Synthesis and characterization of a stable humic-urease complex: application to barley seed encapsulation for improving N uptake

Running title: **Synthesis of humic-urease complex for application to seed encapsulation**

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Abstract

BACKGROUND: Most of the N fertilizers added to the soil are not efficiently used by plants and are lost to the atmosphere or leached from the soil, causing environmental pollution and increasing cost. The barley seed encapsulation in calcium alginate gels containing free or immobilized urease was investigated to enhance plant utilization of soil N.

RESULTS: Urease was immobilized with soil humic acids (HA). A central composite face-centered design was applied to optimize the immobilization process, reaching an immobilization yield of 127%. Soil stability of urease was enhanced after the immobilization. The seed encapsulation with free urease (FU) and humic-urease complex (HUC) resulted in an urease activity retention in the coating layer of 46 and 24%, and in germination rates of 87 and 92%, respectively. Under pot culture conditions, the pots planted with seeds encapsulated with FU and HUC showed higher ammonium N (NH_4^+-N) (26 and 64%, respectively) than the control soil at 28 days after planting (DAP). Moreover, the seed encapsulation with FU and HUC increased the N uptake 83 and 97%, respectively, at 35 DAP.

CONCLUSION: The seed encapsulation with urease could substantially contribute to enhance the plant N nutrition in the early stages of seedling establishment.

KEYWORDS: Enzyme immobilization; Nitrogen availability; Plant growth; Seed coating; Seed encapsulation; Soil urease

INTRODUCTION

The application of N fertilizer is a common practice to increase crop productivity, since N is a limiting factor for plant growth. The global consumption of N fertilizer is closely related with the cereal production. Thus, in example, of the total N fertilizers consumed in world in 2010/11, 55.2% it is estimated to have been applied to cereal production.[1] Barley ranks fourth among the cereals in worldwide production. In Spain is one of the most important crops, representing up to 40% of the total cereal production.[2] However, most of N fertilizers added to the soil are not efficiently utilized by plants. For example, the N use efficiency (NUE) of N fertilizers in the global cereal production had been estimated at 33%.[3] Moreover, the N fertilizers that are not used by plants are lost to the atmosphere or leached from the soil which, besides increasing production cost, has adverse effects on the environment including greenhouse effect and surface and ground water pollution.[4]

Nitrogen occurs in soils in both inorganic and organic forms. Although there is evidence that soluble organic forms of N (such as amino acids) can be taken up by plants, inorganic N forms (NO_3^- and NH_4^+) have classically been considered to dominate plant uptake.[5] In this way, amidohydrolases play an important role in organic N mineralization in soils and thus, N supply for plants. These enzymes catalyze the hydrolysis of C-N bond other that peptide bonds in linear amides.[6] Among amidohydrolases, urease is one of the most studied enzymes. Urease is a ubiquitous enzyme in the soil, where it is produced by a vast number of both eukaryotic and prokaryotic organisms. This enzyme catalyzes the hydrolysis of urea to CO_2 and NH_3. Urea is commonly found in natural environment derived from urine excretion by animals (animal wastes) and the decomposition of nitrogenous compounds (i.e. amino

60 acids, purines) from dead organisms.[7] Moreover, urea is quantitatively the most
61 important N fertilizer.[5] In this way, urease activity of soil has a great importance in
62 agriculture due to urea serves as a readily available N source for the plant growth.

63 Enhance the NUE by plants is essential for a sustainable agriculture. Thereby, an
64 increase in the rate of utilization of soil organic N in the plant rhizosphere could
65 improve the NUE, avoiding or reducing the application of N fertilizers. This increase
66 may be achieved by means of seed coating with stabilized enzymes. The seed coating
67 allows the application of "materials" onto the natural seed coat in such a way that they
68 can affect the seed or soil at the seed–soil interface.[8] Enzymes can be stabilized by their
69 association with humic colloids, which protect them against proteolytic activity and
70 other processes leading to its inactivation in soil.[9,10] Pilar-Izquierdo *et al*.[11,12] reported
71 that the seed coating with alkaline phosphatase stabilized by immobilization in soil
72 humates enhanced the utilization by plants of accumulated soil P. A similar strategy,
73 using stabilized urease, may be helpful to mineralize naturally occurring soil organic N,
74 by hydrolysis of urea compounds to ammonia, and benefit N uptake by plants. Among
75 the techniques that can be used for seed coating (film-coating, pelleting, encrusting and
76 encapsulation),[8,13,14] the encapsulation in calcium alginate capsules[12] have been
77 successfully applied for the coating of barley seeds.[12,15] By means of encapsulation the
78 coating material is enclosed inside the calcium alginate capsule formed around the seed
79 and, after seed drying, a uniform film adhered to the seed surface is formed.[12] Applying
80 this technique no coating material losses or seed agglomeration have been observed,[15]
81 two important problems that can occur when seeds are coated.

82 In this context, the aim of this work was to synthesize a stable HUC and to study the
83 effect of the encapsulation of barley seeds in calcium alginate gels containing free or

84 immobilized urease on N uptake and plant growth. Specifically, this research can be
85 very valuable in order to develop "fertilizers" enriched with stabilized enzymes as a
86 means of bringing about–localized solubilization of soil organic N, avoiding the
87 deleterious effects of excessive application of N chemical fertilizers.

88 **MATERIALS AND METHODS**

89 **Experimental design**

90 Urease (urea amidohydrolase EC 3.5.1.5) from jack bean (*Canavalia ensiformis*)
91 (Calbiochem, San Diego, CA) was immobilized by co-flocculation with HA and Ca^{2+}.
92 A central composite face-centered star points design (CCF) was applied to evaluate the
93 effect of immobilization parameters. The properties and stability of the HUC were
94 studied. Barley seeds (*Hordeum vulgare* L., var. *vanessa*) were coated by encapsulation
95 in Ca-alginate gels containing FU or HUC. A germination test was performed to assess
96 the viability of the encapsulated seeds. A pot culture experiment was carried out to
97 study the effect of the coating on the plant growth and N uptake. The soil used in the
98 experiment was collected from the farm field surface of 0-10 cm at Ribera del Arlanza
99 in Burgos (Spain). Before use, the soil was air-dried at room temperature and then
100 gently ground to pass through a 2-mm sieve. Relevant soil properties are: pH 7.0, total
101 C 26.8 g kg^{-1}, organic C 20.7 g kg^{-1} and total N 13.2 g kg^{-1}.
102 Non-treated and treated seeds were germinated on a wet filter paper at 26 ºC in a
103 growth chamber (Conviron® E7) for 96 h. For each type of seeds, five seedlings were
104 transplanted to a plastic pot (containing 150 g of non-amended soil) at 10 mm below the
105 soil surface, and then the pots were transferred to a controlled environment chamber.
106 The plants were grown under environmental conditions of 20/15ºC photo/dark period

