Temperature-dependent inactivation and catalysis rates of plant-based ureases for engineered biomineralization

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Abstract
Engineered (bio)mineralization uses the enzyme urease to catalyze the hydrolysis of urea to promote carbonate mineral precipitation. The current study investigates the influence of temperature on ureolysis rate and degree of inactivation of plant-sourced ureases over a range of environmentally relevant temperatures. Batch experiments at 30°C demonstrated that jack bean meal (JBM) has a 1.7 to 56 times higher activity (844 μmol urea hydrolyzed g⁻¹ JBM min⁻¹) than the other tested plant-sourced ureases (soybean, pigeon pea and cottonseed). Hence, ureolysis and enzyme inactivation rates were evaluated for JBM at temperatures between 20°C and 80°C. A combined first-order urea hydrolysis and first-order enzyme inactivation model described the inactivation of urease over the investigated range of temperatures. The temperature-dependent rate coefficients (kₜₐₚ) increased with temperature and ranged from 0.0018 at 20°C to 0.0249 L g⁻¹ JBM min⁻¹ at 80°C; JBM urease became ≥50% inactivated in as little as 5.2 minutes at 80°C and in as long as 2238 minutes at 50°C. The combined urea hydrolysis kinetics and enzyme inactivation model provides a mathematical relationship useful for the design of biomineralization technologies and can be incorporated into reactive transport models.

KEYWORDS
enzyme inactivation, enzyme kinetics, jack bean meal, urease, ureolysis-induced calcium carbonate precipitation

1 | INTRODUCTION
Engineered (bio)mineralization or ureolysis-induced calcium carbonate precipitation (UICP) techniques (Equation [1]) have been an increasingly popular area of research for use in ground improvement, construction materials, remediation, and subsurface applications. In fact, ground improvement with mineralization strategies has been studied extensively resulting in a new field of study described as bio-mediated geotechnics. In the subsurface, where temperatures increase with increasing depth, engineered mineralization has the potential to be utilized in place of traditional cement
or grout for remediating wellbore integrity, sealing fractures in concrete and rock formations utilized for fluid storage (e.g., CO$_2$, natural gas, or H$_2$), controlling flow paths for oil and gas recovery, or creating subsurface barriers for water pollution control.\textsuperscript{14-19}

UICP is a process that can be catalyzed by the enzyme urease (from bacteria, plants, or fungi), during which urea is hydrolyzed, which in the presence of calcium ions can induce calcium carbonate precipitation (Equation [1]).\textsuperscript{20-22}

$$\text{(NH}_2\text{)}_2\text{CO} + 2\text{H}_2\text{O} + \text{Ca}^{2+} \rightarrow 2\text{NH}_4^+ + \text{CaCO}_3.$$  

UICP, utilizing the ureolytic bacterium \textit{Sporosarcina pasteurii} or plant-based sources of urease, has been researched and used extensively in practice.\textsuperscript{11,18,22-30} The focus of this investigation were plant-based sources of the enzyme, because of the potential limitations in using bacteria, such as \textit{S. pasteurii}, in higher temperature applications, since \textit{S. pasteurii} has been shown to not grow above 40$^\circ$C.\textsuperscript{31}

One challenge in successfully promoting UICP with urease is that the enzyme is subject to increasingly fast inactivation at elevated temperatures. Thus, accounting for changes in rates of ureolysis-induced mineral precipitation due to different temperatures is important for achieving optimal mineral formation in a desired time frame. Hence, the motivation for this work was to develop simple mathematical relationships describing the kinetics of ureolysis and the inactivation rate of urease over a range of temperatures relevant for UICP applications. These mathematical relationships will be useful for the development of reactive transport models targeting the optimization of UICP-based technologies, for example by designing strategies to increase reagent temperatures to increase rates of ureolysis, or in the case of deeper subsurface applications, developing strategies to protect urease from rapid inactivation at higher temperatures. We have demonstrated previously that such reactive transport models can be essential in the design of successful field-scale applications of the UICP technology.\textsuperscript{32}

