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Original article

Kinetic and thermodynamic parameters of urease in soils with different fertilization regimes

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Abstract

In recent years the growing demand for food led to intensification of agricultural practices, especially by the excessive use of fertilizers, which increased the environmental pollution. Therefore, pollution control by improving the efficiency of fertilizers and reducing their application is of great interest. Urease is one of the most active hydrolases in soil, having an important role in soil N cycle and being used as an indicator of soil quality. The objective of this study was to assess the urease activity in soils with different fertilization treatment, as well as its kinetic and thermodynamic parameters to better understand its driving factors. The results show a better enzymatic activity in soils treated with combined manure and mineral fertilizers. Soil urease has two optimal pH values, in the neutral and basic domains. Enzymatic activity has a steep increase with the temperature in the interval 45–65°C. The KM values increase with temperature from 11 to 23.5 mM, indicating a lower substrate affinity. The activation enthalpies for enzyme-substrate formation as well as for the rate limiting step are 7.59 and 14.18 kcal/mol respectively. The relationship between urease activity and microbial biomass will be further investigated.

Keywords

urease activity, kinetic parameters, activation enthalpy, long term fertilization, hydrolysis rate constant.

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Introduction

Enzymes play an essential role in all processes that take place in organisms and in the environment [1]. They catalyze biochemical reactions that thus take place with an order of magnitude higher than in their absence. Thus, life on Earth could not exist without the rapid unfolding of biochemical processes. Enzymes are involved in many edaphic processes like organic matter decomposition, forming of humic substances, xenobiotic degradation, nitrogen fixation among others [2]. Therefore, ecological functions like biomass production and soil decontamination, are highly dependent on enzymes activity, as they are directly involved in biogeochemical cycles of C, N, P and S. Among them, due to their function, hydrolases – proteases, phosphatases and ureases are the most studied soil enzymes [3]. A major agricultural topic of recent research is food security, and it is closely related to soil health. One of the important indicators of soil quality is soil biological activity. It has been suggested that activity of soil enzymes is a potential indicator of soil quality because of their relationship with soil biology and rapid response to changes in soil management [4].

Urease is a key catalytic enzyme involved in urea hydrolysis, widely distributed in nature in plants, animals and microorganisms [5; 6]. It has an important role in the use of urea fertilizer, is involved in the N cycle, and the changes of its activity can be used as an indirect indicator of N pool and availability in soils. But, by increasing the use of urea as fertilizer, there is an intensification of the activity of soil urease, and an increase of ammonia volatilization, soil alkalinization, and nitrous oxide generation, the 3rd greenhouse gas, or even the degradation of aquatic ecosystems, through eutrophication [7; 8]. Low urease activity, on the other hand, causes environmental pollution through leaching of additional urea. To mitigate these problems the understanding of urease mechanism and kinetics is crucial because it can give information on compounds that could inhibit soil urease activity and reduce urea hydrolysis in case of its use as fertilizer. There is still little knowledge about the kinetic and thermodynamic characteristics of soil urease with amendment of urease inhibitors and different environmental conditions [9].

It has been revealed the influence of individual environmental factors like temperature, water content, nitrogen application rates on urease activity, but the integrated effect of these factors is not clear. In agricultural soils, with different temporal and spatial environmental conditions, the impact of these factors on urea hydrolysis has different consequences. Urea hydrolysis is a first order kinetic process, and the rate constant is an important parameter. Nitrogen transportation and transformation simulation studies use the value of this kinetic parameter at a fixed moisture content, temperature and nitrogen concentration [10.

Previous studies mentioned that there is variation in the stabilities of urease in the heterogeneous soil systems, especially thermal stability, due to the complexation of enzyme with organic colloids or adsorption on clay particles [11]. These differences were attributed to soil pH and adsorptive properties of soils [12].

Therefore, in this study we assessed the kinetic and thermodynamic parameters of soil urease and compared it to extracted urease "*jack bean urease*". There are no comprehensive studies on soil urease activity and kinetics in Romania and this kind of information is important in establishing agricultural strategies with special consideration on urea decomposition rate and its environmental consequences. We expect that this study will generate useful information to assess and develop strategies for sustainable nitrogen management and a useful indicator of soil health.

