

Activatory Effect of Germination on Catalytic Capacity of Urease Extracted from Beans Samples

(Effect of Germination on the Kinetics of Urease from Beans)

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UNDER PEER REVIEW

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29 Abstract

30 **Aim:** This research work was designed to determine the effect of germination on catalytic
31 capacity of urease extracted from seven beans samples.

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33 Federal University of Technology, Minna, Nigeria

34 **Methodology:** Seven samples of beans namely; *Phaseolus lunatus*, *Pachyrhizus tuberosus*,
35 *Glycine max*, *Cajanus cajan*, *Mucuna pruriens*, *Kerstings geocarpa* and *Vigna mungo* were used
36 by comparing urease activity in germinated to ungerminated beans samples.

37 **Result:** The protein level in germinated and ungerminated beans samples as well as, optimum
38 pH, optimum temperature, substrate concentration and kinetic parameters, V_{max} , K_m , and V_{max}/K_m
39 of urease were determined. The pH, temperature and substrate concentration ranged from 5.50-
40 8.0, 30-80°C and 0.1-0.6M respectively. From the results, the protein level in germinated beans
41 samples reduced significantly ($p<0.05$) compared to ungerminated beans samples. The optimum
42 pH and temperature ranged from 6.5-7.0 and 60-70°C respectively for both germinated and
43 ungerminated beans sample. Therefore, germination did not affect the pH and temperature
44 stability of urease when compared to ungerminated beans samples. The results showed that
45 germination significantly ($p=0.05$) increased V_{max} and there was no significant ($p=0.05$)
46 difference in K_m of urease in germinated and ungerminated beans sample studied. However, the
47 catalytic capacity (V_{max}/K_m) of urease in germinated beans was significantly ($p=0.05$) higher than
48 ungerminated.

49 **Conclusion:** This implies that, with germination urease activity can be increased to meet both
50 application of urea as fertilizer (agriculture), clinical analysis of urea in biological fluid and
51 artificial kidney development and other applications.

52 **Key words:** Urease, Germination, Protein, Catalytic capacity.

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Introduction

Urease (EC 3.5.1.5, urea amidohydrolase), a nickel-dependent metalloenzyme, catalyzes the hydrolysis of urea to form ammonia and carbon (IV) oxide. The hydrolysis of one molecule of urea results into the release of two molecules of ammonia and one molecule of carbon (IV) oxide [1]. Several different assays, based mainly on the measurement of the amounts of products released during the reaction, are available for quantifying urease activity.

From the onset of scientific discovery, urea and urease had been subjects of investigation. Urease from jack bean (*canavalia ensiformis*) was the first enzyme ever to be crystallized [2] and the best urease ever [3]. About 50 years later, it was shown that urease contains a metal (Nickel) at the active site. Hence, nickel-dependent ureases have been isolated from many bacteria, fungi [4, 5] and higher plants [6]. However, despite the fact that they are isolated from different sources, their structural conformations [7] and mechanisms of catalysis [1] are quite similar.

A urease apoenzyme (inactive) needs to be activated to facilitate higher urea catalysis. This process of activation requires participation of several accessory proteins that incorporate nickel into the urease forming catalytic site [6, 4].

In the same vein, activation of urease is somewhat important most especially in the area of applications. Today, urease has several clinical; analysis of urea in biological fluid [8] and artificial kidney development [9], industrial; wastewater treatment [8] and environmental remediation [10] and agricultural [11] importance. Although some works had been done on urease activity, however, this work focuses on the study of the mechanism of urease catalyzed-reaction so as to determine its rate of reaction and how it (urease) changes in response to changes in concentration of urea (substrate) in seedlings.

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Materials and Methods

Lima beans (*Phaseolus lunatus*), Mexican yam beans (*Parchyrrhizus tuberrosus*), Soya beans *Glycine max*, Pigeon pea (*Cajanus cajans*), Velvet beans (*Mucuna pruriens*), Kerstingiella (*Kerstings geocarpa*) and Black beans (*Vigna mungo*) seed were purchased from local market in Kaduna Nigeria. Acetone, Nessler’s reagent, Tris buffer, and other chemical used were of analar grade.

Germination

The various beans samples were layered on woolen material and also covered with the same material, and water was applied to moisturize the beans. Under this humid and warm condition, the bean seeds initially swelled, then began to germinate (develop radicles) after 72 hours. The germination process was stopped by freezing the beans for 12 hours followed by 2days sun drying.

Extraction of Urease from Germinated and Ungerminated Beans

Urease was extracted from different beans samples according to the method described by [12]. The beans samples were blended coarsely with laboratory mortar and pestle and 50g of the beans meal was soaked overnight in 100ml extraction buffer (0.025m Tris-acetate buffer, pH 6.5) at 4⁰C (Refrigeration temperature). The soaked beans meals was swirled for 2 minutes and then sieved with four layers of pre-washed and dried white muslin cloth, the filtrate was centrifuged at 15000rev/min for 15mins under refrigeration condition 4⁰C. The clear supernatant was collected while the sediment was washed and discarded. The filtrate from the protein was added to the general pool

Protein Assay

The protein was calculated according to method described by [13], after precipitation with acetone. This method for protein determination is based on the ability of the aromatic amino acid (Tyrosine, Tryptophan and phenylalanine) to absorb light at ultraviolet region of the light spectrum, 235-280nm.

Optimum pH

In order to determine the optimum pH for urease activity from germinated and ungerminated beans samples, enzyme activity were assayed in pH ranging from 5.5 to 8.0 at constant temperature 60°C and 0.2M urea for 30mins.

Optimum Temperature

To determine the optimal temperature up to which the urease enzyme from both germinated and ungerminated beans can withstand temperature, urease enzyme was suspended in Tris-acetate buffer (0.05 M) and optimum pH above, and incubated at different temperatures (30 to 80 °C) for 30 min before the activity was measured.

Determination of the Kinetic Parameters (V_{max} and K_m)

To determine the kinetic parameters (K_m and V_{max}) the substrate concentration was varied (0.1-0.6M), at optimum pH and temperature of urease from germinated and ungerminated beans. The values of K_m and V_{max} were determined using Lineweaver–Burke plots. The catalytic capacity (V_{max}/K_m) was determined for urease from germinated and ungerminated beans samples according to the method of [14].

Result and Discussion

Protien Level in Germinated and Ungerminated Beans

The results of protein level in germinated and ungerminated beans are presented in Table 1. Germination significantly ($p=0.05$) reduced the protein level in the beans when compared to the ungerminated beans samples. However, the result showed that, the effect of germination on protein level of *Cajanus cajan* was significantly ($p=0.05$) higher than other beans samples with 71.36% and 3.49fold decrease in protein level, whereas, the effect of germination on *Parchyrrhizus tuberosus* was least significant ($p=0.05$) with 2.89% and 1.03fold decrease in protein level when compared to others. From these results, it was observed that germination significantly ($p=0.05$) reduced the level of protein in beans samples studied. [17] had shown that, urease in conjunction with arginase utilizes seed protein reserve during germination. Additionally, according to [4], proteins, also act as urease-specific chaperones in the process of nickel fixation into urease active site by playing an activatory role during germination [6, 15, 16], thereby making free protein unavailable. It was therefore accepted that, naturally, as plants grow, the protein levels of the seedlings reduce as a result of biological handling of nickel and nickel trafficking in the urease system. However, the information about the role of proteins in nickel incorporation during urease activation is not limited to plants only. Bacteria such as *K. aerogenes*, *Helicobacter spp.* e.t.c and any other urea metabolizing organism make use of their accessory protein for urease maturation [3, 5]. Based on these facts, there was a reduction in protein level in germinated beans when compared to the ungerminated ones as shown in this study.

In the same vein, the pattern of decrease (folds and %) in protein level differs in the beans samples studied. These variations could be attributed to the fact that, each of the beans samples had different protein level.

Optimum pH and Temperature

The result of optimum pH for urease extracted from germinated and ungerminated beans samples are presented in Figure 1.1 (a-c) to 1.2 (a-c). The result showed that, the optimum pH of urease from both germinated and ungerminated beans samples ranged between 6.5 and 7.0. The results of optimum temperature for urease from germinated and ungerminated beans are shown in Figure 2.1 (a-c) to 2.2 (a-c). Results showed that, the optimum temperature for urease from both germinated and ungerminated beans samples ranged between 65 and 70°C.