Urease is abundant in leguminous seeds, with soybeans (\textit{Glycine max}) reportedly containing 0.012\% urease/dry mass, jack beans (\textit{Canavalia ensiformis}) containing 0.07\% to 0.14\% urease/dry mass\textsuperscript{33} and bacterial cells of \textit{Sporosarcina pasteurii} containing up to 1\% of urease/dry mass.\textsuperscript{34} Enzymes are commonly characterized in terms of (enzyme) units, with one “unit” generally referring to the amount of enzyme needed to convert a certain mass of substrate per time interval. For urease, one unit is often defined as 1 \(\mu\text{mol urea hydrolyzed/minute}\) although sometimes 1 \(\mu\text{mol ammonium being liberated/minute}\) is also used; note that these two definitions are related to each other by a factor 2 (cf. Equation [1]). The activity of different enzyme sources is then generally reported as units per mg (or g) of enzyme source. Observed enzyme activity can thus vary depending on the purity of the enzyme source as well as depending on the reaction conditions including temperature, pH, substrate concentrations, etc.

Ureolysis rates have been determined for many sources of urease, including but not limited to jack beans, soybeans, cottonseeds (\textit{Gossypium hirsutum}), pigeon peas (\textit{Cajanus cajan}) and microbial cells including \textit{S. pasteurii}, the most commonly used bacterium for promoting UICP.\textsuperscript{7,35-39} The activity of purified urease has been reported to be in the range of 2700 to 3500 units per mg of enzyme from jack bean, 650 to 800 units per mg of enzyme for soybean, 3120 units per mg of enzyme for pigeon pea, and 14.5 units per mg of enzyme for cottonseed preparations, and 2500 units per mg of enzyme for \textit{S. pasteurii} (as summarized in Reference 33 Table 2 and references therein). Thermal and chemical inactivation for a variety of enzymes has been described for a range of pH, temperature, pressure and experimental conditions and possible inactivation mechanisms have been examined using activity-time profiles and mathematical relationships ranging from first-order to higher order biphasic models.\textsuperscript{40-46}

After confirming that JBM had the highest urease activity per mass unit relative to ground cottonseed, pigeon pea and soybean meals, a model was developed for JBM mathematically describing urea hydrolysis kinetics during simultaneous inactivation of urease for temperatures between 20$^\circ$C and 80$^\circ$C, which correspond to subsurface depths of up to approximately 2400 m.\textsuperscript{47} A simple combined first-order inactivation and first-order kinetic model described the experimental data well, and the work presented here will contribute toward the improved design of full-scale applications of the UICP technology for well-sealing, soil stabilization and other applications using plant-based enzyme sources.

### 2 | MATERIALS AND METHODS

#### 2.1 | Materials

Jack bean meal (JBM) with an activity specified as \(\geq 750\) units/g (\(\mu\text{mol urea hydrolyzed per minute}\)) at pH 7 and 25$^\circ$C (J0125, Sigma-Aldrich, St. Louis, Missouri) was utilized as a source of urease in these experiments. Soybean flour (SB) (S9633, Sigma-Aldrich, St. Louis, Missouri), cottonseed flour (CS) (C4898, Sigma-Aldrich, St. Louis, Missouri) and pigeon
peas (PP) (North Bay Trading Co. Brule, WI, ground to a flour using a household coffee grinder) were used as alternative plant-based sources of urease. No reported urease activity was included in the specification sheets associated with the plant sources other than for the JBM.

All plant-based urease sources were prepared by adding 5 g L⁻¹ of the ground plant powder into water in a flask on a stir-plate which was mixed at 300 rpm for approximately 16 hours. ACS certified urea (Fisher Scientific, Fair Lawn, New Jersey) was prepared as a 40 g L⁻¹ solution in water which was mixed until the urea pellets were dissolved. All urea and plant-based urease solutions were made with deionized water (DI) and filtered through sterile 0.2 μm disposable Nalgene bottle top filters (ThermoFisher, Rochester, New York) prior to use to remove non-dissolvable meal constituents. Glassware was autoclaved prior to use.