107 temperature and 16 h photoperiods per day. The pots were watered everyday with 15 ml
108 of deionized water. Each experiment was conducted five times. Five plants (one plant of
109 each replicate) were carefully freed from soil at 7, 14, 21, 28 and 35 DAP. After each
110 harvest the plant growth was determined by measuring length, dry weight and N
111 concentration of the shoot. Parallel, soil samples were taken in order to evaluate NH_4^+-
112 N and urease activity in the bulk soil. The inorganic N of soil was extracted according
113 to Keeny and Nelson[16] and the NH_4^+-N present in the solution was evaluated following
114 the method proposed by Keeney and Bremner[17] and modified by Beck.[18]

115 **Enzyme immobilization**

116 Urease was immobilized by co-flocculation with HA and Ca^{2+}. The HA were
117 obtained according to Busto *et al*.[19] The HUC were obtained as described by Pilar *et al*.
118 ,[20] with some modifications. Briefly, 2 mL of HA (ranging from 2 to 6 mg mL^{-1}) was
119 added to 2 mL of the urease solution (0.25 mg mL^{-1}) and 3 mL of phosphate-citrate-
120 borate buffer (PCBB: 100 mM phosphate, 57 mM borate, 36 mM citrate) at different pH
121 value (6.0-10.0). The resulting immobilization solution was mixed by orbital shaking
122 (150 rpm) at different agitation times (from 30 to 180 min) and temperatures (from 4 to
123 50 ºC). The mixture was flocculated by the addition of 2 mL of 0.5 M $CaCl_2$ and shaken
124 gently (150 rpm) for 6 h at 20 ºC. The suspension was centrifuged (15400 g) for 15 min
125 at 4 ºC and the pellet was washed five times with 0.1 M calcium acetate buffer (pH 4.5).
126 The insoluble complexes were resuspended in 10 mL PCBB (pH 7) and assayed for
127 urease activity. The immobilization yield (IY, %) was determined as follows:

128 $$IY\ (\%) = (A_{HUC} - A_H) \times 100/Ao \quad (1)$$

129 where A_{HUC}, A_H and A_0 were the urease activity of the HUC, the HA and the activity
130 added for immobilization, respectively.

131 **Assay of urease activity**

132 Urease activity was measured following the method reported by Kandeler and
133 Gerber[21] with some modifications. Briefly, the activity was determined by incubating 1
134 mL of 50 mM urea (Sigma, St. Louis, MO), 3.5 mL of PCBB (pH 7) and 0.5 mL of the
135 enzyme solution at 37 ºC for 15 min. For the urease activity of coated seeds and soil,
136 samples of five seeds and 1 g of fresh soil, respectively, were added to 4 mL of PCBB
137 (pH 7). After the reaction, 15 mL of 1 M KCl and 10 mM HCl was added to the mixture
138 and shaken gently (150 rpm) for 30 min at room temperature to extract the NH_4^+-N
139 released from urea. Then, 2 mL of 0.5 M $CaCl_2$ was added (to flocculate the organic
140 matter) to the mixture. The resulting solution was shaken gently (150 rpm) for 30 min at
141 room temperature and filtered (Whatman No. 6), and the NH_4^+-N present in the filtrate
142 was determined. In parallel, a standard curve of NH_4^+-N in the range of 0-20 μg mL^{-1}
143 was run. One enzymatic unit was defined as the amount of urease that produces 1 μg of
144 NH_4^+-N from urea over 1 h under the assay conditions.

145 **Properties and stability of the immobilized urease**

146 The Michaelis constants (K_m) of FU and HUC were determined by measuring initial
147 rates of the reaction at urea concentrations ranging from 6 to 30 mM. The K_m values
148 were calculated by using a Lineweaver-Burk plot. The effect of pH on FU and HUC
149 activity was studied using PCBB with pH values ranging from 4 to 10. The optimum
150 temperature for hydrolysis of urea was determined by measuring the urease activity at
151 incubation temperatures from 30 to 80º C.

152 To test the stability in soil, FU (0.0375 mg g^{-1} soil; 1190 U g^{-1} soil) and HUC (0.06
153 g g^{-1} soil; 1684 U g^{-1} soil) were incorporated in samples of soil described above. The
154 soil was held at 20ºC for 30 days and the residual urease activity measured periodically.

To calculate the activity of FU and HUC added to soil, activity of endogenous urease was also measured and subtracted. The residual activity of the FU and HUC was calculated as a percentage of the initial activity measured in soil after their addition.

Seed coating and germination test

Barley seeds were encapsulated in Ca-alginate gels containing FU and HUC using the method described by Pilar *et al.*[15] (modified from Patel *et al.*[22]) and modified as follow: the encapsulation solution contained 6% (w/v) carboxymethylcellulose (CMC) (Sigma, St. Louis, MO), 2% (w/v) calcium chloride and different enzyme/CMC solution ratios (FU: 0.1, 0.2 and 0.4 mg mL^{-1}; HUC: 0.1 g mL^{-1}). The coating urease activity was defined by the ratio of the urease activity of the encapsulated seeds to the activity of the free or immobilized enzyme used in the coating process (FU: 1159 U mg^{-1}; HUC: 213 U g^{-1}). Determinations were replicated five times. The germination test was assayed as described by Pilar-Izquierdo *et al.*[11]

Plant analysis

Length, dry weight and shoot N concentration were determined. Dry weight was determined by oven-drying the shoots at 105 ºC for 48 h. The inorganic N content was evaluated following the method of Hevia and Cioccia.[23] Plant N uptake was calculated by multiplying the dry weight by shoot N concentration.

Statistical methods

The immobilization parameters were optimized using response surface methodology.[24] A CCF was employed in this regard. Optimized conditions and response surfaces were calculated and drawn, respectively, with Statgraphics Centurion 16.1.07.

This software package was also used to fit the second-order model to the independent variables by using eq 2

$$y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i}^{i<j} \sum_{j} b_{ij} X_i X_j + e \quad (2)$$

where y is the dependent variable (response variable) to be modelled, X_i and X_j are the independent variables (factors), b_o, b_i, b_{ii} and b_{ij} are regression coefficients and e is the error. The model was simplified by dropping terms which were not statistically significant ($p>0.05$) by analysis of variance (ANOVA).

Analysis of variance and regression analysis were performed by Statgraphics Centurion 16.1.07 for Windows. The standard errors of the mean (SEM) are given in Tables and Figures. The standard errors of difference (SED) were calculated to test differences in urease activity of the soil, NH_4^+-N in soil and plant growth between encapsulated seeds and control (soil planted with non-treated seeds).

RESULTS

Urease immobilization

Preliminary experiments were carried out to screen the parameters that influence the preparation of the HUC and the experimental domain. From these experiments, four factors were investigated: temperature (X_1), HA concentration (X_2), immobilization pH (X_3) and contact time (X_4). Table 1 presents the experiment matrix, together with the experimental results. The immobilization yield ranged from 53 to 116%. High percentages of immobilization (>110%) were reached when a low level of temperature was used at high or central values of both HA concentrations, immobilization pH and contact time (runs 5 and 9, respectively).