Materials and methods

Description of the research area and sampling

The agricultural field selected for this study belongs to o a long-term experiment established in 1962 and managed by Agricultural Research and Development Station Turda (ARDS Turda), having the following geographical coordinates: 46° 35' 12.3" lat. N, 23° 48' 3.42" long. E. The experiment consists of 7 fertilization treatments (combinations of N, P and manure fertilizers) each in three field replicates with an area of 50 m^2. The rotation system involves a three-crop sequence based on maize, soybean and wheat. Topsoil (0-20 cm) samples were collected in April 2021 being composited out of six subsamples that were extracted by the means of a soil auger.

From each batch (subjected to a certain combination of fertilizers) 5 replicates were taken from a depth of 20 cm. Each replicate was obtained by homogenizing the soil taken from 5-6 injections. The kinetic analysis of urease was performed on the sample taken from the plot fertilized annually with 50 t/ha of N, 25 t/ha of P and 60 t/ha of manure. Before any analysis, samples were processed by grounding and sieving with a sieve with a diameter of 4 mm.

Physicochemical properties

Physicochemical parameters determined for the soil samples were pH, moisture, organic matter, ammonia nitrogen, nitrate nitrogen, phosphorus in the form of phosphate and urease activity. The pH was measured on a suspension soil-water in 1 to 2.5 ratio [13], using a WTW 3000 pH-meter. Soil water content was determined gravimetrically

and organic matter content of soil samples through loss on ignition at 550°C. Inorganic nitrogen compounds were extracted from soils with a solution of potassium chloride (KCl) 0.2 M. Ammonia nitrogen was determined by indophenol-blue method [14], nitrate with the phenol disulphonic acid method, and phosphate by green malachite method [15], using a Helios Gamma UV-VIS spectrophotometer [16]. All analytical results were normalized to dry soil weight.

Urease activity assay

Urease activity was assayed following the procedure described by Tabatabai and Bremner [17]. Five grams of soil were incubated with 10 ml buffer solution (pH in the range 5.4 -11) and 1 ml urea of different concentrations for 2 hours at 37°C. Different urea concentrations between 2 and 160mM were used to assess kinetic parameters, as well as different temperature for determination of thermodynamic parameters. The reaction was stopped by the addition of KCl solution 1M. A volume of 1 mL was taken from the supernatant to determine the ammonium concentration by the indophenol-blue method. The kinetic and thermodynamic parameters were carried out on an average sample made from the 3 replicates taken from lot 4, fertilized annually with 50 t/ha N, 25 t/ha P and 60 t/ha manure.

Michaelis kinetic parameters

The kinetic parameters K_M and V_{0max} were calculated by Hanes-Woolf equation [18]:

$$\frac{[S]}{V_0} = \frac{1}{V_{0max}} \cdot [S] + \frac{K_M}{V_{0max}},$$

where V_{0max} is the enzyme maximum initial velocity, [S] is the concentration of substrate (mM), K_M is the Michaelis constant.

Thus, the variation of K_M with temperature allows the determination of the enthalpy of formation of the ES complex, and the variation of k_2 with temperature on that of the enthalpy of activation of the velocity-determining step, that of the decomposition of the ES complex into products and enzyme. These parameters are represented in the energy diagram in Figure 1. The enthalpy of formation of the ES complex is calculated based on the van't Hoff relation:

$$\frac{dlnK_{eq}}{dT} = \frac{\Delta_r H^{\emptyset}}{RT^2},$$

which, through integration, leads to:

$$lnK = \frac{-\Delta H^{\emptyset}}{RT} + \frac{\Delta S^{\emptyset}}{R}$$

and the plot of lnK = f(1/T) is a straight line with the slope -ΔH/R.