2.2 | Batch kinetic experiments

Batch experiments were carried out in digital shaking water baths operating at the desired experimental temperature (20°C-80°C) at 70 rpm. The initial heating period to reach each temperature was determined by measuring the temperature over time with an Omega CDS107 temperature probe. Time to reach 95% of the target temperature was determined to be less than 3.5 minutes for each treatment. Experiments were carried out in 30 mL glass bottles that contained 10 mL of 5 g L⁻¹ plant-based urease (JBM, SB, CS, or PP) to which 10 mL of 40 g L⁻¹ urea solution were added pre-heated to the desired experimental temperature. This created urease-urea mixtures with final concentrations of 2.5 g L⁻¹ JBM, SB, CS or PP and 20 g L⁻¹ urea.

Batch experiments were run in triplicate for durations of 2 to 8 hours, depending on temperature. Samples (60 μL) were collected for urea analysis every 1 to 15 minutes for up to 2 hours and then every 30 minutes for up to 8 hours. Urea concentrations were determined using a modified Jung assay as detailed in Reference 21. Briefly, the modified Jung assay was performed by measuring absorbance at 505 nm in 96 well plates using either appropriately diluted samples or acidified standards to assess urea concentrations. Samples were diluted in 0.5 M H₂SO₄ to stop enzyme activity and obtain urea concentrations that fell within the linear range of the calibration curve (0 and 2 g L⁻¹). In all batch experiments, less than 10% of the total experimental volume was removed for sampling. A urease-free control with 20 g L⁻¹ urea was also run to assess abiotic hydrolysis within the experimental time; abiotic hydrolysis of urea was not observed at the tested temperatures up to 80°C. The solutions were purposefully not buffered to simulate more closely what would happen during UICP applications, during which the pH would increase initially before calcium carbonate precipitation would occur. The buffer capacity of the JBM solution was measured as described in the supporting information.

2.3 | Inactivation experiments

Enzyme inactivation was determined by exposing 10 mL of 5 g L⁻¹ JBM suspensions to temperatures between 50°C and 80°C for 0.04 hours (2.5 minutes) to 168 hours. Exposed JBM suspensions were cooled down rapidly on ice at pre-determined times and stored at 4°C until utilized in batch experiments to determine the remaining enzyme activity (A). To determine A, each 10 mL sample of thermally-exposed JBM urease suspension was warmed to 30°C and mixed with 10 mL of a 40 g L⁻¹ urea solution at 30°C. Samples were collected over 2 hours and appropriately diluted in 0.5 M H₂SO₄ prior to urea measurement using the modified Jung Assay. Activity (A) was estimated by determining the average urea hydrolysis rate based on the difference in the initial and residual urea concentrations after 2 hours (Equation [2]). Here, U₀ and UₜΔ are the urea concentrations initially and after 2 hours (120 minutes), respectively, and Δt is the time of the kinetic experiment (2 hours).

\[
A = \frac{U_0 - U_{t\Delta}}{\Delta t}.
\] (2)

2.4 | Modeling methods

For comparison with other studies, an apparent first-order rate coefficient (kₐ) was determined (Equation [3]); these initial comparisons did not explicitly account for enzyme inactivation

\[
dU/dt = -k_a[U].
\] (3)
where \( dU \) is the differential change in urea concentration, \( dt \) is the differential change in time, and \( k_a \) is the apparent first-order reaction rate coefficient (min\(^{-1}\)).

Ureases can become inactivated at elevated temperatures above 50°C. Hence, the model was modified to include both changes in urea concentration (\( U \)) and concentration of enzyme source (\( E \)) (Equation [4]); \( k_{\text{urea}} \) is the temperature-dependent first-order ureolysis rate coefficient and \( E \) is temperature- and time-dependent.

\[
\frac{dU}{dt} = -k_{\text{urea}}[U][E].
\] (4)

Here, the reaction equation is second order overall, first-order with respect to \( U \) and first-order with respect to \( E \). The inactivation of the urease enzyme at elevated temperatures was modeled using the normalized activity term (\( a \)) (Equation [5]), a function of temperature and time within this study (Equations [6] to [8]):

\[
a = \frac{E}{E_0} = \frac{A}{A_0},
\] (5)

where \( E_0 \) represents the initial concentration of the active enzyme, \( E \) represents the concentration of the active enzyme at a given time.

