30 mM for all the four soils studied (Table 1). With further increase in substrate concentration, minimal change in enzyme activity was noticed. Similar results were obtained by Rao, (1989), and Vandana, (2012) for soil urease.

The $V_{\text{max}}$ and $K_m$ values were determined using the three linear transformations of the Michaelis-Menten’s equation. Lineweaver – Burk transformation plot of $1/V$ against $1/[S]$, Hanes – Wolf transformation plot of $[S]/V$ against $[S]$ and Eadie – Hofstee transformation plot of $V$ against $V/[S]$ for the four different soils were shown.

From the graphs, it was observed that with all the soils, reasonably linear plots were obtained in all the cases. The values of $V_{\text{max}}$ and $K_m$ obtained from the least square analysis of these plots are presented.

The maximum reaction velocity of soil urease for soils understudy when calculated as μg of NH4+ g−1 h−1 varied from 8.1 to 10.5 and followed the sequence AS II > ASI > VS I > VS II under Lineweaver – Burk plot. The values compared well with those obtained from Hanes – Wolf (0.50 to 0.76) and followed the sequence of Eadie – Hofstee (0.62 to 0.78) plots. In all the three linear plots the same order is followed the sequence is VS II > VS I > ASI > AS II. These values compared well with the findings of Rao, (1989), Vandana, (2012) and Zhang, (2009) found the influence of soil moisture on $K_m$ values. Juan et al., (2010) found higher $K_m$ values for soil urease than observed from pure enzymes. This could be due to the difference in physicochemical characteristics of soils. Higher organic carbon content and clay humus complex traps soil urease and slows down the diffusion to substrate, which prevents the urease from interacting with substrate.

McLaren and Packer, (1970) and Vandana, (2012) were of the view that $K_m$ values may also fluctuate depending upon whether it is in the free or in an absorbed state. While investigating the enzyme splitting of urea in the presence of bentonite, Durand, (1966) obtained higher $K_m$ values for adsorbed enzymes than for free enzyme.

Paulson and Kurtz, (1970) and Vandana, (2012) indicated a much lower apparent affinity of the enzyme for the substrate compared to that of the native enzyme. Different $K_m$ and $V_{\text{max}}$ values for different soil types for soil urease were obtained by (Tabatabai and Bremner, 1971., Nor, 1982., Rao, 1984., Vandana, 2012).

Kinetic constants may also differ with origin of the enzyme. Frankenberger and Tabatabai, (1982) and Stevenson, (1994), reported that urease of plant origin has different kinetic constants than that of the native soil enzyme. Also, urease of microbial origin differed in the properties from that released by soil microflora.
Table.1 Effect of substrate concentration on soil urease activity

| Substrate Concentration (mM) | Urease activity (µg of NH$_4^+$ released g$^{-1}$ soil h$^{-1}$) |
|-----------------------------|--------------------------------------------------------------|
|                             | VS1   | VS2   | AS1   | AS2   |
| 1.0                         | 4.8   | 5.3   | 6.4   | 5.9   |
| 2.0                         | 6.2   | 6.6   | 7.9   | 7.2   |
| 3.0                         | 6.8   | 7.5   | 8.3   | 7.9   |
| 4.0                         | 7.4   | 7.8   | 8.8   | 8.4   |
| 5.0                         | 7.8   | 8.1   | 9.3   | 8.8   |
| 10.0                        | 8.2   | 8.5   | 9.7   | 9.3   |
| 20.0                        | 8.4   | 8.8   | 9.9   | 9.5   |
| 30.0                        | 8.6   | 9.2   | 10.4  | 9.7   |
| 40.0                        | 8.7   | 9.3   | 10.5  | 9.9   |
| 50.0                        | 8.7   | 9.3   | 10.5  | 9.9   |

Table.2 Maximum enzyme reaction velocity ($V_{max}$) and Michaelis Constant ($K_m$) values of soil urease activity

| Soils | Maximum Enzyme Reaction Velocity ($V_{max}$) (µg of NH$_4^+$ released 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  | 9.1 | 9.5 | 9.7 | 0.54 | 0.70 | 0.70 |
| VS II | 8.1 | 8.7 | 9.1 | 0.60 | 0.76 | 0.78 |
| AS I  | 9.5 | 10.3 | 10.5 | 0.51 | 0.58 | 0.63 |
| AS II | 10.0 | 10.1 | 10.3 | 0.49 | 0.50 | 0.62 |

Figure.1 Effect of substrate concentration on soil urease activity
**Figure 2.** Lineweaver - Burk plot of soil urease activity

**Figure 3.** Hanes - Wolf plot of soil urease activity

**Figure 4.** Eadie - Hofstee plot of soil urease activity
In the beginning of the reaction the active sites of the enzymes were not occupied by the substrate molecules hence as we increase the substrate concentration the rate of the reaction increases following first order kinetics and on further increase the rate of the reaction increase slowly as the active sites are nearly saturated following mixed order kinetics and on further increase of substrate concentration the rate of the reaction is independent of substrate concentration and follows zero order kinetics.

The Km value range from 0.49mM to 0.60mM in Lineweaver - Burk Transformation and 0.50mM to 0.76mM in Hanes - Wolf Transformation and in case of Eadie - Hofstee Transformation the Km value range from 0.62mM to 0.78mM. Vertisols showed more Km value than Alfisols. The Vmax value range from 8.1µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 10 µg of NH₄⁺ released g⁻¹ soil h⁻¹ in Line weaver - Burk Transformation and 8.7(µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 10.3 (µg of NH₄⁺ released g⁻¹ soil h⁻¹ in Hanes - Wolf Transformation and in case of Eadie - Hofstee Transformation the max range from 9.1(µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 10.5 (µg of NH₄⁺ released g⁻¹ soil h⁻¹.

Vmax value range from 8.1 (µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 9.7(µg of NH₄⁺ released g⁻¹ soil h⁻¹ in Vertisols and 9.5 (µg of NH₄⁺ released g⁻¹ soil h⁻¹ to 10.5 (µg of NH₄⁺ released g⁻¹ soil h⁻¹ in Alfisols and Alfisols showed more Vmax value than Vertisols.

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