The result showed that, the pH optimum for crude urease in both germinated and ungerminated beans samples shifted towards neutral and was stable within the pH range of 6.5-7.0. The result in this work is supported by other researchers. For instance, optimum pH for urease activity in

Jack beans [18, 19], *Rhizopus oryzae* [20] and surface black soil [21] were found to be 7.0, 7.0 and 6.45 respectively. The stability of urease activity at these pH values may be explained by the structural changes occurring in enzyme proteins caused by pH variation. The tertiary structure of a protein depends on interactions such as hydrogen bonding between enzyme amino acids functional groups. A change in pH can alter the ionization of these amino acid side chains and disrupt the native conformation and in some cases denature the enzyme. Hence, each enzyme has an optimal pH range that helps the maintenance of its native conformation in an environment, where it operates. The degree of ionization of the surface functional groups of amino acid residues is a function of the medium's pH. The main factor involved here is the titration of the ionizable group which maintains surface charge, in the active site, or stabilizes the enzyme resulting in an optimum pH for the enzyme. Observations from this work indicated that, germination did not affect optimum pH for urease activity in germinated beans samples. Because it has been shown that, the incorporation of nickel in the active site of urease during the process of germination alters the conformational state of the inactive (apo-urease) urease enzyme, to form the active urease (Holo-urease) [15]. Since the optimum pH of enzyme only depends on tertiary structure of protein, therefore, germination did not confer any special trait on urease that could influence the optimum pH, owing to the fact that, there was no difference in optimum pH of urease activity in germinated and ungerminated beans samples.

The result showed that, optimum temperature ranged between 60-70°C for both germinated and ungerminated beans samples. From the result, germination did not have effect on the optimum temperature of urease when compared to the ungerminated (Figure 2.1 and 2.2). [22], observed that, urease is a thermostable enzyme. Published works on optimum temperature for urease activities in Jack beans [18], *Rhizopus oryzae* [20], *Yersinia enterocolitica* [23], purified *Chenopodium album* leaves [24] are 60, 55, 65, 60 and 40°C respectively. The temperature dependence of an enzyme-catalyzed reaction exhibits an optimum because the thermodynamic increase of reaction rate is followed by a steep drop caused by thermal denaturation of the enzyme [25].

Substrate Effect and Enzyme Kinetics (V_{max} and K_m)

The results of substrate effect on urease enzyme activity are presented in Figures 3.1 (a-c) and 3.2 (a-c). From the results, urease activity follows the expected Michaelis-Menten curve. The results of reaction velocity (V_{max}) and the Michaelis-Menten's constant (K_m) are presented in Figures 4.1 (a,b) and 4.2 (a,b) and the summary of the kinetic parameters in Table 2. The V_{max} of urease from germinated beans samples was significantly ($p=0.05$) higher than the ungerminated beans, while the K_m showed a corresponding Michaelis-Menten pattern. It has been shown earlier that, germination influences proper assemblage of Ni^{2+} at the active site of urease enzyme (enzyme activation). From the result of this activation, no significant ($p=0.05$) difference in K_m values was observed in urease from germinated beans samples compared to the ungerminated except in *Glycine max*. This showed that the process of activation by germination increased the affinity of urease for its specific substrate urea, thereby making its active sites more accessible to its substrate. However, the K_m values varied between the urease in different beans samples studied. The values of the V_{max} were also different in each of the beans samples, germinated and ungerminated respectively. The effect of germination on K_m had a corresponding relationship with the V_{max} . That is, the activity of the enzyme depends on its affinity for urea. Therefore, the

increase in maximum velocity (V_{max}) observed in urease is due to the effect of germination on the urease active site compared to the ungerminated samples. This observation is further strengthened by the catalytic capacity (V_{max}/K_m) of urease in germinated compared to ungerminated beans samples respectively. Results presented in Table 2 showed clearly that, germination increased the catalytic capacity of urease. It was also deduced from the result that, with increased V_{max} and reduced K_m , the catalytic capacity of the enzyme is heightened. Therefore, the ratio V_{max}/K_m of an enzyme is an index of the catalytic capacity of enzyme through enzymatic reactions [14]. That is, the higher the affinity of the enzyme for the substrate (K_m), the higher the maximum velocity (V_{max}) and this will also increase catalytic capacity of the enzyme. Hence, the relationship of K_m and V_{max} , and the mechanism of nickel fixation into active site of urease by germination are in accordance with the principle of enzyme kinetics. [15] showed that, the K_m and V_{max} of urease from jack beans are 0.25mmol/L and 0.154mmol/mg respectively, whereas, [23] showed that, *Yersinia erocolitica* had K_m and V_{max} of 1.7 ± 0.4 mM and 7.29 ± 0.42 μ mol/min respectively. Comparing these results, urease from germinated beans samples is of better catalytic capacity than urease from these sources.

Although, there were obvious differences in the values of V_{max} and K_m of urease from the various beans samples. The comparison of V_{max} values among the beans samples studied (Table 2) showed that, there was no significant ($p=0.05$) difference in V_{max} of germinated *Glycine max* and *Cajanus cajan*. However, the V_{max} of urease in germinated *Mucuna pruriens* was significantly ($p=0.05$) higher compared to *Vigna mungo*, *Kerstings geocarpa*, *Phaseolus lunatus* and *Pachyrhizus tuberosus*. In general, the V_{max} values were higher in germinated than the ungerminated beans samples because germination had activatory effect on urease from samples studied. However, there was a consistent trend of higher catalytic capacity (V_{max}/K_m) as influenced by germination in all the germinated beans samples.

Hence, since germination increased the catalytic capacity of urease, it indicated that, plants during germination can efficiently hydrolyze both internally generated urea and the one in the soil. It therefore means that, the bulk flow of urea from hydrolysis of arginine by arginase [11] and soil into the plant increase the activity of the enzyme. That is, with an increased urea concentration, there is higher hydrolysis of urea (higher V_{max}) until the active site becomes saturated (optimum substrate concentration), thereby lowering the sensitivity of the enzyme to the substrate. As a result of this phenomenon, with increased substrate concentration, there is lower enzyme sensitivity to substrate at the optimum. Thus, the biochemical characteristics of urease during plant germination showed that the V_{max} is increased so as to metabolize urea generated in the plant and the urea supplied as fertilizer (external). This elucidates the importance of urea in agriculture, most especially in the area of soil fertility. Consequently, enzyme kinetic parameter is a function of substrate concentration and enzyme activity, and Michelis-Menten constants (K_m) for enzymes usually range between 10^{-2} and 10^{-5} mM for most enzymes [25].

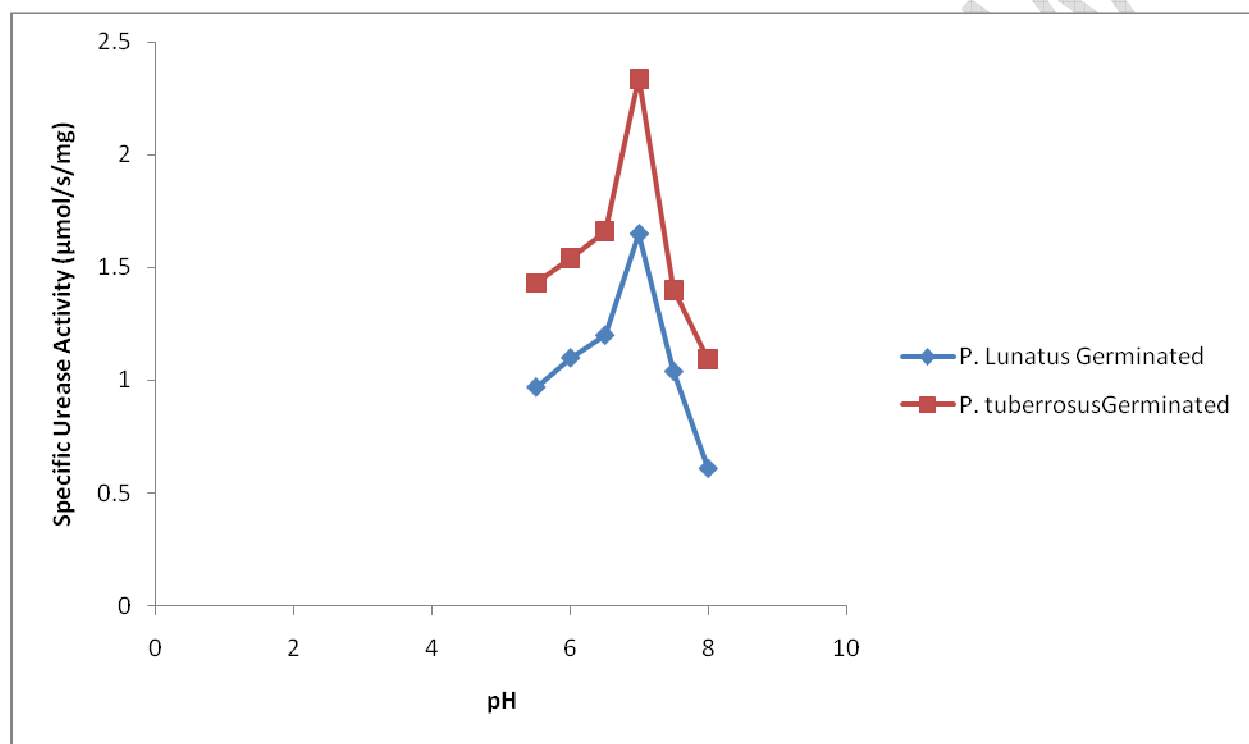


Figure 1.1 a: Effect of pH on urease from germinated beans.