Analysis of variance (ANOVA) was used to determine the adequacy and the significance of the quadratic model. The R^2 value of the model was 0.92, indicating that 92% of the variability in the response can be explained by the model. The adjusted R^2 value of 0.82 suggested that the model was significant. A very low value of the coefficient of variation (CV = 4.44%) clearly indicated a very high degree of precision and a good reliability of the experimental values.

A regression analysis was performed in order to determine the coefficients of the significant effects of each response variable. The effects showing less than 95% significance were omitted. In this sense, eq 3 explains the data obtained in this experiment.

$$IY\ (\%) = -79.794 - 0.217\ X_1 + 0.036\ (X_1)^2 - 0.169\ X_1X_3 - 0.003\ X_1X_4 - 4.796\ (X_2)^2 + 0.043\ X_2X_4 - 0.037\ X_3X_4 \quad (3)$$

The response surface plots for the immobilization yield showed an increase as the temperature was decreased and a slight increase when the immobilization time increased (Figure 1a). The effect of the HA concentration and pH on the response at a fixed temperature and time of 4 ºC and 180 min is illustrated in Figure 1b. The response value reached its highest level at 4.5 mg mL^{-1} HA, whereas pH showed a maximum at pH 8.0. Under these conditions, the model predicted an immobilization yield of 127%. To confirm this result, a validation assay was conducted in the conditions imposed at the optimum. In this assay an immobilization yield of 121% was obtained. This value is in good agreement with the predicted values for the analyzed response, validating the mathematical models attained in the studied region.

Properties and stability of the immobilized enzyme

Figure 2 shows Lineweaver-Burk plots for FU and HUC. The FU followed Michaelis-Menten kinetics over the range of substrate concentration studied. However, the HUC did not follow pure Michaelis-Menten kinetics. The shape of this graph was typical of enzymes exhibiting substrate inhibition at high substrate concentration.[25] In this case, the determination of apparent K_m was possible by using the linear portion of the curve at low substrate concentration.[26] The K_m value for the HUC was higher (129.5 mM) than that of the FU (19.5 mM), increasing it by a factor of 6.6. The pH activity profile of both FU and HUC is plotted in Figure 3a. The FU showed optimal activity at pH 5.5, whereas the optimum pH of the HUC was determined at pH 6.2.

The dependence of the urease activity on temperature is shown in Figure 3b. The optimum temperature of both FU and HUC was found at 60ºC. At temperature values of 70 and 80ºC, the HUC showed the activities of about 8.4 and 9.4% higher than FU, respectively, which can be due to the protective effect of the immobilization support.

The stability of both FU and HUC in soil was studied (Fig. 4). The FU was almost inactivated after 3 days, whereas the HUC retained about 35% and 13% of its initial activity after 7 and 30 days, respectively, of its addition to soil.

Seed encapsulation and germination test

The effect of the barley seed encapsulation with different FU concentration on the coating urease activity was studied. When encapsulation was performed with enzyme/CMC solution ratios of 0.1 and 0.2 mg mL^{-1} the urease activity retained in the coating layer was similar (31 and 33%, respectively). However, at the highest enzyme concentration assayed (0.4 mg mL^{-1}), a significant increase ($p < 0.01$) in the coating urease activity was observed (46%). The seed encapsulation with immobilized urease was carried out at a fixed HUC/CMC solution ratio of 0.1 g mL^{-1}. Under this assay

condition, the coating urease activity was 24%. This enzyme concentration was chosen in order to achieve an adequate level of urease activity in the coating layer. In this way, Pilar-Izquierdo *et al.*[12] found that the seed encapsulation with higher concentrations of humic-enzyme complexes resulted in a remarkable increase in the thickness of the seed-coating layer, which limited the substrate diffusion. As far as the germination of seeds is concerned, the seeds encapsulated with FU and HUC germinated 88% (for all enzyme concentration assayed) and 92%, respectively, in comparison with non-treated seeds.

Effect of seed encapsulation on urease activity and NH_4^+-N of soil

The effect of seed encapsulation on urease activity and NH_4^+-N of the soil is shown in Table 2. A significant increase ($p < 0.05$) in the urease activity in the bulk soil of pots planted with encapsulated seeds was observed at the end of the study period. Thus, at 35 DAP, the soil urease activity of pots planted with seeds encapsulated with FU and HUC was increased 15 and 21%, respectively, in comparison with control soil. No significant differences between the seed coating with FU and HUC were found. The NH_4^+-N in soil was increased with time to maximum rates at 28 DAP in all pots, and then decreased to a concentration similar to that at the start of the experiment. Moreover, this increase was significantly higher in pots planted with treated seeds from 21 DAP. Thus, the NH_4^+-N in soil planted with seeds encapsulated with FU and HUC increased 56 and 21%, respectively, at 21 DAP, and 26 and 64%, respectively, at 28 DAP. However, at the end of the study period, only the pots planted with seeds encapsulated with HUC showed a significant increase (23%) in the NH_4^+-N in soil.

Effect of seed encapsulation on plant growth and N uptake

The effect of seed encapsulation with FU and HUC on plant growth and N uptake is shown in Table 3. The seed encapsulation significantly increased the shoot length during the first four weeks of plant growth. Thus, at 7 DAP, plants grown from seeds encapsulated with both FU and HUC showed higher shoot length (38%) than the control. However, although control plants grew more slowly, they reached a shoot length similar to the plants grown from encapsulated seeds at the end of the study period. The shoot dry weight of plants grown from coated seeds, like the shoot length, was significantly increased ($p < 0.01$) during the first three weeks of growing. Moreover, the seed encapsulation with FU increased the shoot dry weight 15%, in comparison with control plants, at the end of the study period. The shoot N content was significantly higher ($p = 0.00$) in plants grown from seeds encapsulated with FU and HUC than in control plants, showing an increase of 55 and 87%, respectively, after 35 DAP. Moreover, this increase was significant higher in plants grown from seeds encapsulated with HUC than that with FU since 21 DAP to the end of the experiment. A decrease in the shoot N concentration with time was observed in all pots, which as expected, since is known that the N content of tissues decreases with the life cycle of plant.[27] Finally, the seed encapsulation with urease significantly increased ($p = 0.000$) the N uptake by plants. Thus, at 35 DAP, the plants grown from seeds encapsulated with FU and HUC showed an increase in the N uptake of 83 and 97%, respectively.

DISCUSSION

Stabilized extracellular soil enzymes, in particular hydrolases such as urease, play an important role in the availability of nutrients for plants. Soil enzymes can be stabilized through association with clay minerals, particulate organic matter and HA.[10,30] In this

291 way, soil urease is crucial for the N cycle and its association with HA is a fundamental
292 requirement for its stability.[28]

293 The chemical complexity of the humic substances still prevents a full understanding
294 of their interactions with reactive biomolecules presents in the environment, such as
295 extracellular enzymes.[29] However, several mechanisms have been proposed for the
296 stability of humic-enzyme complexes. Thus, humic compound may adsorb enzymes by
297 ion exchange, covalent complexation and hydrogen bonding.[30] Moreover, enzymes can
298 be trapped within the humic net and also immobilized at the surface by adsorption
299 forces.[31] Recent studies applying nuclear magnetic resonance spectroscopy indicate that
300 enzymes are stabilized by encapsulation in humic aggregates[32] or by non-covalent
301 interactions, such as van der Waals, H-bonds and π-π bonds.[29] Anyway, insufficient
302 data are available to draw definitive conclusions about the nature of the interaction in
303 the samples examined here.