The temperature dependence of the rate constant k_2 with temperature, according to the well-known Arrhenius equations [19]:

$$k = A \cdot e^{-\frac{E_a}{RT}},$$

where E_a is the activation energy (kJ/mol), A is the preexponential factor, R is the gas constant ($J \cdot mol^{-1} \cdot K^{-1}$) and T is the absolute temperature (K). The plot of lnk_2 = f(1/T) allows the determination of the activation energy and activation enthalpy, according to the equation: $\Delta H_2^* = Ea - RT$.

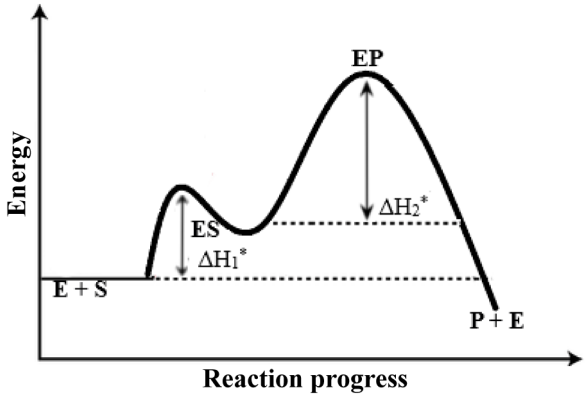

Fig. 1. Energy diagram of the enzymatic reaction

Results and discussion

Physicochemical characterization of soils

Prior to the evaluation of urease, the physicochemical characterization of the samples taken from the 7 plots subjected to different fertilization treatments was carried out. It was reported that physical, chemical and biological composition of soils, together with type of crops and management practices influence soil enzymatic activities [20]. Table 1 shows the average results obtained from the analysis of the 3 replicates from each plot.

Table 1. Average values of the physicochemical parameters of soil samples taken from plots with different fertilization treatments

Tratament (t/ha/year)	pH	N-NH_4^+ µg/g soil	N-NO_3^- µg/g soil	P-PO_4^{3-} µg/g soil	MO%	U%
1: 150N, 60P	5.08	0.92	1.88	11.25	8.89	18.19
2: 120N, 50P, 20t manure	5.10	0.94	1.76	18.08	8.93	16.19
3: 80N, 30P, 40t manure	5.17	0.82	3.02	23.64	9.42	17.34
4: 50N, 25P, 60t manure	5.08	1.11	2.81	19.98	9.01	19.13
5: 20t manure	5.19	2.06	1.99	10.23	9.24	19.05
6: 40t manure	5.14	2.99	1.30	11.14	9.05	16.46
7: not fertilized	5.27	1.07	0.97	2.69	8.65	18.24

The analysis of the results indicates that the soil has a slightly acidic reaction, the pH values being between 5.08-5.27. In general, agricultural soils are characterized by a more acidic reaction than untilled soils because of the application of fertilizers. The humidity varies in the range

of 16.19-19.13%, and the organic matter in the range of 8.65-9.42%. In terms of nutrient content (N and P), the not fertilized soil is differentiated, having the lowest inorganic nitrogen (DIN) content, of 2.04 μg N/g dry soil and reactive phosphorus (in the form of PO_4^{3-}) of 2.69 μg P/g dry soil. Soils treated only with manure have, as expected, the highest ammonia nitrogen content (above 2 μg $N\text{-}NH_4^+$/g dry soil) but the lowest phosphorus content (except for not fertilized soil).

Determination of urease activity in agricultural soils subjected to different fertilization treatments

The results obtained from the analysis of the urease activity are presented in Table 2. As can be observed, the enzymatic activity (expressed as maximum initial reaction rate) presents small differences for the 7 fertilization treatments, varying between 0.44 and 0.61 mM/h, at 37°C, pH = 8, $[S]_0$ = 80 mM. The higher values of enzymatic urease were obtained for soil samples of plots 3 and 4, fertilized with high amounts of organic fertilizer combined with mineral fertilizers, which is in accordance with data described in literature. Some authors reported a decrease of urease activity with long-term nitrogen fertilization [3]. This was explained by the absorption of inorganic nitrogen by soil microorganisms [21]. But urease activity was also reported to increase with addition of nitrogen and organic fertilizers [22]. It was also noted that application of both organic and mineral fertilizers enhances urease and phosphatase activities in soils cultivated with beans [23]. The increase was attributed to a combined effect of higher degree of enzyme stabilization with formation of organo-mineral complexes and increase of microbial biomass because of organic carbon content [24]. Soil sampled from plot 4 are characterized by the highest water content and high organic matter.