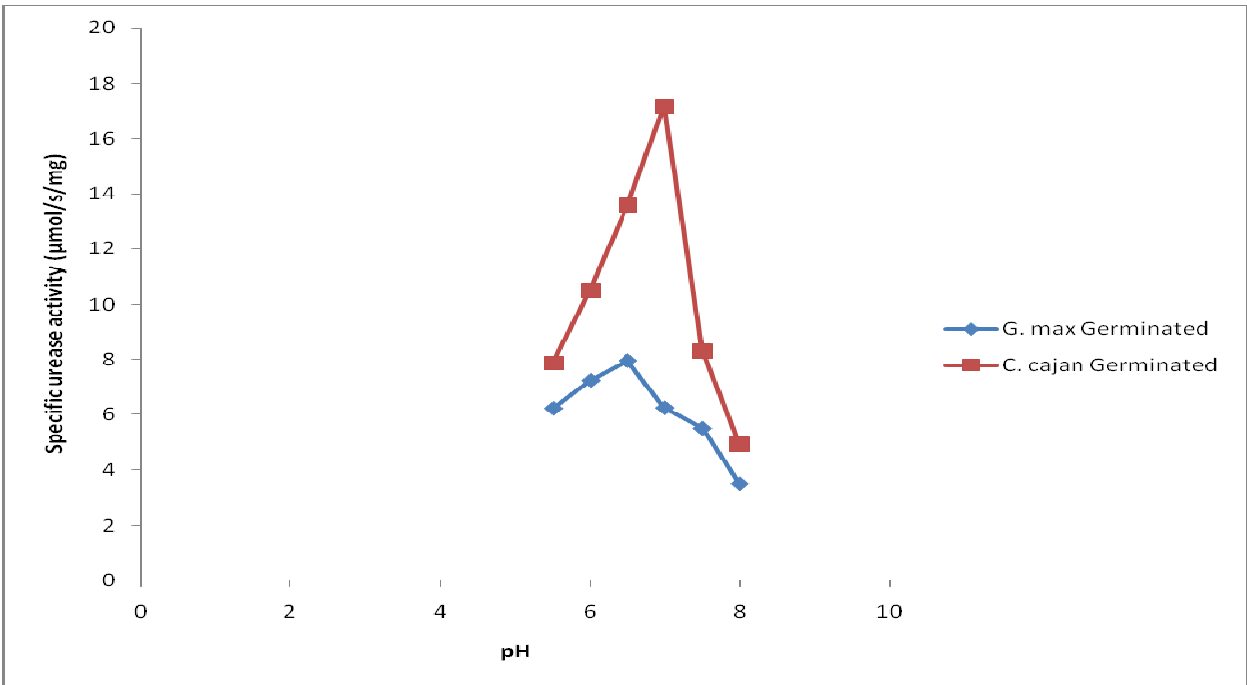


Figure 1.1b: Effect of pH on urease from germinated beans.

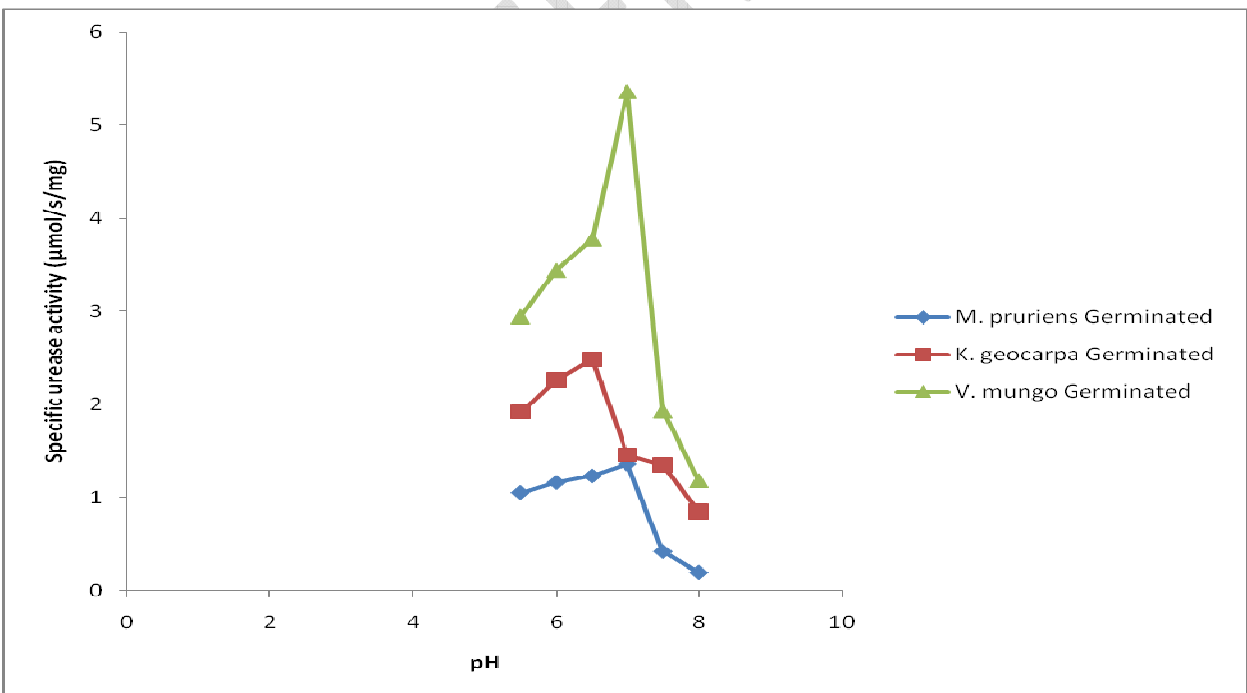
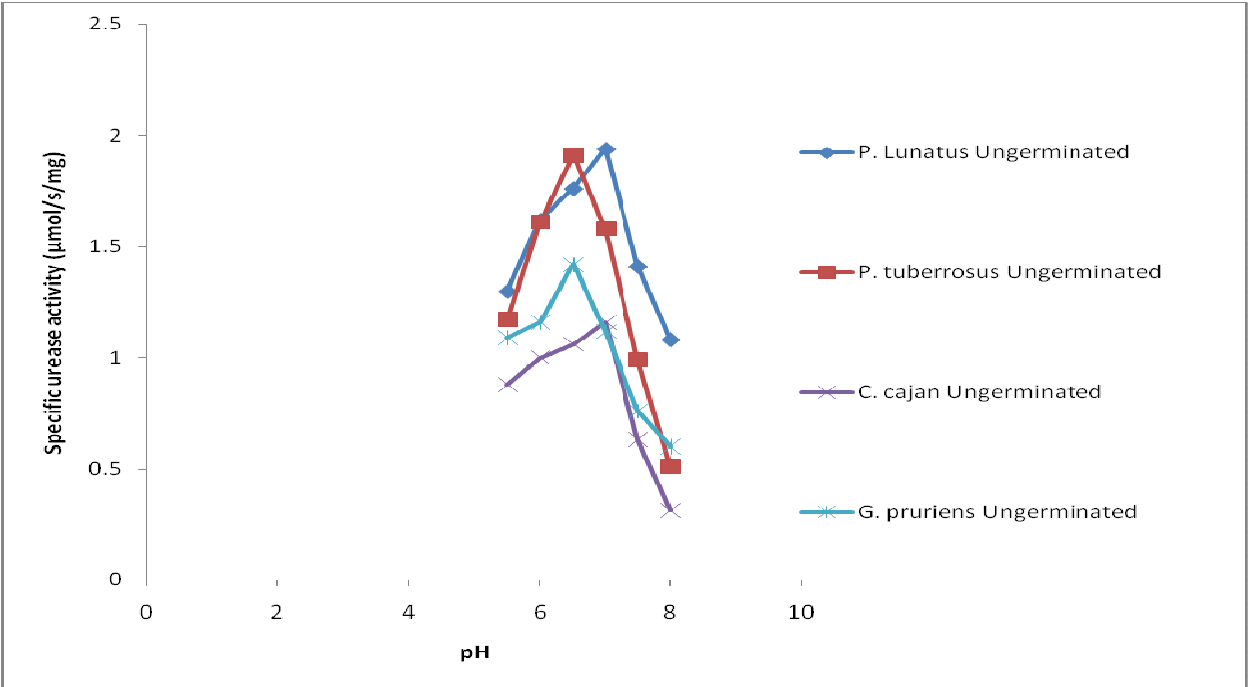