304 It is well known that immobilization can affect enzyme activity and catalytic
305 properties. In the present study, the binding of urease to soil HA increased the enzyme
306 activity, reaching immobilization yields higher than 100% under optimal conditions.
307 Busto *et al.*[19] also observed immobilization yields higher than 100% in humate-β-
308 glucosidase and humate-carboxymethyl cellulase complexes by 180 and 350%,
309 respectively. In contrast, Pilar *et al.*[18] reported a loss of activity in phosphatase-humate
310 complexes with respect to the initial enzyme concentration. The enzymes attached to the
311 humic matrix have a microenvironment quite different than that of free enzymes in
312 solution,[23] because they are affected by different diffusion, charge and steric effects.[31]
313 Therefore, the activity of the humic-enzyme complexes toward substrates may be
314 different from that of the free enzyme.

With regard to the catalytic properties of the immobilized urease, the increase in the K_m value observed after immobilization indicate a lower affinity of the immobilized enzyme by its substrate in comparison with the soluble enzyme, which may be caused by steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding or by diffusion limitations of the substrate.[33] In general, enzymes bound to soil components have higher K_m values than the free enzymes.[34] The shift in the pH-activity curves towards alkaline region observed upon immobilization has been attributed to the microenvironment created by the charged carriers. Tan[35] reported that dissociation of COOH in humic matter starts at pH 3.0, and the humic molecule becomes negatively charged. As a result of a negative surface charge the pH in the microenvironment of the enzyme will be lower than the bulk pH. A higher bulk pH is required in providing an optimum pH in the microenvironment of the enzyme and hence a shift to higher values is encountered.[36] The higher stability of the immobilized urease added to the soil, in comparison with their soluble counterpart, suggests that the association of the enzyme with the HA protects it from degradation in soil. Therefore, given the increase in the activity and stability of urease immobilized in the humic support, the seed coating with the HUC synthesized is expected to improve the NUE in the plant rhizosphere.

The values of enzyme activity retained to the seed coating layer may be related with the coating process. Firstly, a leakage of the enzyme can occur during the encapsulation process, probably before the alginate capsule was formed,[37] which could result in a decrease in the calculated coating yield. Thus, Vikartovska *et al*.[38] reported enzyme losses of about 56% during the first step of the encapsulation of glucose oxidase in polyelectrolyte complex capsules. Moreover, the enzyme added during the seed

339 encapsulation is enclosed inside the capsule formed, which could cause diffusional
340 restrictions so that not all the activity of the immobilized enzyme was expressed.[39]
341 Germination of coated seeds can be affected by the coating composition, coat
342 thickness and species, among others, which may increase, decrease or not affect the
343 germination rates.[40] In this work, a slight decrease in the germination rate of
344 encapsulated seeds was observed. These results were similar to those reported by Pilar-
345 Izquierdo *et al*.[12] when barley seeds were encapsulated with phosphatase free and
346 immobilized in HA. This decrease in the germination rate could be attributed to the
347 coating acting as an oxygen barrier or the hardness of the Ca-alginate capsule formed
348 around the seed. Film-coating polymers decreased seedling emergence in wet soils with
349 low oxygen diffusion rate.[41] Moreover, a delay of 24 h in germination of encapsulated
350 seeds in comparison with control was observed. In this way, Ester *et al*.[42] found that the
351 germination of seed film-coated with an insecticide was delayed, although after a few
352 days the growth of plants from film-coated seeds was similar to control plants. Since the
353 FU/CMC solution ratio had no effect on the germination percentage of encapsulated
354 seeds, 0.4 mg mL^{-1} was selected as the optimal enzyme concentration taking into
355 account the coating urease activity.
356 Increases in NH_4^+-N concentration with time in soils incubated under laboratory
357 conditions have been reported.[4,43] Moreover, Abbasi *et al.*[4] also observed a similar trend
358 in the NH_4^+-N concentration in a control soil, in which this concentration increased with
359 time to a maximum and then declined to background level at the end of the experiment.
360 The increase in the NH_4^+-N with time indicates that the mineralization of organic N
361 present in soil has occurred. Moreover, the significantly higher NH_4^+-N concentration
362 observed in pots planted with encapsulated seeds from 21 DAP, in comparison with

363 control soils, may be attributable to the urease activity added in the seed coating layer.
364 Thus, although increases in the urease activity of bulk soil were only detected at the end
365 of the study period, a higher rhizosphere urease activity in the first weeks of the pot
366 experiment could be expected. On the other hand, the decrease in the soil NH_4^+-N
367 concentration observed in all pots at the end of the study period could be partially due to
368 the nitrification of NH_4^+-N to NO_3^--N. Nitrification is often considered the major fate of
369 NH_4^+ in agricultural soil.[44] Thus, increases in the NO_3^--N in soils incubated under
370 laboratory conditions have been reported.[4,45] Furthermore, the decrease in the soil NH_4^+-
371 N could be also due to the increase observed in the plant N uptake. In addition, the
372 changes in the NH_4^+-N concentration observed in the pots with time could be related
373 with the microbial activity.[44,46]

374 **CONCLUSION**

375 In this research the synthesis of a stable HUC and the application of FU or HUC to
376 barley seed encapsulation for improving plant utilization of soil N was studied. The
377 results obtained in the pot experiment showed the beneficial effect of the seed
378 encapsulation by increasing the shoot N content and the N uptake. No significant
379 differences in the N uptake of plants grown from seeds encapsulated with FU and HUC
380 were found at the end of the study period. Nevertheless, althought the shoot N content
381 in plants grown from seeds encapsulated with HUC was higher than in plants grown
382 from seeds encapsulated with FU, at 35 DAP, the value of N content was in the same
383 order of magnitude. These results, together with the higher NH_4^+-N concentration found
384 in the pots planted with encapsulated seeds, could be explained by a higher urease
385 activity in the root vicinity as a consequence of the coating applied on the seeds.

The seed encapsulation with urease could potentially enhance the N plant nutrition, without the massive addition of N fertilizers. However, further research to evaluate the NUE is necessary. Moreover, it would be necessary to carry out field experiments to evaluate the effect of the seed encapsulation with urease on the plant growth and grain yield under natural conditions.

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REFERENCES

1 Heffer P, Assessment of fertilizer use by crop at the global level 2010-2010/2011. *International Fertilizer Industry Association (IFA)*. Paris, France. (2013).

