ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

An Improved Method for Determining Urease Activity from Electrical Conductivity Measurements

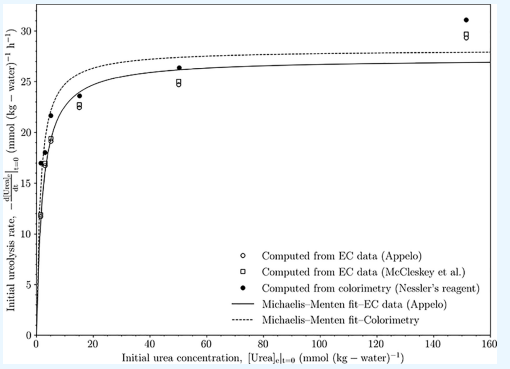
Vinay Krishnan,* Hamed Khodadadi Tirkolaei, and Edward Kavazanjian, Jr.

Cite This: *ACS Omega* 2023, 8, 13791−13798

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: We present an improved approach to evaluating the activity of urease from electrical conductivity (EC) measurements. In this approach, chemical equilibrium modeling via PHREEQC is used in conjunction with empirical equations for computing EC to develop a function that relates the increase in EC during urea hydrolysis in a closed reactor to the concentration of ammonium species present (and concentration of urea remaining) in the reaction solution. By applying this function to data from continuous measurement of EC during urea hydrolysis, we obtain a profile of the concentration of the urea substrate with time, which is then used to determine the urease activity. The activity of commercially available urease extracted from jack beans was determined using this method and compared well to the activity determined using Nessler's reagent, a commonly used colorimetric assay. This EC-based method is inexpensive and can be used for accurate determination of urease activity for a variety of applications.



INTRODUCTION

The measurement of the activity of urease has applications in diverse areas such as medical diagnosis (e.g., detection of *Helicobacter pylori* and other urease-producing bacteria), agricultural engineering, and environmental engineering.[1,2] Recently, urease activity measurement has also become of interest to geotechnical engineering researchers who work on microbially and enzymatically induced carbonate precipitation. Urease catalyzes the hydrolysis of urea to produce ammonia and carbamate. The carbamate spontaneously decomposes into ammonia and carbonic acid, as shown in the following reaction sequence.[2] The ammonium-ammonia and carbonic acid-bicarbonate equilibria result in an increase in pH of the reactor.

$$CO(NH_2)_2(aq) + H_2O \xrightarrow{\text{urease}} NH_3(aq) + NH_2COOH\ (aq) \quad (1)$$

$$NH_2COOH\ (aq) + H_2O \rightarrow NH_3(aq) + H_2CO_3(aq) \quad (2)$$

$$2NH_3(aq) + H_2CO_3(aq) + H^+ \rightarrow 2NH_4^+ + HCO_3^- \quad (3)$$

The most common method of measuring urease activity is to add a colorimetric assay agent for ammonia (e.g., Nessler's reagent) to a solution of hydrolyzed urea. The absorbance of the solution at a certain wavelength (usually ≈ 400−450 nm) is measured using a spectrophotometer and is proportional to the total concentration of ammonium species in the solution. This experimental procedure is rather tedious and provides a limited set of data points to evaluate the initial urea hydrolysis rate (i.e., the rate of ureolysis immediately following the addition of urease to a solution of urea). In comparison, it has been known for a long time that the increase in electrical conductivity (EC) during urea hydrolysis can be correlated with the concentration of hydrolyzed urea and thereby used to evaluate the urea hydrolysis rate.[3]

Sastri and Sreenivasaya emphasized that the advantage of using EC measurements to evaluate urease activity is that a large number of data points can be collected in the early stages of urea hydrolysis, thereby providing a precise estimate of the initial urea hydrolysis rate. These investigators studied the hydrolysis of a 1% urea solution (≈ 167 mM) without a buffer and reported an increase in conductance of 86 mS (cell constant not reported) after 90 min of reaction.[4] A similar study was conducted by Croston et al. using a 3% urea solution (≈ 500 mM) in 0.02 M phosphate buffer.[5] Chin and Kroontje measured the increase in EC following "full" hydrolysis in urea solutions of concentrations ranging from 5 to 2000 ppm (without buffer). The estimated increase in EC was 14 μS/cm for a 5 ppm (83 μM) urea solution and 3715 μS/cm for a 2000 ppm (33.3 mM) urea solution.[6] Whiffin measured the EC