To predict changes in (\( a \)) over time at different temperatures three inactivation models of differing complexity were considered, one single-step and two multi-step inactivation models (graphically represented in Table 1).\(^{46,49,50}\) All \( k \) values in these models are temperature-dependent and detailed explanations of the higher order inactivation mechanisms in the Supplemental Information. The derivations and inactivation models are described thoroughly by Sadana.\(^{50}\)

### 2.4.1 Single step inactivation model

The single step inactivation model was the most-simple model investigated within this study; in Equation [6], \( a \) is the normalized activity after exposure to temperature (\( T \)) for time \( t \) and \( k_d \) is the first-order thermal inactivation rate coefficient at a given \( T \).

\[
a = e^{-k_d t}.
\] (6)

The normalized activity (\( a \)) over time for a given exposure temperature (50°C-80°C), for exposure times up to 168 hours, was determined by taking samples and determining the remaining activity using batch ureolysis tests which were conducted at 30°C. The inactivation rate coefficients (\( k_d \)) for each temperature (50°C and 80°C) were then determined by linearly regressing the residual activity vs time on a semi-natural log plot, with the slope being \( k_d \).

The natural logarithm of \( k_d \) was then plotted against \( 1/T \) and linear regression was used to obtain a relationship for the temperature-dependency of the inactivation coefficient (\( k_d(T) \)). Since enzyme inactivation below 50°C was slow

| Model         | Mechanism                              |
|---------------|----------------------------------------|
| First order   | \( E \rightarrow E_d \)                |
| Series-parallel | \( E \rightarrow E_1 \rightarrow E_d \) |
| Series-type   | \( E \rightarrow E_1 \rightarrow E_d \) |

Note: Here \( E \) represents the native form of the enzyme, \( E_1 \) represents an isomerized form of the enzyme with a different activity than \( E \), and \( E_d \) represents the inactivated form of the enzyme without any catalytic activity.
(\(k_d\) values were very small), experiments would have required exposures of 168 hours (7 days) or longer to observe significant reduction in enzyme activity, which might have introduced artifacts due to non-temperature exposure-related degradation of the enzyme; hence, the residual enzyme activities (\(A\)) for temperatures below 50°C were calculated from the Arrhenius-type equation developed in this work.

### 2.4.2 Multiple step inactivation models

The higher order models investigated included a series-type and series-parallel type model which include multiple inactivation pathways.\(^{46,49,50}\) In a series-parallel model (Table 1 and Equation [7]), the native enzyme follows one of two paths toward the inactivated form, (a) a two-step series path that assumes the inactivation of the native enzyme (\(E\)) to the completely inactivated form \(E_d\) via a partially inactivated isozyme (\(E_1\)) and (b) a single step path toward complete inactivation (\(E_d\)).\(^{50}\) The series-type inactivation model (Table 1 and Equation [8]) follows a two-step inactivation pathway through a partially inactivated isozyme (\(E_1\)) to a completely inactivated form (\(E_d\)).\(^{50}\) In each of these higher order models, kinetic coefficients \(k_1, k_2,\) and \(k_3\) are the inactivation coefficients from the native form (\(E\)) to the isozyme form (\(E_1\)) to the inactive form (\(E_d\)).

\[
a = \left[\left(1 + \frac{\beta k_1}{k_1 - k_2 - k_3}\right) e^{-(k_1 + k_3)t} - \left(\frac{\beta k_1}{k_2 - k_1 - k_3}\right) e^{-k_3t}\right],
\]

\[
a = \left[\left(1 + \frac{\beta k_1}{k_2 - k_1}\right) e^{-k_1t} - \frac{k_1 \beta}{k_2 - k_1} e^{-k_3t}\right].
\]

The parameters associated with the series-parallel and series inactivation models (\(\beta, k_1, k_2,\) and \(k_3\)) were estimated using “fmincon,” a nonlinear regression code within MATLAB, which minimizes the difference (sum of the squared residuals) between experimental and predicted data. Constraints were added that would only evaluate a specific range of numerical values, for example, reversibility of the reactions was not permitted (ie, no negative \(k_1, k_2,\) or \(k_3\) values).