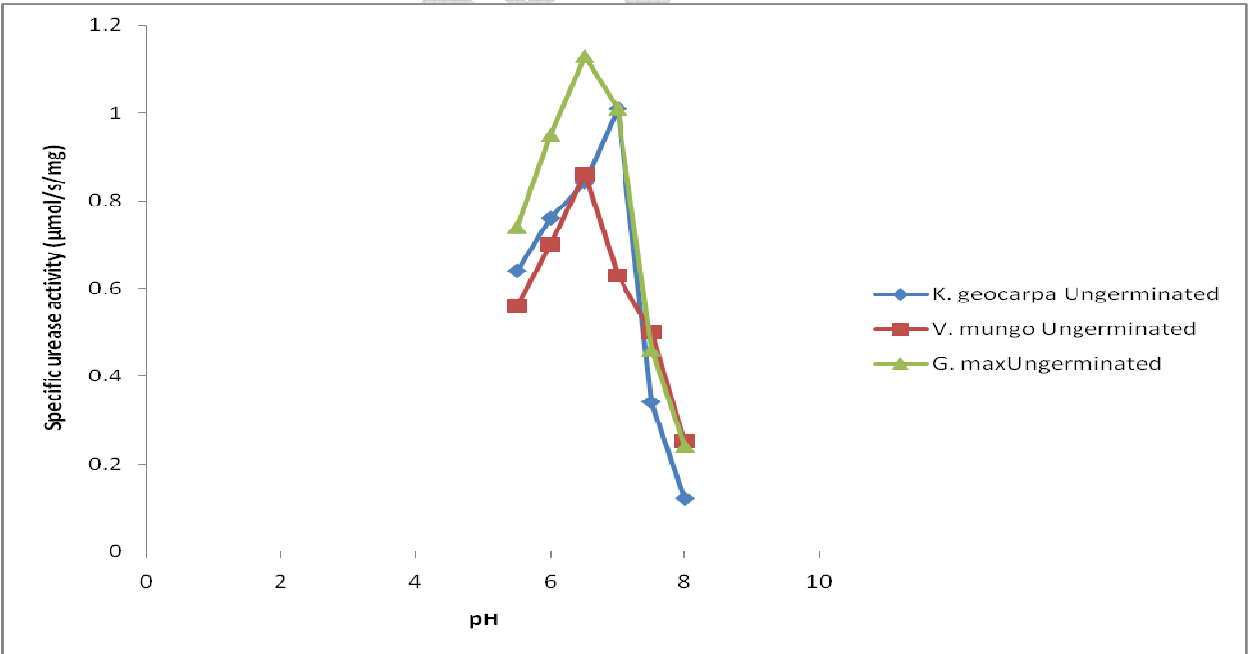
Figure 1.1c: Effect of pH on urease from germinated beans.



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320 Figure 1.2a: Effect of pH on urease from ungerminated beans.

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323 Figure 1.2b: Effect of pH on urease from ungerminated beans.

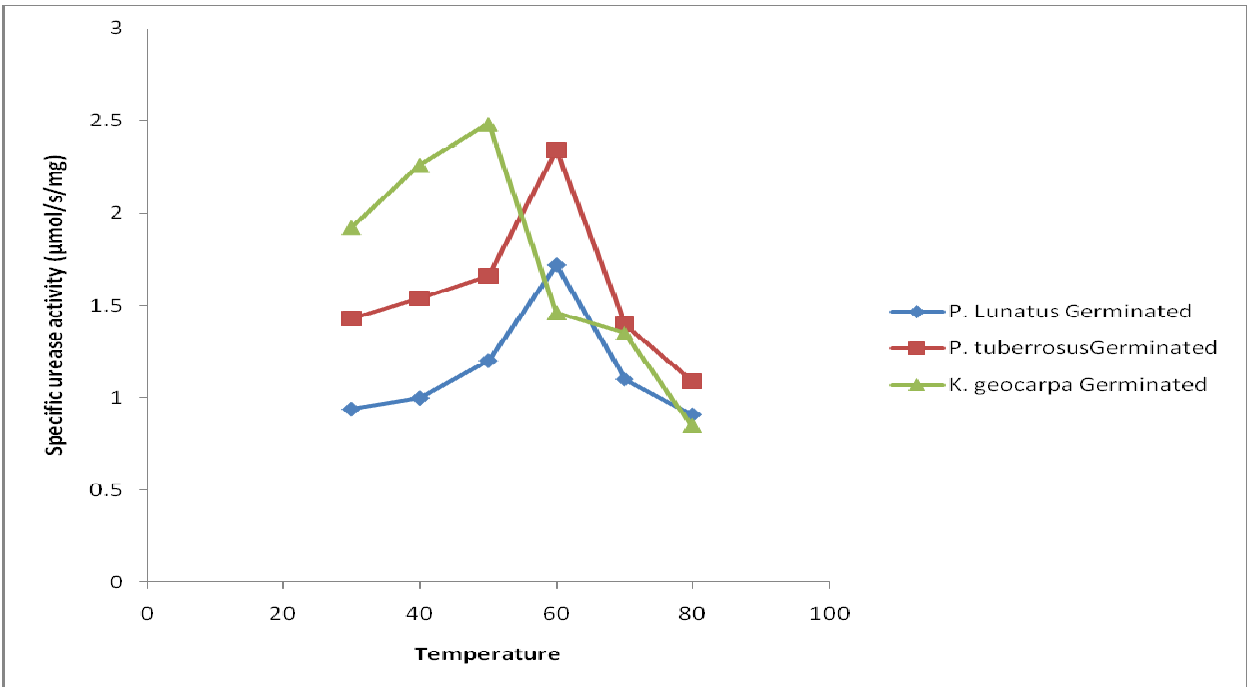


Figure 2.2a: Effect of temperature on urease from germinated beans.

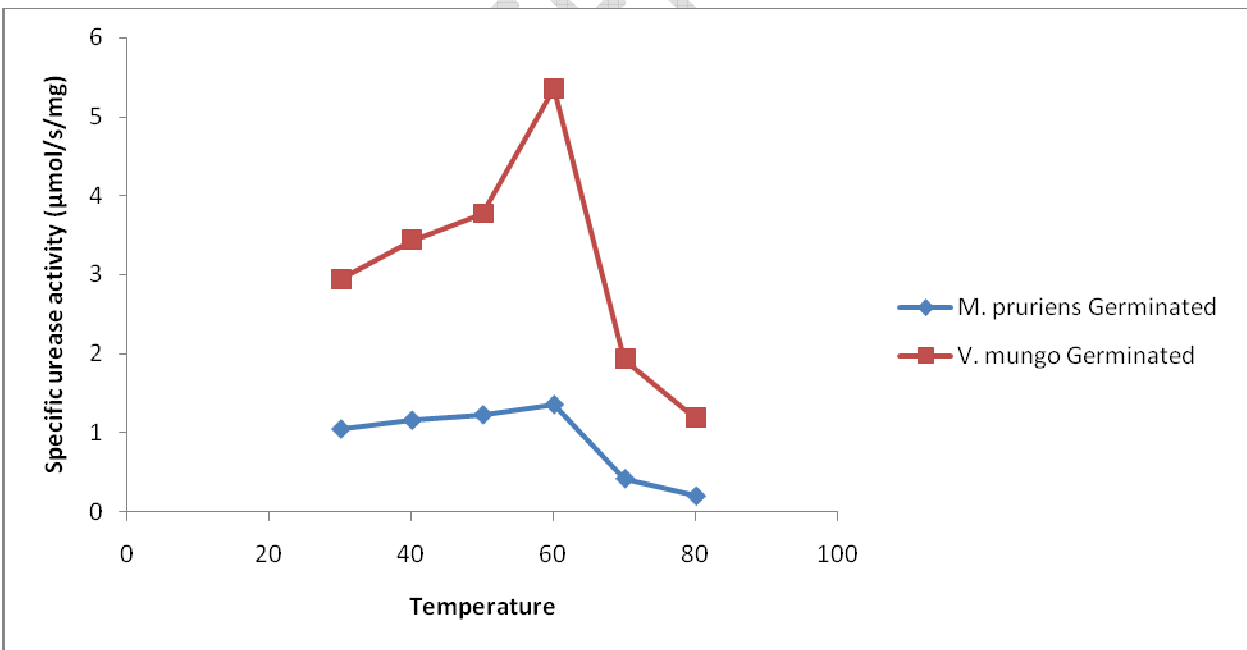


Figure 2.2b: Effect of temperature on urease from germinated beans.