Received: December 26, 2022
Accepted: March 24, 2023
Published: April 6, 2023

following "full" hydrolysis in urea solutions of concentrations ranging from 25 to 250 mM (without buffer). The urease used by Whiffin for these experiments was produced from *Bacillus pasteurii* and was highly purified (Sigma-Aldrich catalog number U7127, powder, ≥100,000 U/g at pH 8.2). The reported EC following hydrolysis was 1.99 mS/cm for a 25 mM urea solution and 22.9 mS/cm for a 250 mM urea solution. Using linear regression, Whiffin concluded that the increase in EC was *linearly proportional* to the concentration of hydrolyzed urea, with the ratio of the concentration of hydrolyzed urea (in mM) to the increase in EC (in mS/cm) given by 11.1 mM/(mS/cm).[7]

In current practice for estimating urease activity, the change in EC following the first few minutes of ureolysis is recorded (in mS/cm) and multiplied by 11.1 to obtain the concentration of hydrolyzed urea (in mM). The rate of urea hydrolysis is then given by this concentration of hydrolyzed urea divided by the elapsed time. However, in typical cases, the concentration of hydrolyzed urea in the first few minutes is small and, as observed from the data by Chin and Kroontje, the ratio of the concentration of hydrolyzed urea (in mM) to the increase in EC (in mS/cm) at low concentrations of hydrolyzed urea is less than 11.1. For example, the ratio obtained from a 5 ppm urea solution is 83 μM/(14 μS/cm) = 5.9 μM/(μS/cm). Therefore, the Whiffin method overestimates the initial urea hydrolysis rate.

In this study, we seek to improve upon the Whiffin technique by using chemical equilibrium modeling to develop a generalized relationship between the concentration of urea hydrolyzed in a closed reactor and the corresponding increase in EC. In this approach, for a given concentration of hydrolyzed urea, the concentration of various ionic species present in the reactor is computed. Then, from the computed concentrations and molal ionic conductivities, the EC of the reactor solution is computed using empirical equations.[8,9] For a given set of reaction conditions (e.g., urease type and concentration), a one-to-one correspondence (not necessarily linear) between the concentration of hydrolyzed urea and the increase in EC is obtained. This function can then be used to map the measured increase in EC of a partially hydrolyzed urea solution back to its ionic composition. We compute this function for urea hydrolysis conducted in a closed reactor at constant temperature (≈ 25 °C). By applying the function to data from continuous measurement of EC during urea hydrolysis (using a commercially available urease extracted from jack bean), we obtain profiles of concentration of urea substrate with time, which is then used to determine the urease activity. The activity estimated using this method is validated via colorimetry using Nessler's reagent.

MATERIALS AND METHODS

Chemical Equilibrium Modeling. The speciation reactions involved in the hydrolysis of urea were modeled using PHREEQC (batch version 3.5.2).[10] The model considered a closed reactor consisting of a solution of fully hydrolyzed urea (solute) in 1.0 kg of water (solvent). The hydrolyzed urea solution was modeled by providing (as model input) the total molality of species in the C(+4) oxidation state (CO_3^{2-}, HCO_3^-, H_2CO_3, CO_2 (aq), CO_2 (g)) and the total molality of species in the N(−3) oxidation state (NH_4^+, NH_3 (aq), NH_3 (g)). The PHREEQC input file was programmed to run multiple simulations, and the total molality of species in the C(+4) oxidation state and the total molality of species in the N(−3) oxidation state were varied between simulations. However, per the stoichiometry of urea hydrolysis, the ratio of the total molality of species in the N(−3) oxidation state to the total molality of species in the C(+4) oxidation state equaled 2.0 for every simulation. The headspace in the reactor had a fixed volume of 1.32 L and was initially composed of 0.78 atm N_2 (g), 0.21 atm O_2 (g), 0.0004 atm CO_2 (g), and 0.0125 atm H_2O (g) (≈ 40% relative humidity). Note that the atmospheric CO_2 (g) is in addition to species in the C(+4) oxidation state from urea hydrolysis. N_2 (g) and O_2 (g) were input in the redox-uncoupled state, i.e., these gases participated only in gas−liquid equilibria and not in any redox reactions. The input volume of headspace is representative of the experimental setup used in this study and may be varied as warranted. The temperature of the reactor was set to 25 °C.