Kinetic and thermodynamic studies were performed on soils from plot 4. The choice of soil samples from this plot was based on the values obtained for urease activity which were the highest and the fertilization treatment is the most complex, including, in addition to mineral fertilizers, the largest amount of organic fertilizer.

Table 2. Values of the maximum initial velocity at pH = 8 and 37°C of the catalyzed reaction of urease

Plot (treatment)	C (μg $N\text{-}NH_4^+$)	V0 (mM/h
1	10.98	0.571
2	10.74	0.558
3	11.23	0.583
4	11.72	0.609
5	8.53	0.443
6	10.63	0.552
7	10.18	0.529

Characterization of kinetic and thermodynamic parameters of urease in agricultural soil

1. Variation of the initial reaction rate with pH

Variation of the enzymatic rate of reaction with pH was investigated on the pH range 5-10. Phosphate buffer solutions have been used for the pH range 5-8 and borate buffer solutions for the range 8.4 -10. Although the assays are usually performed in phosphate buffer and/or in borate buffer, in the literature it is indicated that the phosphate buffer at pH < 7.5 manifests inhibition by the $H_2PO_4^-$ ion [25], as well as the borate buffer manifests inhibition with increasing pH [26]. Boric acid exhibits maximum inhibition at pH 5 and minimum at pH 10 [27]. Phosphoric and boric acid are competitive inhibitors, admittedly, with low inhibitory activity.

The variation of the soil urease activity with pH shows two maximum values corresponding to pH 7.4 and 10.8 (Figure 2), as opposed to the dynamics mentioned in literature for the purified *jack bean urease* which shows only one maximum, with a bell shape and an optimal pH 7-7.5, according to Krajewska (2009a). The bell shape is specific to many enzymes, and in *jack bean urease* the existence of three functional groups has been demonstrated, with pKa of 5.3, 6.6 (associated with a molecule of His in catalytic active center), and 9.1 (either W2 or WB) [28]. Optimal pH reported in literature for soil urease is between 6.5-7, but values above 8.8 and even below 5.8 have been reported [29]. The optimal pH depends on the buffer used, but also on the nature of the enzyme. We assume that the differences from the *jack bean enzyme,* for which we obtained a maximum value at pH of 7.2 using the same buffers, are due to the different origin of the enzymes in the soil. According to various reports, urease can originate from multiples sources, including bacteria, fungi, plant and animal tissues and animal waste [30]. The hypothesis is supported by the fact that the soil we took into analysis was fertilized with 60 t/ha/year of manure. The same literature study mentions that microbial urease is controlled by N fluxes in the soil. At the same time, even the optimal pH value of jack bean urease can be considerately higher, if the enzyme gets immobilized on certain types of clay minerals as shown by Lai and Tabatabai [31].

2. Variation of the initial reaction rate with the substrate and temperature

In this study the variation of soil urease activity with temperature shows a maximum at a temperature of 65°C (pH=10, $[S]_0$ = 160 mM), like many enzymes. From 15 to 45°C, the increase of the initial rate is about 1.8 times over each 10°C, followed by a steep increase from 45°C to 65°C, of 3.5 times (Figure 3). Other studies [32] showed different response of urease activity to temperature [33], with two

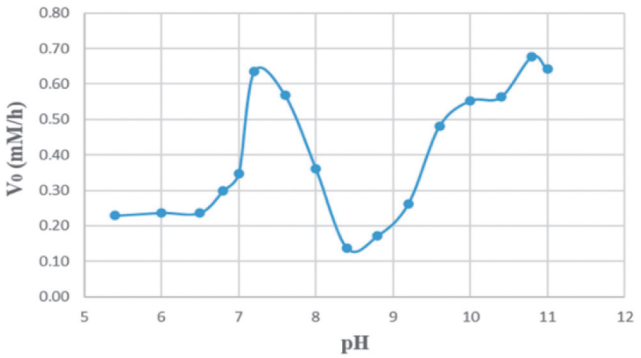

Fig. 2. Variation of the initial rate of the soil urease catalyzed reaction with pH