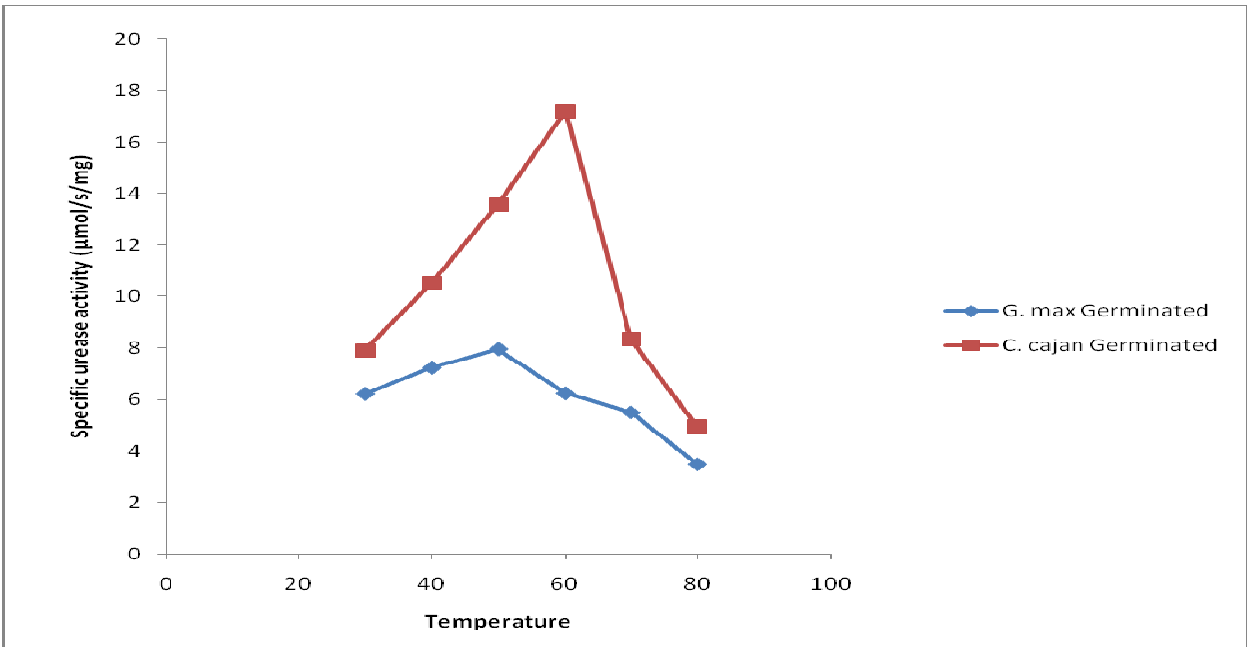


Figure 2.2c: Effect of temperature on urease from germinated beans.

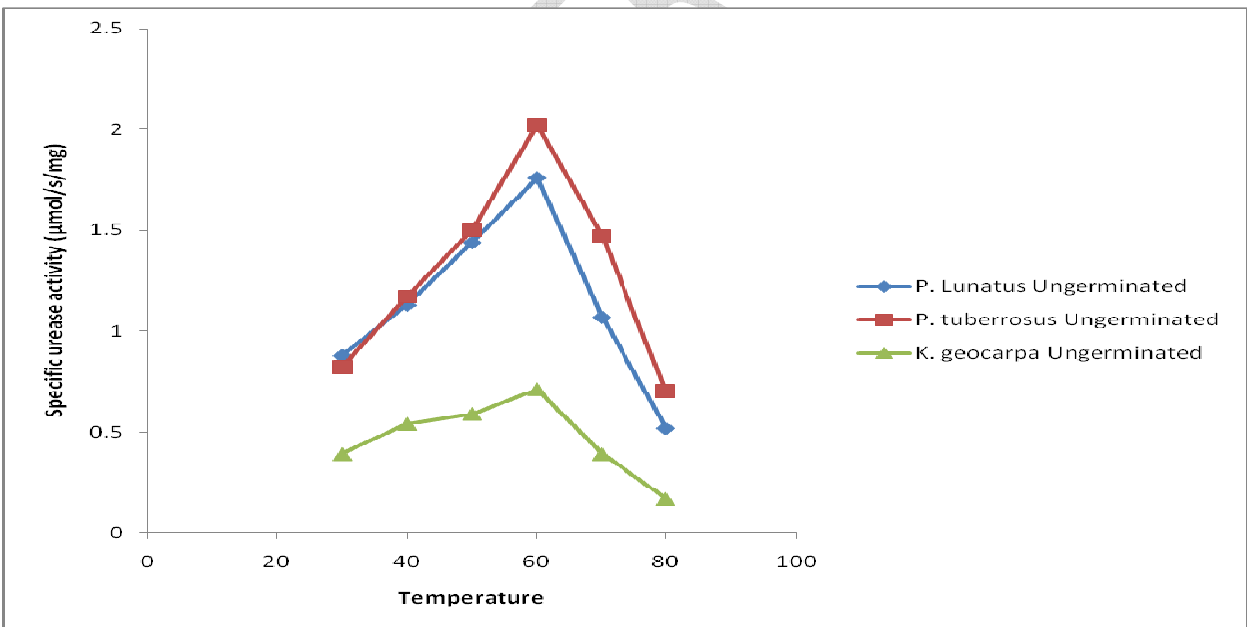


Figure 2.3a: Effect of temperature on urease from ungerminated beans.

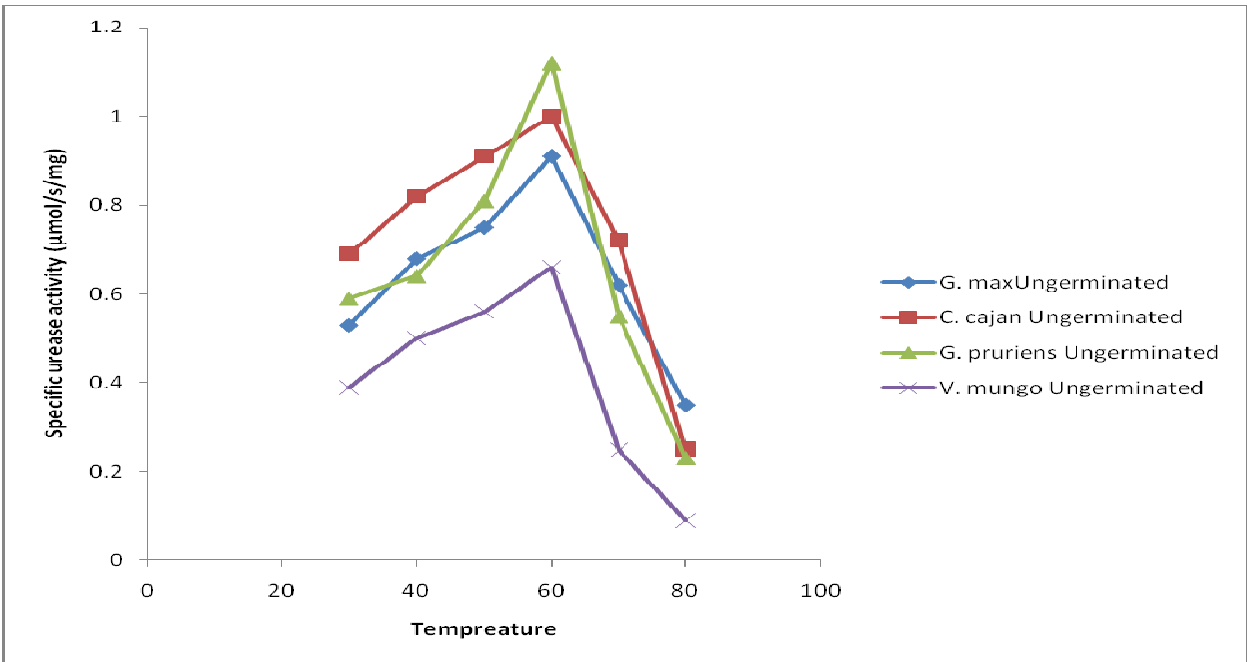


Figure 2.3b: Effect of temperature on urease from ungerminated beans.