Even in ureolytic reactors without a buffer, the addition of urease itself imparts a small buffering capacity. For example, highly purified ureases extracted (or purified) using a buffer (e.g., KH_2PO_4/K_2HPO_4) may have small amounts of buffer salt precipitated with the protein. Urease enzyme sources that are not highly purified are typically composed of various other proteins, sugars, and stabilizing additives. At neutral to alkaline pH, proteins, free amino acids, and other amine compounds such as amino sugars may act as a buffer due to protonation/deprotonation of the amine group. As an example, major proteins present in urease derived from jack bean (*Canavalia ensiformis*) include canavalin, concanavalin A, concanavalin B, and urease itself. Canavalin is a 7S seed storage protein, concanavalin A is a lectin, and concanavalin B is a carbohydrate-binding protein belonging to glycosyl hydrolase family 18. The actual buffering behavior of proteins is quite complex and depends on cooperativity, access of individual titratable sites to solvent water, and changes in protein structure over time (e.g., protein folding, aggregation).

An evaluation of the amino acid sequences of canavalin (UniProtKB accession number P50477), concanavalin A (UniProtKB accession number P02866), and concanavalin B (UniProtKB accession number P49347) indicates that, barring the initial signal peptide sequence, long hydrophobic amino acid blocks are not present. Therefore, it is reasonable to assume that the majority of amino acids present in these proteins are accessible to the solvent water. As such, because the amount of protein added to the reactor is relatively small (≈ 60 mg/L), the buffering capacity can be approximated in the PHREEQC model using a simplified approach. In this approach, the amino acids in the proteins are assumed to act independently of each other. A single pK_a value (≈ 9.3) is assumed for the amine groups in the protein, and the buffering behavior is assumed to follow the classical Henderson−Hasselbalch equation. It is assumed that the proteins (other than urease) do not participate in any reactions besides the protonation/deprotonation of the amine groups. The moles of amino acids (i.e., titratable sites) is estimated by dividing the mass of protein in the enzyme by the average molecular mass of amino acids (110 g/mol).

For each simulation, PHREEQC computed the activities of aqueous species and fugacities of gaseous species such that the equations governing chemical equilibria, element balance, and charge balance were satisfied. Based on the computed molalities of ionic species, PHREEQC also computed the EC of the reactor solution using equations given in Appelo.[9] The EC of the reactor solution was also computed using a different set of equations given by McCleskey et al..[8] The

https://doi.org/10.1021/acsomega.2c08152
ACS Omega 2023, 8, 13791−13798

equations to compute EC using Appelo and McCleskey et al. are summarized in the subsequent section.

For each simulation, the increase in EC following ureolysis (ΔEC_m) was estimated by subtracting the EC computed from a baseline trial without any hydrolyzed urea and protein buffer (i.e., deionized water in equilibrium with the atmosphere in the headspace). Note that the model assumes a diffusion coefficient of zero for the proteins (regardless of protonation/deprotonation). Therefore, the proteins do not contribute to the EC of the reactor solution. The model did not consider any unhydrolyzed urea or salts present in the urease enzyme source. As the EC of unhydrolyzed urea is negligible, the contribution of urea to changes in the EC during urea hydrolysis can be ignored. Similarly, as the concentration of urease enzyme added in typical urea hydrolysis experiments is relatively small, the contribution of salts in the urease enzyme source to the EC is small (relative to the increase in EC during the first 3 to 5 min of urea hydrolysis) and may be assumed to be constant. In other words, ions in the urease enzyme source do not significantly affect the increase in EC during urea hydrolysis. Thus, given a set of reaction conditions (e.g., urease source and concentration, headspace volume), a one-to-one correspondence between the concentration of hydrolyzed urea ($[\text{hydrolyzed urea}]_m$) and ΔEC_m can be established. The PHREEQC codes used in this study are included in the Supporting Information.