or three times increases from 15°C to 35°C or only 0.15-0.62 times for the same temperature interval [34]. The optimal temperature mentioned in the literature was 60°C [35]. By choosing a high concentration of substrate, the thermal stability of the enzyme is increased. At temperatures above 65°C, the initial velocity decreases because the enzyme is largely denatured. It was reported that, even if the inactivation of urease activity occurs in 65-70°C, it is not completely destroyed up to 105°C [36], its greater thermal stability being explained by adsorption on clay colloids or complexation with organic colloids [11]. Other studies showed that soil enzymes are generally more resistant to thermal denaturation in the heterogeneous soil systems [37]. The effect of temperature on soil enzyme activities leads generally to changes in substrate affinity, enzyme stability and kinetics through its influence on size and activity of microbial biomass. Urease activity has been shown to be dependent on soil temperature and moisture content, but not on soil nitrogen concentration [38], but the influence of nitrogen application rate was also mentioned in literature [34]. Many studies revealed the influence of single factors on urease activity, however the effect of interaction between factors should not be ignored. Our results also suggest that fertilization treatment influences urease activity, which was greater in soils treated with the highest amounts of organic fertilizer (Table 2).

Regarding the influence of the substrate concentration on the maximum initial velocity, the urea concentration range 2-160 mM was used. The working temperatures were

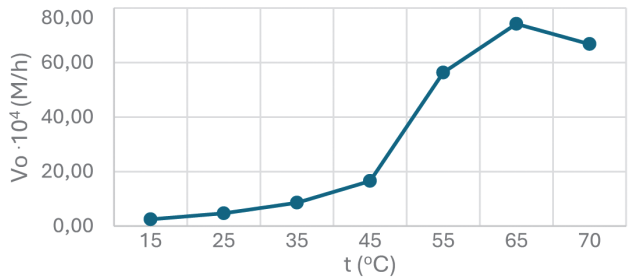

Fig. 3. Variation of the initial rate of reaction catalyzed by soil urease with temperature

in the range of 15-55°C, but due to the low enzymatic activity at low temperatures, it was difficult to obtain a clear variation of the kinetic parameters (K_M and V_{0max}). Thus, only the results at temperatures of 35, 45 and 55°C were interpreted, respectively. The parameters kept constant were pH=10, t = 2h. The graphical representation clearly shows that a Michaelis-Menten kinetic is observed, reaching a plateau at high concentrations of urea (Figure 4**)**.

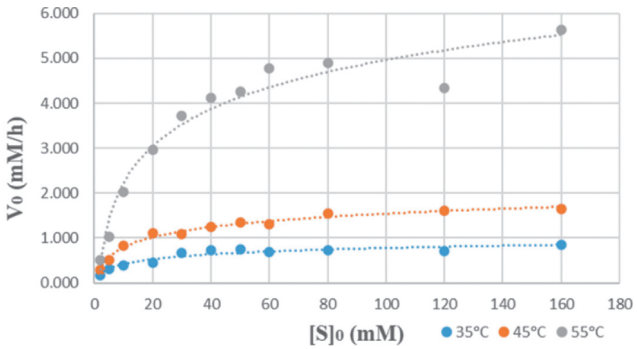

Fig. 4. Variation of the initial rate of the reaction catalyzed by soil urease with substrate and temperature

The Hanes-Woolf linearization (Figure 5) equation was used to determine the kinetic parameters K_M and V_{0max}.