2 Mateo EM, Gimeno-Adelantado JV, Soria JM, García-Esparza MA, Mateo-Castro M and Jiménez M, Barley, a potential source of ochratoxin A in food in the framework of climate change, in *Industrial, Medical and Environmental Applications of Microorganisms*, ed. by Méndez-Vilas A. Wageningen Academic Publishers, The Netherlands, pp. 255-260 (2014).

3 Raun WR and Johnson GV, Improving nitrogen use efficiency for cereal production. *Agron J* **91**:357-363 (1999).

4 Abbasi MK, Hina M and Tahir MM, Effect of *Azadirachta indica* (neem), sodium thiosulphate and calcium chloride on changes in nitrogen transformations and inhibition of nitrification in soil incubated under laboratory conditions. *Chemosphere* **82**:1629-1635 (2011).

5 Miller AJ and Cramer MD, Root nitrogen acquisition and assimilation. *Plant Soil* **274**:1-36 (2004).

6 Ekenler M and Tabatabai MA, Arylamidase and amidohydrolases in soils as affected by liming and tillage systems. *Soil Till Res* **77**:157-168 (2004).

7 Wang WH, Köhler B, Cao FQ and Liu LH, Molecular and physiological aspects of urea transport in higher plants. *Plant Sci* **175**:467-477 (2008).

8 Scott JM, Seed coatings and treatments and their effects on plant establishment. *Adv Agron* **43**:43-83 (1989).

9 Burns RG, Interaction of enzymes with soil mineral and organic colloids, in *Interactions of Soil Minerals with Natural Organics and Microbes*, ed. by Huang PM and Schnitzer M. Soil Science Society of America, Madison, pp. 429-451 (1986).

10 Burns RG, Deforest JL, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, Weintraub MN and Zoppini A, Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biol Biochem* **58**:216-234 (2013).

11 Pilar-Izquierdo MC, Ortega N, Perez-Mateos M and Busto MD, Barley seed coating with free and immobilized alkaline phosphatase to improve P uptake and plant growth. *J Agr Sci* **150**:691-701 (2012).

12 Pilar-Izquierdo MC, Busto MD, Ortega N and Perez-Mateos M, Seeds encapsulated in calcium-alginate gels with phosphatase and humate-phosphatase complexes for improving phosphorus bioavailability. *Agron J* **105**:1565-1570 (2013).

13 Taylor AG, Seed treatments, in *Encyclopedia of Applied Plant Sciences*, ed. by Thomas B, Murphy DJ and Murray BG. Elsevier Academic Press, Amsterdam, pp. 1291-1298 (2003).

14 Sarrocco S, Raeta R and Vannacci G, Seeds encapsulation in calcium alginate pellets. *Seed Sci Technol* **2**:649–661 (2004).

15 Pilar MC, Ortega N, Perez-Mateos M and Busto MD, Alkaline phosphatase-polyresorcinol complex: characterization and application to seed coating. *J Agr Food Chem* **57**:1967-1974 (2009).

16 Keeney DR and Nelson DW, Nitrogen-inorganic forms, in *Methods of Soil Analysis. Part 2 Chemical and Microbiological Properties (2nd Edition)*, ed. by Page AL, Miller RH and Keeney DR. American Society of Agronomy, Madison, pp. 643-698 (1982).

17 Keeney DR and Bremner JM, Comparison and evaluation of laboratory methods of obtaining an index of soil nitrogen availability. *Agron J* **58**:498-503 (1966).

18 Beck TH, Die N-Mineralisierung von Böden im Laborbrutversuch. *Z Pflanz Bodenkunde* **146**:243-252 (1983).

19 Busto MD, Ortega N and Perez-Mateos M, Stabilisation of cellulases by crosslinking with glutaraldehyde and soil humates. *Bioresource Technol* **60**:27-33 (1997).

20 Pilar MC, Ortega N, Perez-Mateos M and Busto MD, Kinetic behaviour and stability of *Escherichia coli* ATCC27257 alkaline phosphatase immobilised in soil humates. *J Sci Food Agr* **83**:232-239 (2003).

21 Kandeler E and Gerber H, Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol Fert Soils* **6**:68-72 (1988).

22 Patel AV, Pusch I, Mix-Wagner G and Vorlop KD, A novel encapsulation technique for the production of artificial seeds. *Plant Cell Rep* **19**:868–874 (2000).

23 Hevia P and Cioccia AM, Application of a colorimetric method of the determination of nitrogen in nutritional studies with rats and humans. *Nutr Rep Int* **38**:1129-1136 (1988).

24 Ortega N, Perez-Mateos M, Pilar MC and Busto MD, Neutrase immobilization on alginate-glutaraldehyde beads by covalent attachment. *J Agr Food Chem* **57**:109-115 (2009).

25 Busto MD, Ortega N and Perez-Mateos M, Studies on microbial β-D-glucosidase immobilised in alginate gel beads. *Process Biochem* **30**:421-426 (1995).

26 Parr SR, Some kinetic properties of the β-D-glucosidase (cellobiase) in a commercial cellulase product from *Penicillium funiculosum* relevance in the hydrolysis of cellulose. *Enzyme Microb Tech* **5**:448-462 (1983).

27 Barbazán M, Ferrando M and Zamalvide JP, Dry matter accumulation and nitrogen in anual Winter grass used as cover crops in vineyards. *Agrociencia* **6**:10-19 (2002) (In Spanish).

28 Dong LH, Yang JS, Yuan HL, Wang ET and Chen WX, Chemical characteristics and influences of two fractions of Chinese lignite humic acids on urease. *Eur J Soil Biol* **44**:166-171 (2008).

29 Mazzei P, Oschkinat H and Piccolo A, Reduced activity of alkaline phosphatase due to host-guest interactions with humic superstructures. *Chemosphere* **93**:1972-1979 (2013).

30 Nannipieri P, Sequi P and Fusi P, Humus and enzyme activity, in *Humic Substances in Terrestrial Ecosystems*, ed. by Piccolo A. Elsevier, Amsterdam, pp. 293-328 (1996).

31 Ruggiero P, Dec J and Bollag JM, Soil as a catalytic system, in *Soil Biochemistry, vol. 9*, ed. by Stotzky G and Bollag JM. Marcel Dekker, New York, pp. 79-122 (1996).

32 Tomaszewski JE, Schwarzenbach RP and Sander M, Protein encapsulation by humic substances. *Environ Sci Technol* **45**:6003-6010 (2011).

33 Yan J, Pan G, Ding C and Quan G, Kinetic and thermodynamic parameters of β-glucosidase immobilized on various colloidal particles from a paddy soil. *Colloids Surface B* **79**:298-303 (2010).

34 Kaleeswari RK, Role of phosphatase enzymes in phosphorus nutrition of crops. Agric Rev **28**:149-153 (2007).

35 Tan KH, Chemical processes, in *Handbook of Processes and Modeling in the Soil-Plant System*, ed. by Benbi DK and Nieder R. Haworth Press, Binghamton, pp. 27-56 (2003).

36 Reshmi R, Sanjay G and Sugunan S, Immobilization of α-amylase on zirconia: A heterogeneous biocatalyst for starch hydrolysis. *Catal Commun* **8**:393-399 (2007).