Estimation of EC. Based on the computed molalities of ionic species in the reactor, PHREEQC estimates the EC of the solution using eq 4 (see section on notations for definitions):

$$EC = \sum_i \Lambda^0_{m,i} \gamma_{EC,i} m_i \tag{4}$$

The molar ionic conductivity at infinite dilution (assumed equal to the molal ionic conductivity at infinite dilution) is computed using eq 5:

$$\Lambda^0_{m,i} = \frac{z_i^2 F^2}{RT} D_{w,i} \tag{5}$$

The factor $\gamma_{EC,i}$ is computed using eqs 6 and 7:[9]

$$\gamma_{EC,i} = \exp\left(\frac{-a_{1r} A |z_i| \sqrt{I}}{1 + Ba}\right) \tag{6}$$

$$a = \sqrt{I} \frac{a_{2i}}{1 + I^{0.75}} \tag{7}$$

McCleskey et al. provided an alternative method to compute EC.[8] This method is presented in eqs 8 and 9:

$$EC = \sum_i \lambda_{m,i} m_i \tag{8}$$

$$\lambda_{m,i} = \lambda^0_{m,i} - \frac{A_i' I^{0.5}}{1 + B_i' I^{0.5}} \tag{9}$$

In this study, the EC of a hydrolyzed urea solution was computed using equations of both Appelo (i.e., Eqs (4−7)) and McCleskey et al. The constants D_w, a_1, and a_2 for the major ions involved in urea hydrolysis are listed in Table S1 of the Supporting Information. The constants λ_m^0, A', and B' for the major ions are listed in Table S2 of the Supporting Information.

Experimental Setup. Urea hydrolysis experiments were conducted in a closed reactor bottle of volume 1.16 L. The concentration of urea used in the experiments ranged from 1.5 to 150 mM. The urease concentration was 0.30 g/L for all experiments. The urease was produced from jack bean (Fisher Scientific catalog number U2125, powder). The total volume of the reaction solution was 0.5 L; thus, the ratio of the headspace volume to the mass of the solution equaled ≈ (1.16−0.5 L)/(0.5 L × 1 kg/L) = 1.32 L/kg of solution. Probes measuring pH, EC, and temperature were connected to a multi-parameter analyzer and data logger (Consort C3010 Benchtop Multiparameter Analyzer) and immersed in the reaction solution. The pH, EC, and temperature of the reaction solution were recorded at regular intervals, ranging from 5 to 30 s at the initial stages of ureolysis. At later stages of the ureolysis, the recording interval was increased. The parameters were recorded until the EC attained a constant value (i.e., stopped increasing). The room temperature was maintained at 25 ± 1 °C. A detailed description of the experimental setup is included in Section S2 of the Supporting Information.

Additional experiments were conducted to verify the accuracy of the EC-based method using a colorimetric assay. In a separate reactor bottle, a solution of urea (1.5−150 mM) was prepared. Urease enzyme was added to this solution at a concentration of 0.30 g/L. Following the addition of the urease enzyme into the reactor, samples of partially hydrolyzed urea solution were retrieved after approximately 2, 5, and 10 min of hydrolysis and diluted. The dilution factor ranged from 2 to 10-fold. Then, 2 mL of the diluted solution was added to a cuvette containing 100 μL of Nessler's reagent. The absorbance of the solution at a wavelength of 412 nm (OD_{412}) was measured, and the ammonium concentration was estimated using a calibration curve that was developed by measuring the absorbance of known concentrations of ammonium chloride following Nesslerization. The equation of the calibration curve for Nesslerization is provided in Section S3 of the Supporting Information. Note that given the relatively low concentrations of hydrolyzed urea at the time of sampling and the high solubility of ammonia in water, the effect of the sampling process on the gas−liquid equilibrium was minimal.

The protein concentration of the urease enzyme used in this study was estimated by Khodadadi Tirkolaei et al. as 199 mg of protein/g of enzyme using a commercial protein assay.[11] Thus, the concentration of amino acids is given by

$$\frac{0.30 \frac{\text{g − enzyme}}{\text{L}} \times 0.199 \frac{\text{g − amino acid}}{\text{g − enzyme}}}{110 \frac{\text{g − amino acid}}{\text{mol − amino acid}} \times 10^{-3} \frac{\text{mol − amino acid}}{\text{mmol − amino acid}}} = 0.54 \frac{\text{mmol − amino acid}}{\text{L}} \tag{10}$$