$$\frac{[S]}{V_0} = \frac{1}{V_{0max}} \cdot [S] + \frac{K_M}{V_{0max}}$$

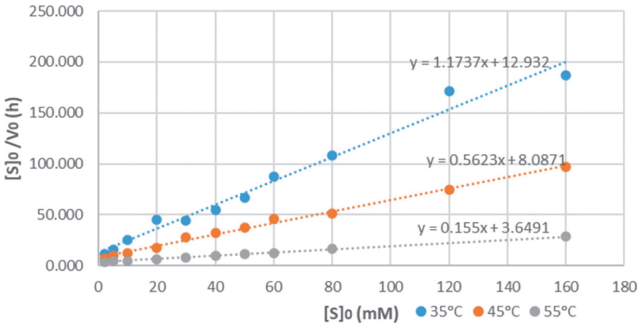

Fig. 5. Hanes-Woolf linearization of the initial rate variation of the catalyzed reaction by soil urease

The values of V_{0max} for temperatures in the range 35-55°C were obtained from the graph slope and K_M from the free term of equation.

The K_M values obtained in our study vary between 11–23.5 mM, increasing with temperature, and fall within the very wide range of variation of this constant mentioned in the literature, between 1.2-330 mM (Table 3).

Table 3. Kinetic parameters (K_M, V_{0max} and k_2) at different temperatures of the reaction catalyzed by the urease enzyme

T (K)	1/T (K^{-1})	$K_M \cdot 10^3$ (M)	V_{0max} (M/h)	lnK_M	k_2 (s^{-1})	lnk_2
308	0.0032	11.02	0.852	-4.508	3.156	1.149
318	0.0031	14.38	1.778	-4.242	6.585	1.885
328	0.0030	23.54	6.452	-3.749	23.896	3.174

This constant is an indication of enzyme affinity for substrate, a lower value of K_M indicating a higher affinity. Its value is increasing with incubation temperature, probably because of the reduced amount of enzyme active sites covered by humus accompanied with temperature [9]. The same authors hypothesized that soil urease could be trapped by higher soil organic C content and slow down the diffusion of substrate, impeding the enzyme-substrate interaction. The humic substances present in the soil modify the K_M and V_{0max} values in relation to the purified enzyme, which explains the large variations of the K_M constant depending on the organic matter content and the type and quantity of clay in the soil [29]. The formation of different inhibitor-urease complexes has the same effect, as well as conformational changes in enzyme structure, decreasing the accessibility of its active sites [39]. These large variations have not been fully explained, but it has been found that the enzyme can be stabilized by the formation of urease organo-mineral complexes or clay-enzyme associations in some soils. Fidaleo and Lavecchia [40] concluded that value of K_M is influenced by temperature, substrate properties, pH and ionic strength.

The values of the kinetic parameters V_{0max}, K_M and k_2 of the soil urease hydrolysis reaction at different temperatures allowed the determination of activation parameters of the reaction.

Enthalpy of ES complex formation (ΔH_1) was obtained by the variation of $\ln K_M$ with temperature (Figure 6), and the enthalpy of activation of the rate-determining step (ΔH_2^*) was obtained from the variation of $\ln k_2$ with temperature (Figure 7), which correspond to the decomposition of the ES complex into products and enzyme (Figure 1).

The value obtained for the enthalpy of formation of the ES complex is 31.75 kJ/mol, more than 3 times higher than that we obtained for the formation of the complex with the purified "*jack bean urease*". This value suggests that the process is more endothermic in the case of the enzyme in the soil. Our results are very close to values reported

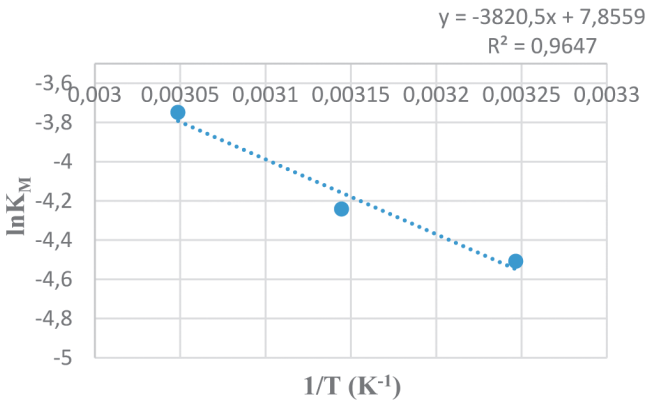

Fig. 6. Plot of $\ln K_M$ with temperature, for enthalpy determination of the ES complex formation