### 3 RESULTS AND DISCUSSION

#### 3.1 Ureolysis rates of different plant-sourced ureases

The rates of ureolysis of the four plant sources (JBM, SB, PP and CS) were initially compared at 30°C and 60°C using batch experiments containing the same mass of meal without further purification. This initial comparison occurred based on the apparent first-order rate coefficient (\(k_a\)) for each enzyme source, that is, not explicitly accounting for inactivation (cf. Equation (3), Figure 1). JBM exhibited an approximately 1.7 times higher ureolysis rate at 30°C and 3.3 times higher ureolysis rates at 60°C than SB, the second-most active source; urease activity decreased in the order JBM > SB > PP > CS. The reaction progress curves, used to calculate the \(k_a\) values presented in Figure 1, are presented in the Supplemental Information (Figure S1). Since the urease content of each meal is unknown, no comparison of the specific (ie, per urease molecule) activity for the different plant sources can be provided. At 30°C JBM was the most active with 844 units per gram (\(\mu\)mol of urea hydrolyzed per minute per gram of meal), SB with 504, PP with 201 and CS with 15 units per gram of meal. At 60°C JBM was the most active again with 2252 units per gram of meal, SB with 676, PP with 206, and CS with 27 units per gram of meal. The urease activities observed for JBM (844 and 2252 units per g of JBM at 30°C and 60°C, respectively) are in agreement with the manufacturer’s information that states >750 \(\mu\)mol of urea hydrolyzed per minute per g of JBM at 25°C and pH 7 (Sigma-Aldrich Product Specification). These results also agree with the order of plant urease activities as summarized and reported in Krajewska et al\(^{33}\) with the exception that PP was reported to have a higher activity than SB and CS. JBM was chosen over the other plant-based urease sources or purified enzymes for further study, because its high activity and reasonable cost indicate its potential for successful use in engineering applications.
3.2 | Temperature-dependent kinetics of urea hydrolysis by jack bean meal

3.2.1 | Apparent, temperature-dependent urea hydrolysis rates from batch kinetic experiments

Results from batch experiments containing JBM performed at temperatures between 20°C and 80°C are shown as the change in urea concentration over time (Figure 2). In all treatments, the pH increased to around 9.3 very quickly, that is, after approximately 1% of the added urea (ie, 0.2 g L⁻¹ of the 20 g L⁻¹) had been hydrolyzed; the pH remained around 9.3 for the remainder of the experiment. At 30°C, JBM was observed to hydrolyze 60% +/− 5% (average +/− SD, n = 3) of urea within 2 hours. Urea hydrolysis rates increased with increasing temperatures to an optimum around 60°C where complete hydrolysis of 20gL⁻¹ urea was achieved within 60 minutes at 60°C. For temperatures <60°C, urea hydrolysis occurred at slower rates; for temperatures >60°C, ureolysis was faster initially (eg, during the first 30 minutes) but slowed down rapidly afterward, presumably due to the inactivation of urease at elevated temperatures. For instance, urea hydrolysis occurred quickly at temperatures above 70°C but ceased after approximately 30 minutes at 75°C and 20 minutes at 80°C, with approximately 40% and 68% of urea remaining, respectively, and no statistically significant decrease being observed, suggesting that JBM urease had been completely inactivated.