Data Analysis. The unit for representing the concentration of urea and ionic species used in this study is molality; the conversion of molarity to molality for urea solutions is detailed in Section S4 of the Supporting Information. In each experiment, the increase in EC following ureolysis (ΔEC_e) was estimated by subtracting from the measured EC of hydrolyzed urea: (i) the baseline EC reading of the urea solution prior to the addition of enzyme (<5 μS/cm) and (ii) the EC of urease solution prepared at the concentration used in the reactor experiments (≈ 15 μS/cm for 0.30 g/L of urease). Then, by applying the relationship between ΔEC_m

https://doi.org/10.1021/acsomega.2c08152
ACS Omega 2023, 8, 13791−13798

Table 1. Summary of Urea Hydrolysis Experiments

$[urea]_{e,t=0}$ (mmol/kg water)	$[hydrolyzed\ urea]_{e,t\to\infty}$ (mmol/kg water)[a]	ΔEC_e at "end" of urea hydrolysis (μS/cm)	$[hydrolyzed\ urea]_{e,t\to\infty}/\Delta EC_{e,end}$ ((μmol/kg water)/(μS/cm))	predicted $[hydrolyzed\ urea]_m/\Delta EC_m$[b] ((μmol/kg-water)/(μS/cm))	computed ureolysis decay constant (k, h^{-1})[c]
1.47	1.47	200.8	7.32	7.18, 7.27	7.96, 8.08
3.04	3.04	403.5	7.54	7.48, 7.58	5.51, 5.58
5.08	5.08	659.7	7.70	7.63, 7.73	3.77, 3.82
15.1	15.1	1852	8.15	7.85, 7.98	1.49, 1.51
50.1	50.2	5870	8.55	8.08, 8.30	0.493, 0.499
151.5	152.4	16,070	9.48	8.40, 8.82	0.194, 0.196

[a]The theoretical concentration of fully hydrolyzed urea ($[hydrolyzed\ urea]_{e,t\to\infty}$) is slightly higher than the initial concentration of urea ($[urea]_{e,t=0}$) due to the consumption of solvent water during ureolysis. The two parameters are related as follows: $[hydrolyzed\ urea]_{e,t\to\infty} = [urea]_{e,t=0}/(1 - [urea]_{e,t=0} \times M_{water})$, where M_{water} is the molar mass of water (0.018015 kg/mol). [b]Values of $[hydrolyzed\ urea]_m/\Delta EC_m$ predicted by the PHREEQC model assuming $[hydrolyzed\ urea]_m = [hydrolyzed\ urea]_{e,t\to\infty}$. For this prediction, the input concentration of amino acids in the PHREEQC model is 0.54 mM. The first entry denotes values predicted using Appelo and the second entry denotes values predicted using McCleskey et al. [c]The first entry denotes values computed using Appelo and the second entry denotes values computed using McCleskey et al.

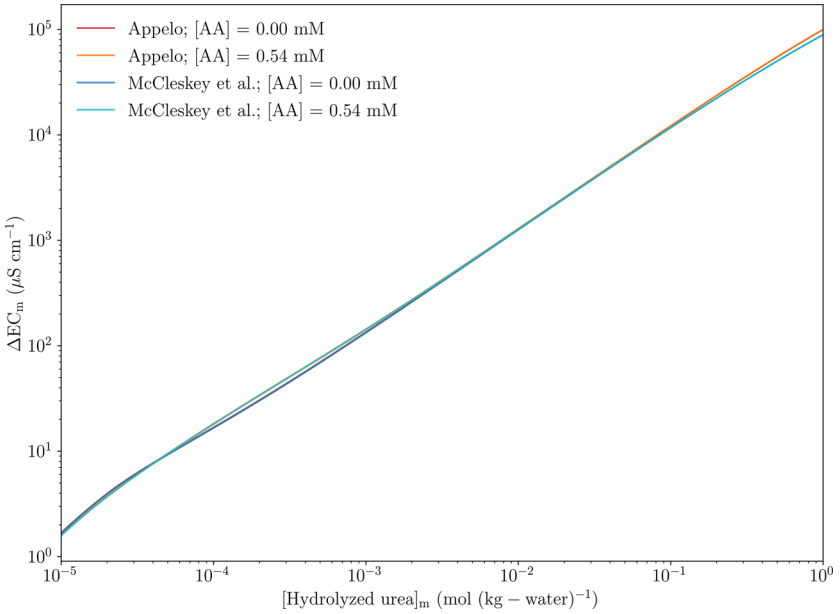


Figure 1. ΔEC_m vs $[hydrolyzed\ urea]_m$ ([AA]: amino acid concentration).

and $[hydrolyzed\ urea]_m$ derived from the PHREEQC model to the dataset of measured ΔEC_e values, we obtained a dataset of the experimental concentration of hydrolyzed urea ($[hydrolyzed\ urea]_e$) with time. Given $[hydrolyzed\ urea]_e$, we computed (i) the moles of urea hydrolyzed and moles of urea remaining and (ii) the mass of solvent water consumed in urea hydrolysis and the mass of water remaining. From these computed parameters, we obtained a dataset of experimental urea concentration ($[urea]_e$) with time. A detailed description of the calculation of $[urea]_e$ from $[hydrolyzed\ urea]_e$ is provided in Section S5 of the Supporting Information.