37 Kurayama F, Suzuki S, Bahadur NM, Furusawa T, Ota H, Sato M and Suzuki N, Preparation of aminosilane-alginate hybrid microcapsules and their use for enzyme encapsulation. *J Mater Chem* **22**:15405-15411 (2012).

38 Vikartovská A, Bučko M, Mislovičová D, Pätoprstý V, Lacík I and Gemeiner P, Improvement of the stability of glucose oxidase via encapsulation in sodium

alginate-cellulose sulphate-poly (methylene-co-guanidine) capsules. *Enzyme Microb Tech* **41**:748–755 (2007).

39 Tanriseven A and Doğan S, Immobilization of invertase within calcium alginate gel capsules. *Process Biochem* **36**:1081–1083 (2001).

40 Gorim L and Asch F, Effects of composition and share of seed coatings on the mobilization efficiency of cereal seeds during germination. *J Agron Crop Sci* **198**:81-91 (2012).

41 Kim SH and Taylor AG, Germinability of film-coated snap bean seed as affected by oxygen diffusion rate under different soil moisture contents. *Korean J Crop Sci* **49**:46–51 (2004).

42 Ester A, de Vogel R and Bouma E, Controlling *Thrips tabaci* (Lind.) in leek by film-coating seeds with insecticides. *Crop Prot* **16**:673-677 (1997).

43 Yanyu S, Changchun S, Guisheng Y, Yingchen L, Rong M and Jiaoyue W, Effects of N addition on soil enzyme activities in marshland ecosystem of Northeast China: an incubation experiment. *International Conference on Agricultural and Biosystems Engineering. Advances in Biomedical Engineering* **1-2**:5-8 (2011).

44 Robertson GP and Groffman PM, Nitrogen transformations, in *Soil Microbiology, Ecology and Biochemistry (4th Edition)*, ed. by Paul EA. Elsevier Academic Press, Amsterdam, pp. 421-446 (2015).

45 Abbasi MK, Shah Z and Adams WA, Mineralization and nitrification potentials of grassland soils at shallow depth during laboratory incubation. *J Plant Nutr Soil Sc* **164**:497-502 (2001).

523 46 Myrold DD and Bottomley PJ, Nitrogen mineralization and immobilization, in
524 *Nitrogen in Agricultural Systems*, ed. by Schepers JS and Raun WR. Soil Science
525 Society of America, Madison, pp. 157-172 (2008).
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Table 1. Experimental design and results according to the central composite face-centered design.

Assay	Variable level*				HUC activity†	IY (%)‡	
	X_1	X_2	X_3	X_4		Exptl	Predicted
1	4	6	6	30	730	85	80
2	4	2	6	180	2174	93	92
3	4	2	10	30	2347	101	101
4	4	2	10	180	2055	88	87
5	4	4	8	105	1326	110	114
6	4	2	6	30	1986	85	83
7	4	6	6	180	780	109	114
8	4	6	10	30	825	98	102
9	4	6	10	182	961	116	114
10	27	2	8	105	1402	59	67
11	27	6	8	105	645	74	71
12	27	4	8	180	1139	93	93
13	27	4	6	105	606	78	85
14	27	4	8	30	1072	88	95
15	27	4	10	105	971	78	78
16	50	6	6	30	692	80	84
17	50	2	6	180	2100	90	89
18	50	2	10	30	2141	92	90
19	50	2	10	180	1270	53	53
20	50	4	8	105	1167	96	99
21	50	2	6	30	2450	106	103
22	50	6	6	180	835	99	95
23	50	6	10	30	678	78	75
24	50	6	10	180	539	59	64
25 (cp)	27	4	8	105	1123	92	87
26 (cp)	27	4	8	105	1106	90	87
27 (cp)	27	4	8	105	1199	99	87

*Variable level X_1, X_2, X_3 and X_4 were temperature (ºC), HA concentration (mg mL^{-1}), immobilization pH and contact time, respectively.
†In U mg^{-1} HA
‡IY = Immobilization yield. Soil HA solution: A_H = 99 U mg^{-1} HA; urease activity added for immobilization: A_0 = 4453 U

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Table 2. Urease activity and NH_4^+-N of soil planted with non-treated seeds (control) and seeds encapsulated with FU and HUC. Values are presented as the mean (n=3) and SEM (in parenthesis)*

		Seed treatment					
Parameter	**DAP**	**Control**	**FU**	**HUC**	**SED (4DF)†**	**SED (4 DF)‡**	**SED (4 DF)§**
Urease activity ($U\ g^{-1}$ dried soil)	7	178 (28)	176 (36)	147 (13)	ns	ns	ns
	14	148 (18)	156 (26)	176 (11)	ns	ns	ns
	21	137 (4)	148 (5)	150 (9)	ns	ns	ns
	28	154 (0)	142 (1)	158 (7)	1.1	ns	1.1
	35	156 (6)	180 (6)	189 (4)	8.6	6.9	ns
NH_4^+-N ($mg\ NH_4^+$-N kg^{-1} dried soil)	7	2.7 (0.11)	2.9 (0.18)	2.9 (0.12)	ns	ns	ns
	14	3.2 (0.01)	2.7 (0.12)	3.3 (0.06)	0.1	ns	0.1
	21	3.4 (0.11)	5.3 (0.27)	4.1 (0.22)	0.3	0.2	ns
	28	7.3 (0.29)	9.2 (0.28)	12.0 (0.01)	0.4	0.3	0.3
	35	2.6 (0.25)	2.8 (0.13)	3.2 (0.06)	ns	0.3	0.1

* Soil urease activity at t=0: 132 (7) $U\ g^{-1}$ dried soil. NH_4^+-N at t=0: 2.5 (0.06) $mg\ NH_4^+$-N kg^{-1} dried soil.

† SED to compare the coated seeds with FU and control at different DAP.

‡ SED to compare the coated seeds with HUC and control at different DAP.

§ SED to compare the coated seeds with FU and HUC at different DAP.

ns, not significant; DF, degrees of freedom.