3.2.2 | Temperature-dependent inactivation of JBM urease

Since inactivation of urease was observed at elevated temperatures, the temperature-dependent rate of inactivation was evaluated separately in batch experiments. JBM was exposed to temperatures between 50°C and 80°C for various times and at pre-determined times the remaining enzyme activity was determined. JBM urease showed more than 50% inactivation after 2238 minutes (37.5 hours) during exposure at 50°C, 835 minutes (13.9 hours) at 55°C, 317 minutes (5.3 hours) at 60°C, 125 minutes (2.1 hours) at 65°C, 62 minutes (1 hour) at 70°C, 26 minutes (0.4 hour) at 75°C and 5 minutes (0.1 hour) at 80°C (Figure 3). Illeová et al.⁵¹ noted that urease lost 50% of its activity after 3000 minutes (50 hours) at 55°C and suffered complete loss of activity in less than 60 minutes (1 hour) at 87.5°C. In a more recent study, Illeová et al.⁴¹ noted that that the urease lost 50% of activity after 360 minutes (6 hours) at 65°C and 3 minutes (0.05 hour) at 85°C.⁴¹ In the Illeová et al. studies, the jack bean enzyme had been purified and suspended in either a 0.1 M or 0.05 M phosphate buffer at pH 7.0, respectively, which could explain the differences observed in the study presented here.

Results of the first-order inactivation model (Equation [6]) for urease inactivation between 50°C and 80°C were plotted using an Arrhenius-type plot. The resulting ln(k_d) values as plotted vs 1/T are shown in Figure 4 along with the resulting Arrhenius-type equation (also listed in Table 2, R² = 0.99). The coefficients obtained from each model for temperatures between 20°C and 80°C are summarized in the Supporting information (Table S2) along with the correlation coefficients for their respective fits.
FIGURE 2  Urea concentrations (g L$^{-1}$) over time (minutes) in the presence of 2.5 g L$^{-1}$ JBM. Temperatures ranged from 20$^\circ$C to 50$^\circ$C in top panel, 55$^\circ$C to 65$^\circ$C in the middle panel and 70$^\circ$C to 80$^\circ$C in the bottom panel.
**Figure 3** Residual ureolytic activity ($\ln[A/A_0]$) of jack bean meal exposed to various temperatures for different amounts of time (up to 5760 minutes [96 hours]); top panel: $50^\circ C$ to $60^\circ C$, bottom panel: $65^\circ C$ to $80^\circ C$.

**Figure 4** Temperature-dependency of inactivation coefficients $\ln(k_d)$ ($k_d$ in min$^{-1}$; $T$ in K). Data from enzyme inactivation experiments were fitted to a first-order inactivation model across the temperature range of $50^\circ C$ to $80^\circ C$ ($323-353$ K). Confidence intervals (Lower Confidence Level [LCL], Upper Confidence Level [UCL], 95%) of the rate estimates were determined from different exposure periods. UCL and LCL values were natural log transformed and error bars were generated. Also, confidence bands (CB, 95%), represented by the dashed lines, were generated around the regression for the mean response ($\ln(k_d)$), represented by the dotted line, as a function of $1/T$ for temperatures between ($50^\circ C$ and $80^\circ C$).
**TABLE 2** Arrhenius-type equations and correlation coefficients ($R^2$) for the temperature-dependency of rate coefficients obtained from mathematical modeling using first-order, series–parallel and series-type models (units for $k$ [min$^{-1}$], $T$ [K])

|            | First-order | Series–parallel | Series-type |
|------------|-------------|-----------------|-------------|
| Equation   | $\ln k_d = -21772(1/T) + 59.2$ | $\ln k_1 = -12705(1/T) + 26.8$ | $\ln k_3 = -18876(1/T) + 50.5$ |
| $R^2$      | 0.99        | 0.56            | 0.98        |

![FIGURE 5](image)

**FIGURE 5** Comparison of predicted urea concentration from series-type (dash-dot-dot), series–parallel (dash-dot) and first-order (dashes) models and experimental ureolysis data for 30°C (diamonds, blue), 60°C (triangles, red), and 80°C (circles, black). The model fits for each temperature overlap almost perfectly, hence, the lines are not easily distinguishable.