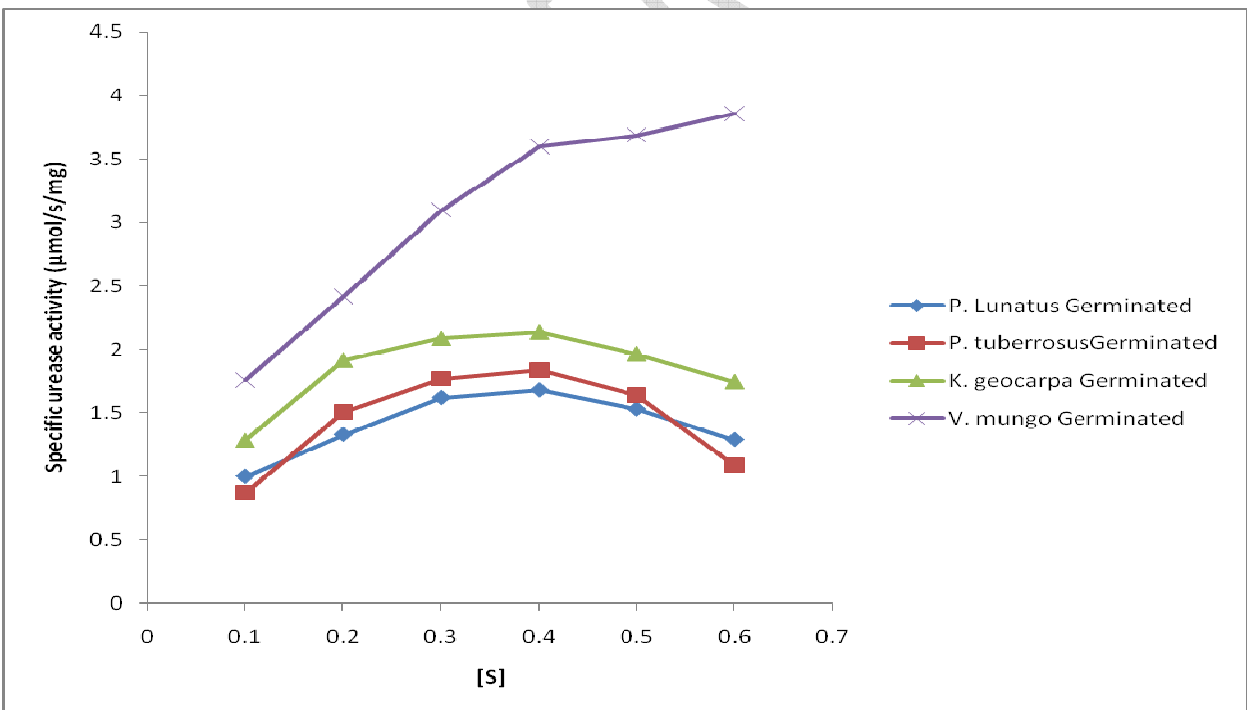


Figure 3.1a: Effect of substrate concentration on urease from germinated beans

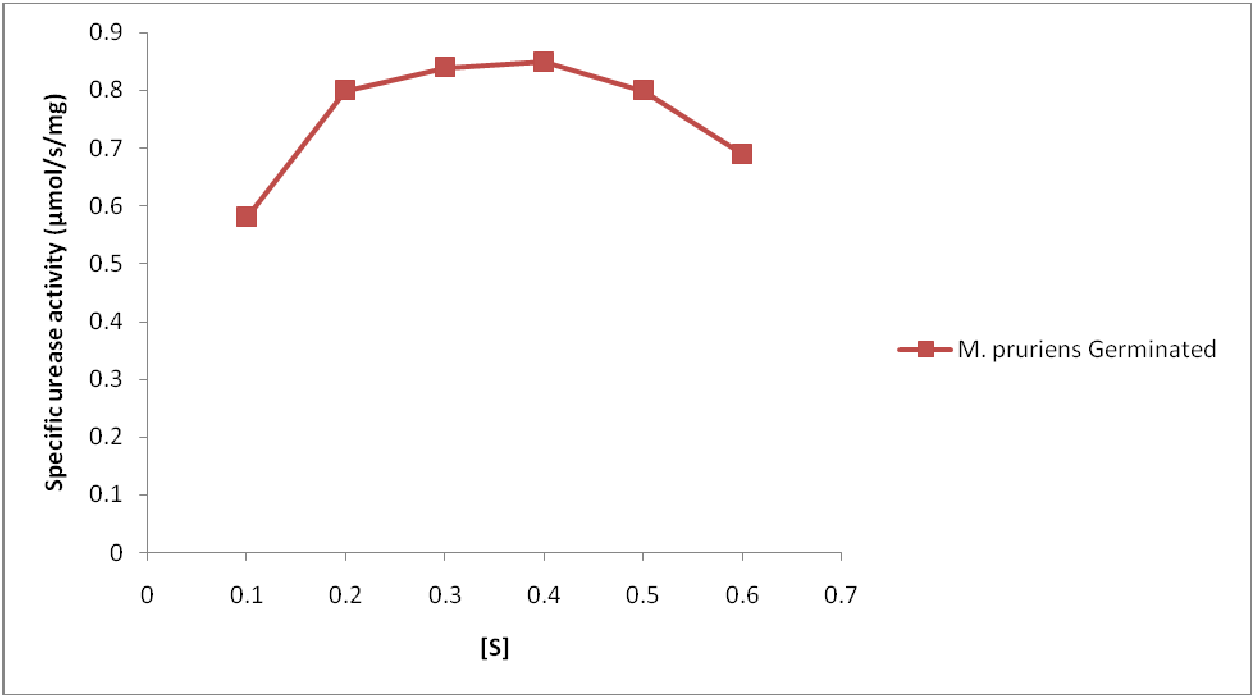


Figure 3.1b: Effect of substrate concentration on urease from germinated beans

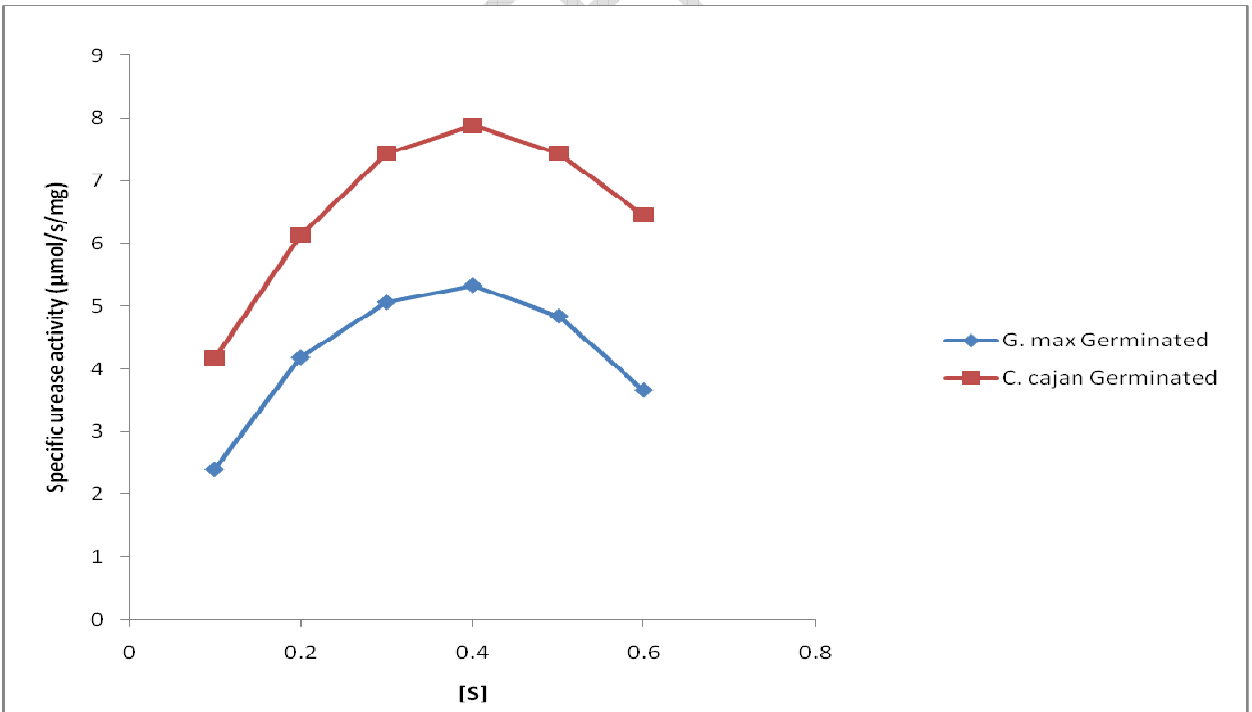


Figure 3.2c: Effect of substrate concentration on urease from germinated beans

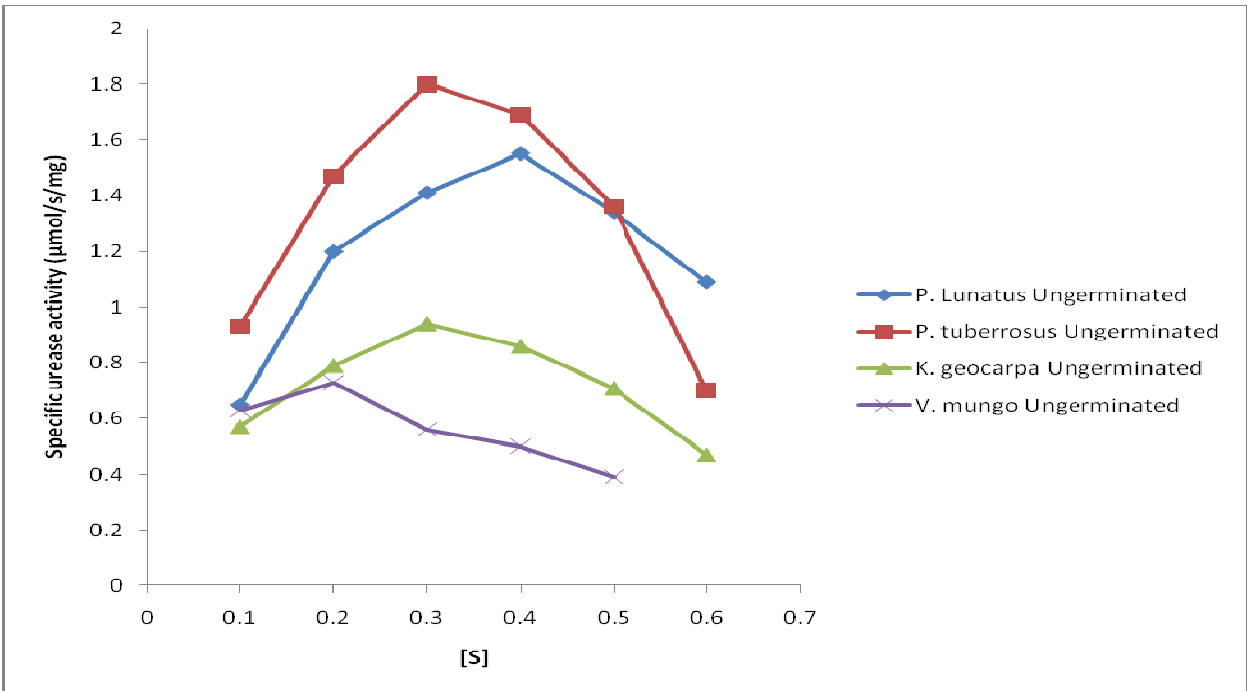