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Table 3. Influence of the seed encapsulation with FU and HUC on the plant growth (dry weight, length and shoot N content) and N uptake. Values are presented as the mean (n=5) and SEM (in parenthesis)

		Seed treatment					
Parameter	**DAP**	**Control**	**FU**	**HUC**	**SED (8 DF)***	**SED (8 DF)†**	**SED (8 DF)‡**
Shoot length (mm)	7	93 (5.5)	129 (4.4)	128 (4.2)	7.0	6.9	ns
	14	203 (8.4)	265 (9.4)	244 (5.8)	12.6	10.2	9.4
	21	261 (16.1)	306 (12.0)	314 (13.9)	16.2	17.7	ns
	28	291 (17.8)	328 (8.7)	321 (6.6)	13.2	11.9	ns
	35	316 (11.5)	319 (7.5)	314 (7.7)	ns	ns	ns
Shoot dry weight (mg plant^{-1})	7	13.1 (0.67)	18.2 (0.77)	18.1 (0.57)	1.0	0.9	ns
	14	27.6 (1.02)	43.6 (2.20)	34.5 (1.84)	2.4	2.1	2.2
	21	49.0 (2.26)	63.6 (4.56)	63.1 (3.45)	5.1	4.1	ns
	28	67.1 (4.05)	72.3 (2.41)	72.0 (2.66)	ns	ns	ns
	35	88.1 (1.55)	101.4 (4.25)	92.6 (3.43)	4.5	ns	4.3
Shoot N (g kg^{-1} dried weight)	7	42.6 (1.55)	64.4 (2.39)	49.5 (1.07)	2.8	1.9	2.4
	14	22.8 (0.57)	30.0 (0.57)	30.8 (0.63)	0.8	0.8	ns
	21	23.6 (0.76)	30.6 (0.49)	36.7 (1.12)	0.9	1.4	0.5
	28	20.0 (0.72)	23.8 (0.92)	26.9 (0.52)	1.2	0.9	0.9
	35	7.5 (0.40)	11.6 (0.31)	14.0 (0.33)	0.5	0.5	0.3
N uptake (mg plant^{-1})	7	0.56 (0.020)	1.15 (0.043)	0.88 (0.016)	0.05	0.03	0.04
	14	0.63 (0.016)	1.32 (0.025)	1.04 (0.021)	0.03	0.03	0.03
	21	1.17 (0.038)	1.99 (0.032)	2.38 (0.073)	0.05	0.08	0.03
	28	1.33 (0.048)	1.67 (0.064)	1.98 (0.039)	0.08	0.06	0.06
	35	0.62 (0.033)	1.24 (0.034)	1.26 (0.030)	0.05	0.04	ns

* SED to compare the coated seeds with FU and control at different DAP.

† SED to compare the coated seeds with HUC and control at different DAP.

‡ SED to compare the coated seeds with FU and HUC at different DAP.

ns, not significant; DF, degrees of freedom.

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FIGURE CAPTIONS

Figure 1. Response surface plot for immobilization yields as a function of: a) temperature and contact time (HA concentration, 4 mg mL^{-1}; pH, 8); b) immobilization pH and HA concentration (temperature, 4 ºC; contact time, 180 min).

Figure 2. Lineweaver-Burk plot for free (FU) and immobilized (HUC) urease activity.

Figure 3. Effect of pH (a) and temperature (b) on free (FU) and immobilized (HUC) urease activity.

Figure 4. Stability in soil of free (FU) and immobilized (HUC) urease.

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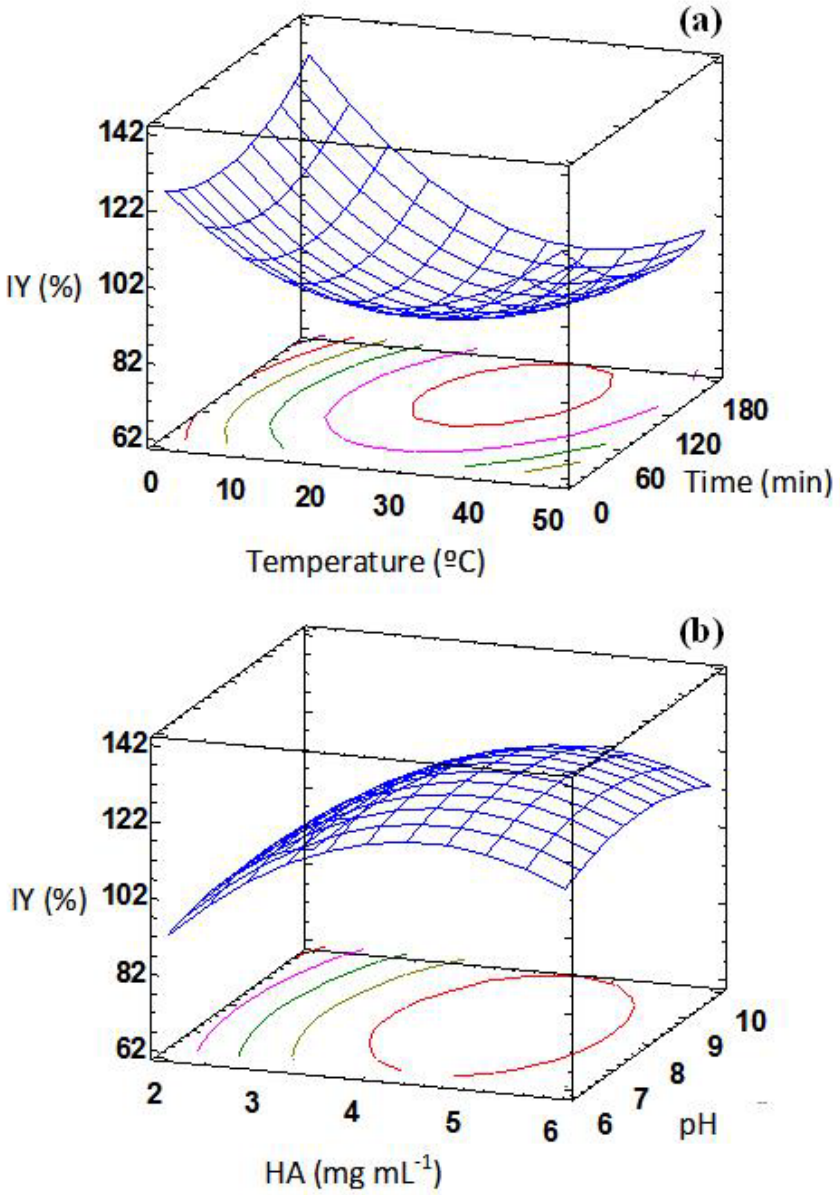

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575 Figure 1. Response surface plot for immobilization yields as a function of: a)
576 temperature and contact time (HA concentration, 4 mg mL^{-1}; pH, 8); b) immobilization
577 pH and HA concentration (temperature, 4 ºC; contact time, 180 min).