### 3.2.3 Temperature-dependent inactivation and urea hydrolysis kinetic modeling

The combined inactivation and urea hydrolysis models (summarized in Table 1) predicted the temperature-dependent urea hydrolysis results well over the temperature range from 20°C to 80°C. Each model predicts the urea hydrolysis data well with correlation coefficients ($R^2$ values) of 0.93 or greater except for the 75°C estimates ($R^2 < 0.81$, refer to detailed discussion in supporting information below Figure S9; the fit results are summarized in Table S2). The comparison of the model results and experimental data are plotted at three temperatures (30°C, 60°C, and 80°C) for easy comparison (Figure 5). It becomes obvious that the three different models are not easily distinguished from each other for any of the temperatures, indicating that each model is adequate for predicting urea hydrolysis rates at temperatures between 20°C and 80°C. A discussion about the $k_{urea}$ values can be found in the supporting information and the $k_{urea}$ values are plotted in Figure S10. In addition, the urea hydrolysis data and first-order model results for all temperatures between 20°C and 80°C are shown in Figure S9.

It has been suggested that enzyme inactivation kinetics can be described using a first-order inactivation model, which describes a one-step irreversible inactivation of the enzyme from its native form to an inactivated form. In other cases, higher order or more complex inactivation models, including series-parallel and series-type models, might better describe the pathway. For purified urease, Illeová et al proposed a thermal inactivation model between the
temperatures of 55°C and 87.5°C using a biexponential model, with at least three different reaction steps, including reversibility.\textsuperscript{51} It can be difficult to fully determine the mechanisms responsible for inactivation which may follow a hexamer to monomer transformation with a possible active trimer intermediate depending on the solution chemistries and experimental conditions.\textsuperscript{44,46,49,51,53} First-order models have therefore been used to adequately describe inactivation kinetics for enzymes under broad temperature ranges\textsuperscript{46,50} and first-order models seem sufficient to describe the combined process of urea hydrolysis and urease inactivation for subsurface applications.

To further analyze the appropriateness of the three models, the resulting natural log of the kinetic coefficient(s) were plotted as a function of 1/T, comparing the fit of the trendlines (Figures S2-S8), and the temperature-dependent equations and their correlation coefficients are summarized in Table 2. Our objective was to have an acceptable fit of the experimental data, while minimizing model complexity. The developed kinetic relationships are to be added to an existing reactive transport model,\textsuperscript{32,54-57} and since there is no strong justification from the fitted data for a more complex model, the first-order model is proposed. The rate coefficients, $k_1$, $k_2$, and $k_3$ would be expected to increase as temperature increases if they had a significant influence on the model behavior as seen with $k_d$ (Figure S2-S8).\textsuperscript{50} However, temperature dependencies were only clearly observable for the rate-controlling coefficients ($k_3$ in the series-parallel model, discussed in more detail below).

The estimates for the activity ratio ($\beta$) determined within MATLAB decreased as temperature increased (Figures S5 and S8); the literature suggests that for urease, $\beta = 1$ at lower temperatures (25°C), and that $\beta$ may be greater than or less than one for a range of temperatures.\textsuperscript{50} However, $\beta$ also did not significantly influence the fit of the models; for the series-type inactivation model $k_3$ was found to be greater than $k_1$, meaning the isozyme form of the enzyme was rapidly degraded; for the series-parallel model, $k_2$ and $k_3$ were found to be greater than $k_1$, hence the inactivation of both the native and isozyme form of the enzyme occurs so rapidly that the value of $\beta$ does not significantly affect the fit of the model.

Indeed a more in-depth analysis of the rate-controlling parameters for the models revealed that for the series-parallel model, the estimated $k_1$ values (rate constant describing the transformation of the native enzyme to the isozyme) were approximately two orders of magnitude smaller than the values for $k_3$ (rate constant describing the transformation of the native form to the completely inactivated form), indicating that the series inactivation pathway proceeded more slowly than the direct inactivation pathway. This suggests that the series-parallel model behaved similarly to the direct inactivation first-order model. Within the series model, the first inactivation step from the native enzyme to the isozyme form was rate-controlling, while the second step could be considered instantaneous relative to the first step because the estimates for $k_2$ for all temperatures were always higher than for $k_1$ (Table S2 and Figures S2 and S3). The two rate-controlling coefficients ($k_3$ in the series-parallel model and $k_1$ in the series-type model), representative of the steps mentioned in the rate limiting analysis, are the same magnitude and exhibit a similar temperature-dependency, indeed resembling the first-order model and its rate-controlling coefficient $k_d$ (cf. Table 2, which shows that similar equations describe the temperature-dependency of $k_d$ for the first-order inactivation model, $k_3$ for series-parallel, and $k_1$ for the series-type model).