Figure 3.2a: Effect of substrate concentration on urease from ungerminated beans

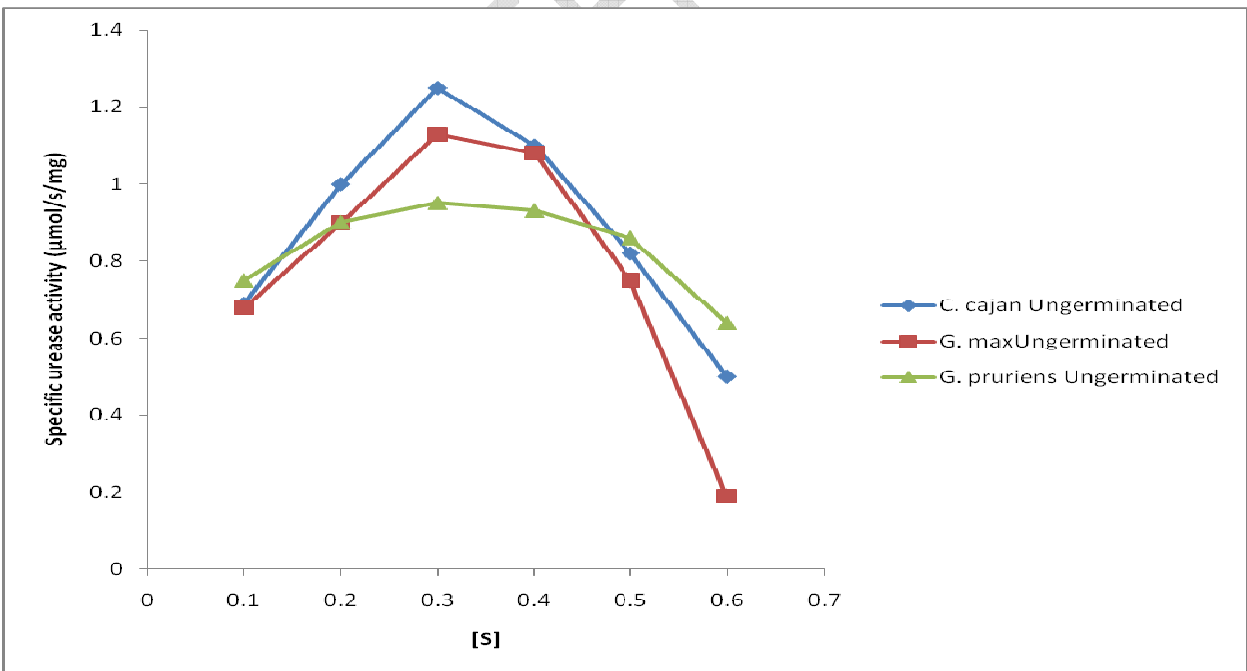


Figure 3.2b: Effect of substrate concentration on urease from germinated beans

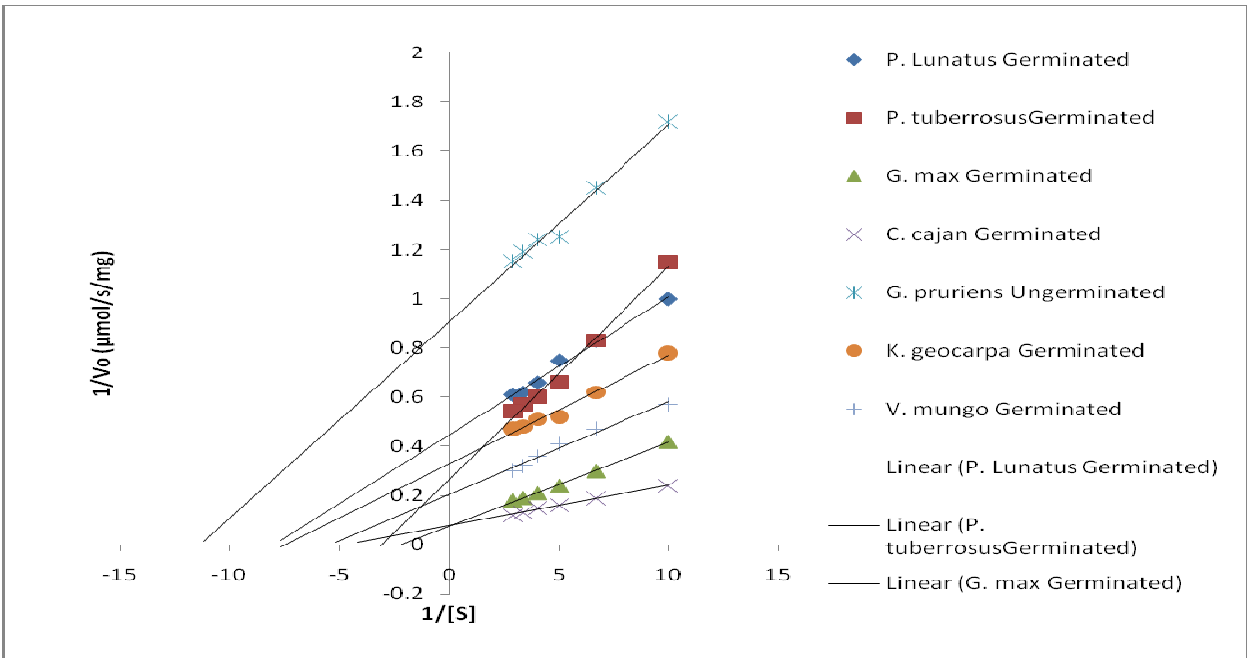


Figure 4.1a: Double reciprocal plot of rate V^{-1} vs $1/[S]$ for urease from germinated beans.

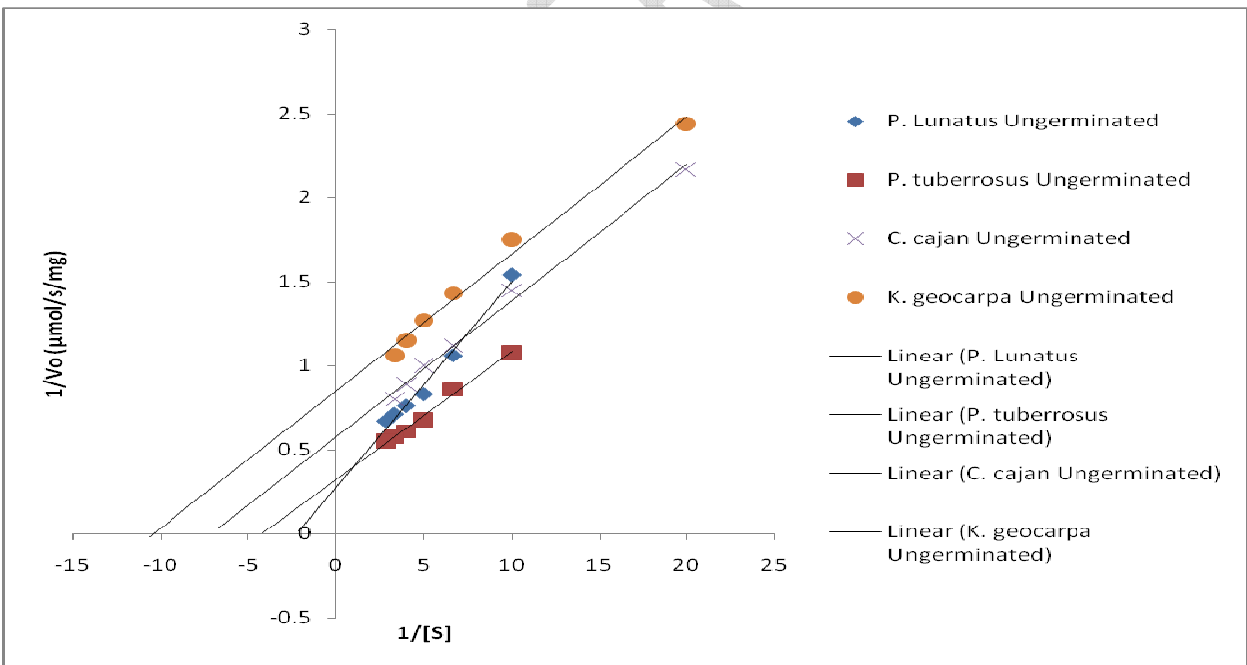


Figure 4.2a: Double reciprocal plot of rate V^{-1} vs $1/[S]$ for urease from ungerminated beans.