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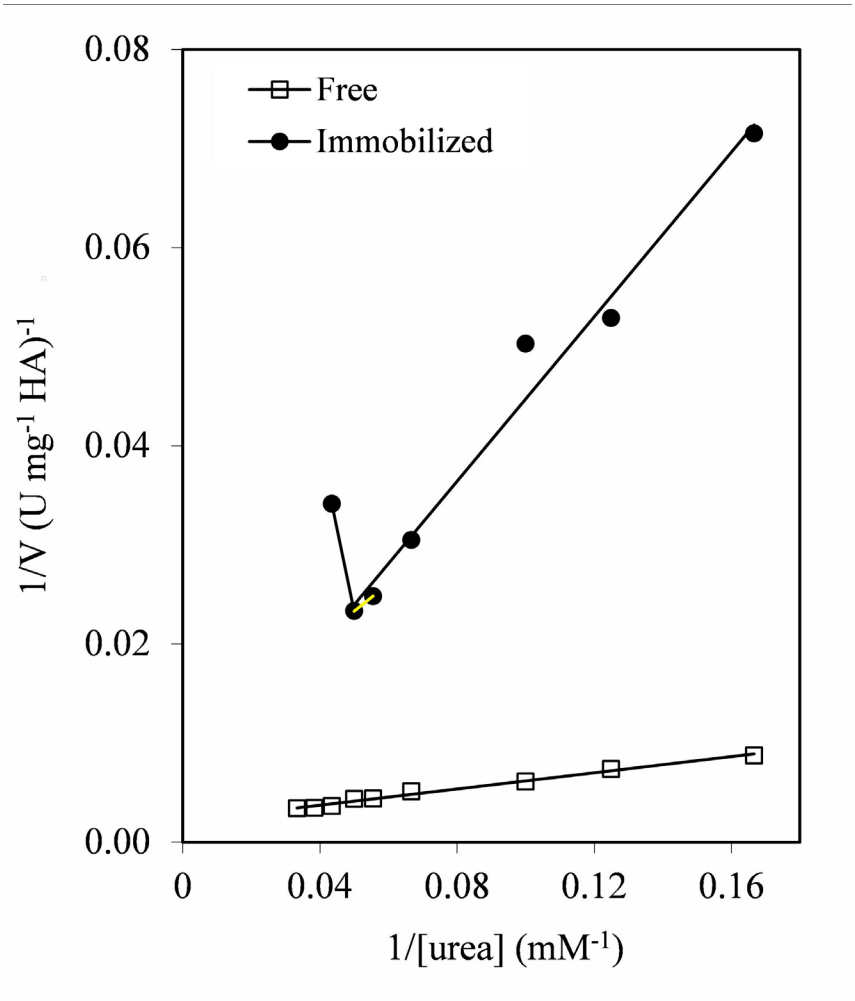
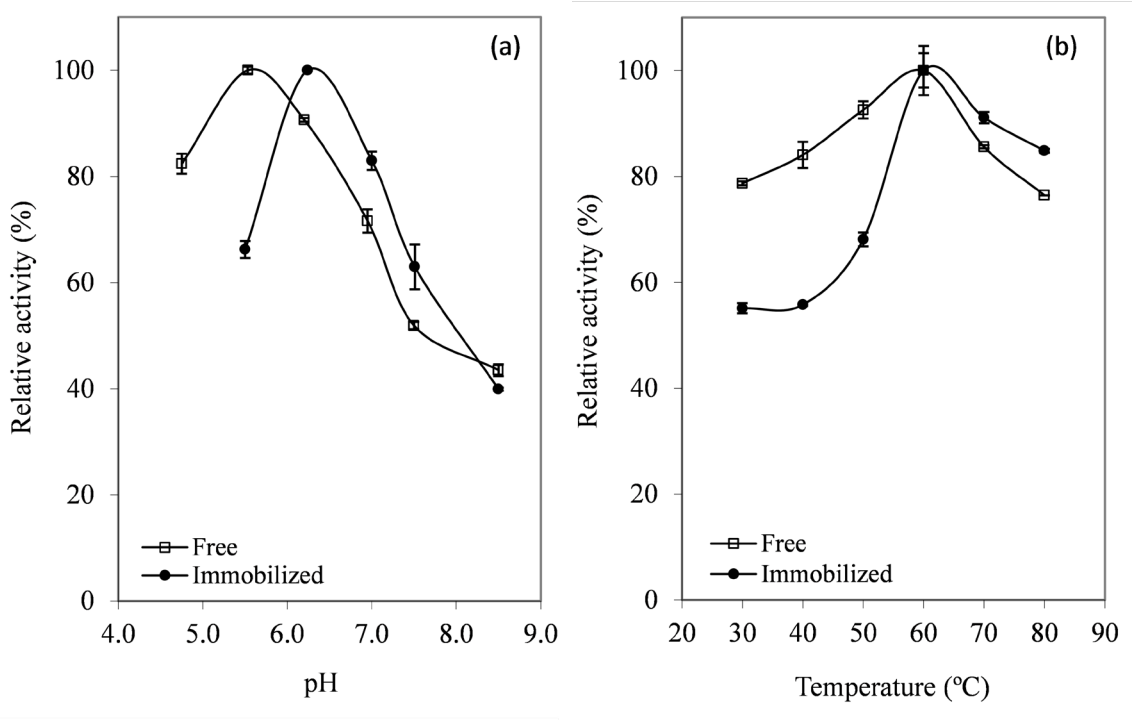


Figure 2. Lineweaver-Burk plot for free (FU) and immobilized (HUC) urease activity.



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590 Figure 3. Effect of pH (a) and temperature (b) on free (FU) and immobilized (HUC)
591 urease activity.

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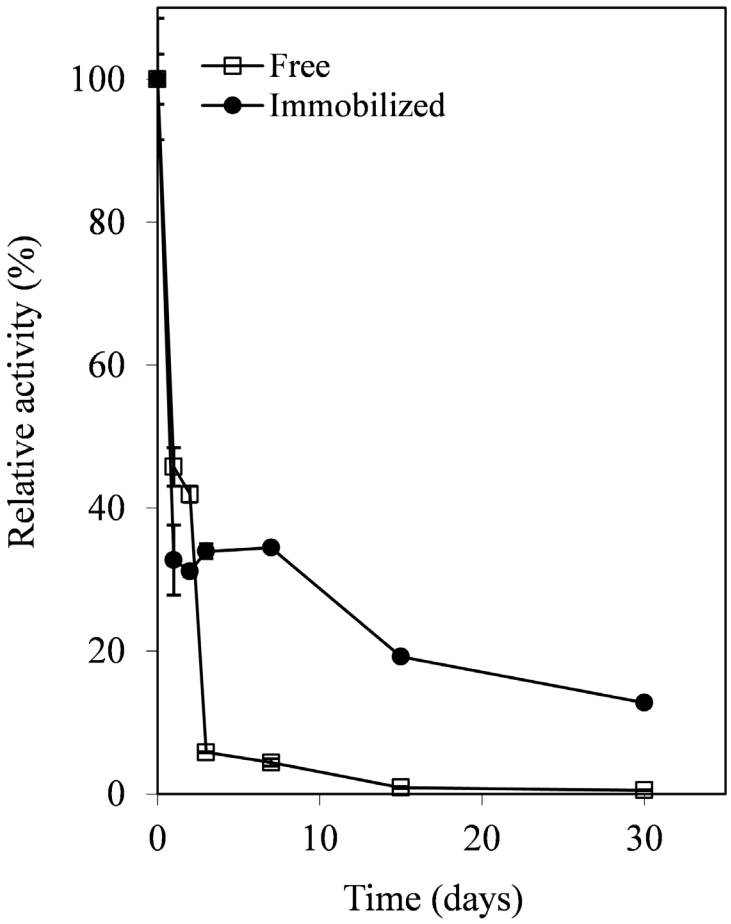

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606 Figure 4. **S**tability in soil of free (FU) and immobilized (HUC) urease.

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