4 | CONCLUSIONS

This work investigated urea hydrolysis kinetics of various plant-based ureases over a range of temperatures (20°C-80°C) using experimental and modeling approaches. JBM exhibited the highest activity among the urease sources investigated at both low (30°C) and higher temperatures (60°C) (ie, 844 and 2252 μmol urea hydrolyzed min\textsuperscript{-1} g\textsuperscript{-1} of meal, respectively). At 30°C, JBM had an approximately 1.7x greater activity than soybeans (SB), 4.2x greater activity than PP and 56x greater activity than cotton seeds (CS); at 60°C JBM exhibited an approximately 3.3x greater activity than SB, 11x greater activity than PP and 83x greater activity than CS (Table S1). JBM was chosen for an in-depth analysis to determine the rates of ureolysis across a temperature range of 20 to 80°C as well as inactivation rates for temperatures >50°C. The highest ureolysis rates in this study were observed at 60°C with 20 g L\textsuperscript{-1} urea hydrolyzed by 2.5 g L\textsuperscript{-1} JBM in less than 1 hour. Greater initial ureolysis rates were observed at temperatures greater than 60°C, but due to the inactivation of the enzyme (see Table S2 for representative enzyme half-lives at elevated temperatures) urea was hydrolyzed less efficiently than in the 60°C case. More than 50% inactivation of the urease enzyme was observed as quickly as 5.2 minutes at 80°C and as slowly as 2238 minutes at 50°C.

Three enzyme inactivation schemes were fitted to the experimental data, and a simple first-order inactivation model, in which the native form of the enzyme is inactivated through a single-step, adequately described the urease inactivation data across the temperature range of 20°C to 80°C. While enzyme inactivation might occur through a multi-step process,
the outcome of the biphasic models was dominated by one rate-limiting step explaining why a simple first-order inactivation model was sufficient for predicting the progress of ureolysis at temperatures up to 80°C. The first-order model, would be less computationally expensive to implement into reactive transport models, such as the ones developed by us and others.²⁻⁵⁴⁻⁵⁷ Control and prediction of urea hydrolysis and urease inactivation rates have important implications for the deeper terrestrial subsurface where temperature generally increases with increasing depth and for which more temperature-tolerant strategies may need to be developed.

The current study investigated the rates of enzyme-mediated ureolysis that can contribute to the precipitation of calcium carbonate, recognizing that the presence of calcium was not investigated in this study. Future work should focus on the thermal stability of microbiologically produced ureases, kinetics of the reactions under different chemical conditions such as at different pH values, in the presence of divalent cations or CO₂-affected brine, and the kinetics of thermally induced urea hydrolysis, since thermochemical urea hydrolysis could represent a possible alternative to microbiologically or enzyme-induced urea hydrolysis for UICP.

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PEER REVIEW INFORMATION
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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

NOMENCLATURE
- apparent first-order ureolysis rate coefficient (min⁻¹)
- first-order ureolysis rate coefficient (L g⁻¹ JBM min⁻¹)
- inactivation rate coefficient (first-order model) (min⁻¹)
- coefficient of isomerization (min⁻¹)
- coefficient of inactivation of isomerized form (min⁻¹)
- coefficient for direct inactivation in series-parallel model (min⁻¹)
- ratio of specific enzyme activities in series-parallel and series-type models
- concentration of urea (g L⁻¹)
- initial concentration of urea (g L⁻¹)
- concentration of urea after a given time (Δt) (g L⁻¹)
- normalized activity of enzyme (unitless)
- activity of urease (g L⁻¹ minute⁻¹)
- initial activity of urease (g L⁻¹ minute⁻¹)
- temperature (°C, K, specified, which unit is used)
- time (min)
- concentration of initial native form of enzyme (g L⁻¹)
DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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