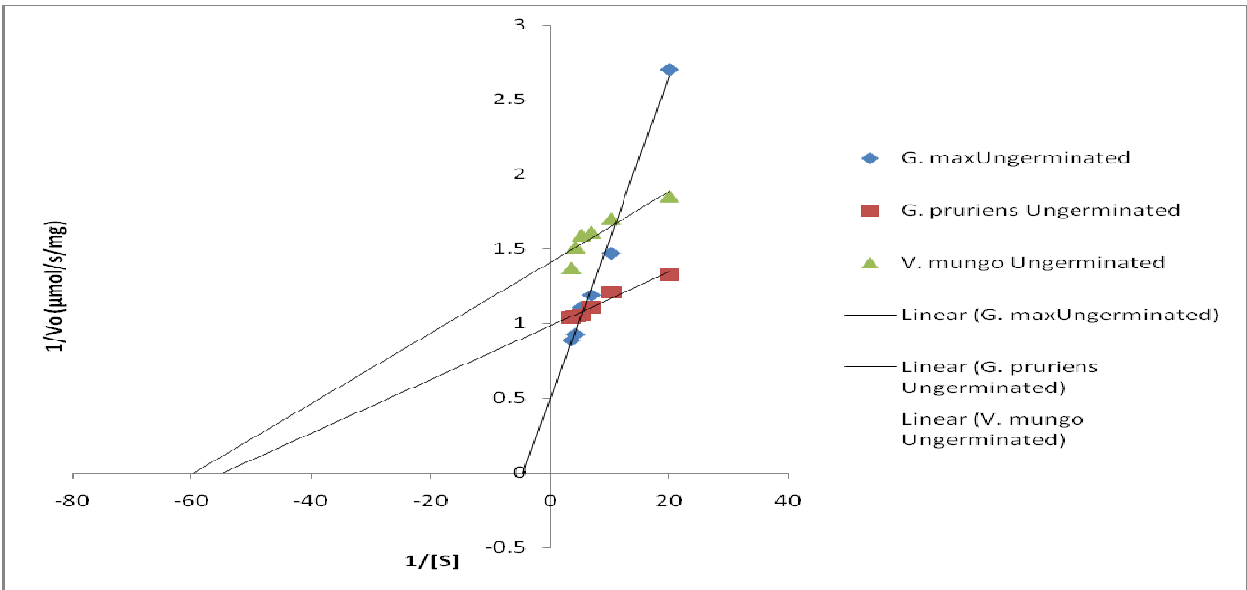


Figure 4.2b: Double reciprocal plot of rate V^1 vs $1/[S]$ for urease from ungerminated beans.

Table 1: Protein Level in Germinated and Ungerminated Beans Samples

Down columns, values with ns, *, ** and *** are statistically different from *Glycine max* at $p < 0.05$, < 0.01 and < 0.001 level of significance respectively. Across rows, values with different letters (superscripts) are statistically different at $p < 0.05$. Each data is mean $\pm$ SD of three replicates. NS: not significant.

Beans samples	Germinated	Ungerminated	% Decrease	Fold decrease
<i>Glycine max</i>	60.13 $\pm$ 8.89 ^b	90.17 $\pm$ 2.11 ^a	33.32	1.50
<i>Parchyrhizus tuber</i>	43.73 $\pm$ 0.47 ^{a***}	455.03 $\pm$ 7.65 ^{a***}	2.89	1.03
<i>Phaseolus lunatus</i>	46.93 $\pm$ 9.25 ^{a***}	51.00 $\pm$ 4.16 ^{ans}	7.98	1.09
<i>Cajanus cajan</i>	15.13 $\pm$ 2.14 ^{bns}	52.83 $\pm$ 2.02 ^{ans}	71.36	3.49
<i>Mucuna pruriens</i>	91.13 $\pm$ 5.54 ^{b***}	95.57 $\pm$ 7.68 ^{a**}	4.65	1.05
<i>Kerstings geocarpa</i>	29.30 $\pm$ 2.08 ^{b***}	67.17 $\pm$ 2.14 ^{ans}	56.38	2.29
<i>Vigna mungo</i>	22.37 $\pm$ 6.04 ^{b***}	72.90 $\pm$ 2.08 ^{ans}	69.31	3.26

Table 2: Kinetic Characteristic of Urease from Germinated and Ungerminated beans

Down columns, values with ns, *, ** and *** are statistically different from *Glycine max* at $p < 0.05$, < 0.01 and < 0.001 level of significance respectively. Across rows, values with different letters (superscripts) are statistically different at $p < 0.05$. Each data is mean $\pm$ SD of three replicates. NS: not significant.

Beans samples	V_{max} (U/mg)		K_m (mM)		Catalytic capacity	
	Germinated	Ungerminated	Germinated	Ungerminated	Germinated	Ungerminated
<i>Glycine max</i>	11.67 $\pm$ 0.73 ^b	1.64 $\pm$ 0.17 ^a	0.38 $\pm$ 0.04 ^b	0.15 $\pm$ 0.03 ^a	30.71	10.93
<i>Parch. tuber</i>	4.09 $\pm$ 0.47 ^{a***}	3.68 $\pm$ 0.88 ^{a***}	0.30 $\pm$ 0.10 ^{ans}	0.35 $\pm$ 0.15 ^{a**}	13.63	10.51
<i>S. stenocaarpa</i>	2.37 $\pm$ 0.38 ^{b***}	4.71 $\pm$ 0.23 ^{a***}	0.19 $\pm$ 0.06 ^{a*}	0.16 $\pm$ 0.02 ^{ans}	12.47	29.44
<i>P. lunatus</i>	2.23 $\pm$ 0.16 ^{a***}	1.87 $\pm$ 1.63 ^{ans}	0.15 $\pm$ 0.05 ^{a***}	0.29 $\pm$ 0.04 ^{a**}	14.87	6.45
<i>Cajanus cajan</i>	12.49 $\pm$ 0.56 ^{bns}	2.04 $\pm$ 0.65 ^{ans}	0.21 $\pm$ 0.01 ^{a*}	0.20 $\pm$ 0.11 ^{ans}	59.48	10.20
<i>Mucuna pruriens</i>	6.68 $\pm$ 0.81 ^{b***}	3.39 $\pm$ 0.20 ^{a**}	0.28 $\pm$ 0.05 ^{ans}	0.29 $\pm$ 0.22 ^{a*}	25.69	12.11
<i>Kerst. geocarpa</i>	3.06 $\pm$ 0.26 ^{b***}	1.32 $\pm$ 0.08 ^{ans}	0.14 $\pm$ 0.04 ^{a**}	0.13 $\pm$ 0.01 ^{ans}	21.86	10.15
<i>Vigna mungo</i>	4.95 $\pm$ 0.71 ^{b***}	1.02 $\pm$ 0.06 ^{ans}	0.18 $\pm$ 0.06 ^{a**}	0.046 $\pm$ 0.004 ^{b*}	27.50	22.17

Conclusion

In this study, it was observed that germination reduced the protein level and increased the catalytic capacity of urease when compared to ungerminated beans samples. In view of what other researchers have done, seed reserved protein was used to assemble the active site of urease by incorporation of nickel. It was discovered that, the optimum pH and temperature were not affected by germination, since both germinated and ungerminated beans samples have similar optimum pH and temperature. The results of this experiment also showed that, the K_m and V_{max} were greatly influenced by germination in that, germination increased V_{max} and reduced K_m . Therefore the catalytic capacity of the enzyme was increased. These express the significance of the application of urease in agriculture, clinical chemistry (blood urea analysis can efficiently be carried out with urease enzyme) and industry. The mechanism that activated urease activity during germination makes urease an important enzyme in agriculture and also suggests urea as a very good way of increasing soil fertility, owing to urease ability to change in response to change in urea concentration. And also, the incidence of leaf necrosis (dryness of leaf tips) which could have reduced surface area for photosynthesis due to excess urea in plant is equally masked by increase in urease activity.

The urease kinetics observed in *Mucuna pruriens* compared to *Glycine max* and *Cajanus cajan* showed that, the legume could be a very good and cheaper source of plant urease for industrial or clinical purposes most especially because of its nutritional disadvantages.

Acknowledgement

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516

517

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