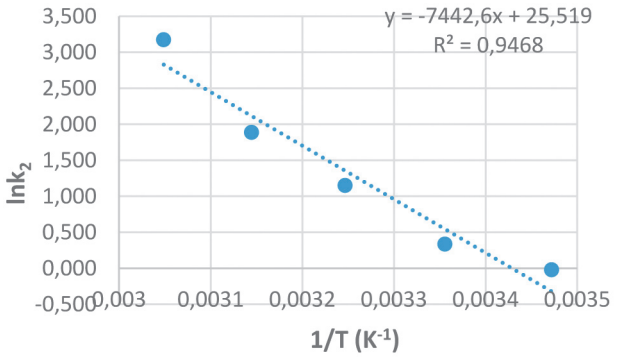

Fig. 7. Plot of lnk2 with temperature for determination of enthalpy of activation of the rate-determining step (ΔH_2^*)

in literature, which vary between 37.96 to 49.70 kJ/mol in loamy sand soil and from 32.04-44.34 kJ/mol in silty clay soil [41]. Other researchers [31] reported values of activation enthalpy of about 36 kJ/mol, whereas Juan et al. (2010) mentioned values between 19.08-21.64 kJ/mol. Same authors [31] hypothesized that a large enthalpy of activation is an indication that for the formation of activated state, many stretching, squeezing and probably breaking of chemical bonds are necessary. Moyo et al. [32] concluded that values reported in literature for activation energy for soil urease are dependent on pH, urea concentration, water content and even assay techniques used that can influence differently the energy requirements for the formation of enzyme-substrate.

Proceeding further to calculate the activation energy of the rate-determining step (ΔH_2^*), the formation of reaction products from the ES complex, an enthalpy of activation of 59.29 kJ/mol was obtained. This value is significantly higher than that obtained for "*jack bean urease*" ((ΔH_2^* = 17.01 kJ/mol), indicating that the energy barrier of this process is higher, so the reaction occurs more slowly in the case of urea hydrolysis under the action of the enzyme in the soil.

Comparing the values obtained in this study with those mentioned by other studies (Table 4), we find that the results fall within the (very wide) ranges of variation mentioned in the literature.

Table 4. Comparative values of the kinetic and thermodynamic parameters of the hydrolysis reaction of urea catalyzed by the purified and native enzyme in soil [29] [42; 43]

	K_M (mM)	V_{0max} (µM/s)	ΔH_1^* (kcal/mol)	ΔH_2^* (kcal/mol)
Jack bean urease this study	19.8	2.77	2.05	4.07
Soil urease this study	14.38	10.84 µg N/g soil/h	7.59	14.18
Literature - jack bean)	2.7-45	0.75	-	1.5-9.6
Literature - soil enzyme (free bound)	1.3-330	14-143 µg N/g soil/h	-	5.7-9.8

There are obviously differences in the units of measurement used to express the maximum reaction rates in the case of the enzymatic reaction catalyzed by the purified enzyme and the one catalyzed by the enzymes in the soil. As for the activation enthalpy of the limited rate step of products formation, in the case of purified urease our value is consistent with those reported in the literature, while in the case of the analyzed soil urease, the obtained value is slightly higher.

Conclusion

Results of our study showed that urease activity was stimulated by the application of organic fertilizer in combination with mineral fertilizers. The lowest enzymatic activity was obtained for plots fertilized with only manure in low amounts. Soil urease activity has two optimal pH values, at neutral and basic domains, which suggest different originate sources, which must be investigated. Temperature has a great effect on enzyme activity, influencing biochemical transformation of nutrient catalyzed by soil urease. The increase of K_M value with temperature shows a decrease in enzyme affinity for urea. The larger activation enthalpy we obtained for the soil urease indicates a lower probability of the activated complex enzyme-substrate of progressing towards reaction products.

Future studies are required to analyze the relationship between urease and microbial biomass for different agricultural ecosystems, leading to better understanding of soil health and development of N management strategies.

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Conflicts of interest

The authors declare no conflict of interest.

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