Using the dataset of $[urea]_e$ with time, a first-order decay function ($d[urea]_e/dt = -k[urea]_e$, where k is a decay constant) was fitted to the data obtained from the first 5 min of the urea hydrolysis reaction. For experiments where the initial urea concentration was 5 mM or less, the decay function was fitted to data obtained from the first 3 min of the reaction. The parameter k was estimated for each experiment from the best-fit decay function curve. Note that the decay function does not represent the rate law for urea hydrolysis; rather, it is simply an exponential regression for computing the initial ureolysis rate. The value of k was different for each experiment. The initial hydrolysis rate for each experiment ($= k[urea]_{e,t=0}$) was then plotted against the initial urea concentration ($= [urea]_{e,t=0}$). The Michaelis–Menten rate expression was fitted using nonlinear least squares regression to this plot to estimate the peak reaction velocity (V_{max}) and the initial urea concentration at which the initial hydrolysis rate was half of V_{max} (i.e., K_m). The Michaelis–Menten rate expression is given as follows:

$$\left.\frac{d[urea]_e}{dt}\right|_{t=0} = -\frac{V_{max}[urea]_{e,t=0}}{K_m + [urea]_{e,t=0}} \quad (11)$$

An equivalent regression approach would be to fit the following expression to a plot of the computed value of k for each experiment versus the initial urea concentration (where $\hat{k}$ denotes the fitted value of k).

$$\hat{k} = \frac{V_{max}}{K_m + [urea]_{e,t=0}} \quad (12)$$

The same procedure was repeated for the experiments where $[urea]_e$ was estimated using Nessler's reagent. Finally, the urease activity was estimated by dividing V_{max} by the urease concentration. The computations for all the experiments are

https://doi.org/10.1021/acsomega.2c08152
ACS Omega 2023, 8, 13791−13798

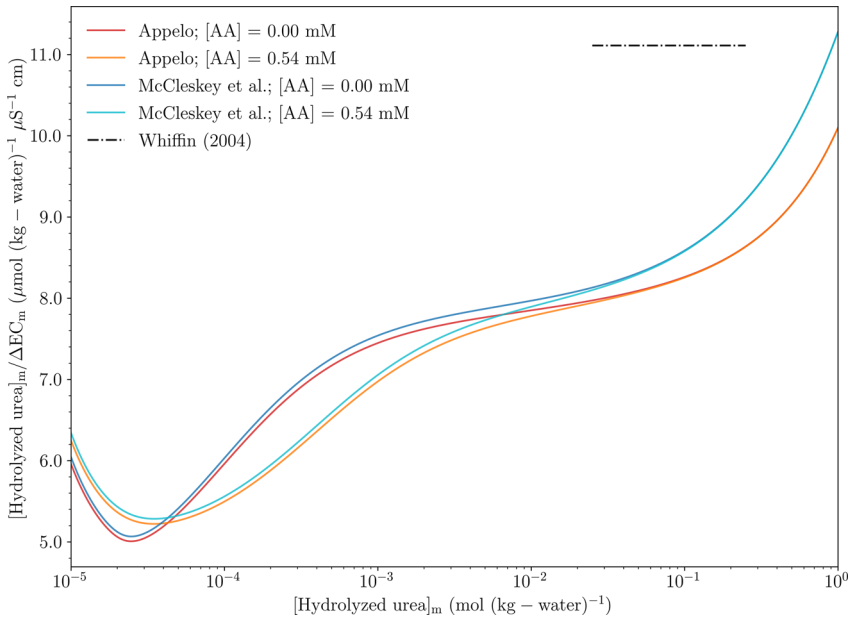


Figure 2. $[\text{Hydrolyzed urea}]_m/\Delta EC_m$ vs $[\text{hydrolyzed urea}]_m$ ([AA]: amino acid concentration). The ratio estimated by Whiffin for urea concentrations of 25 to 250 mM is also shown.

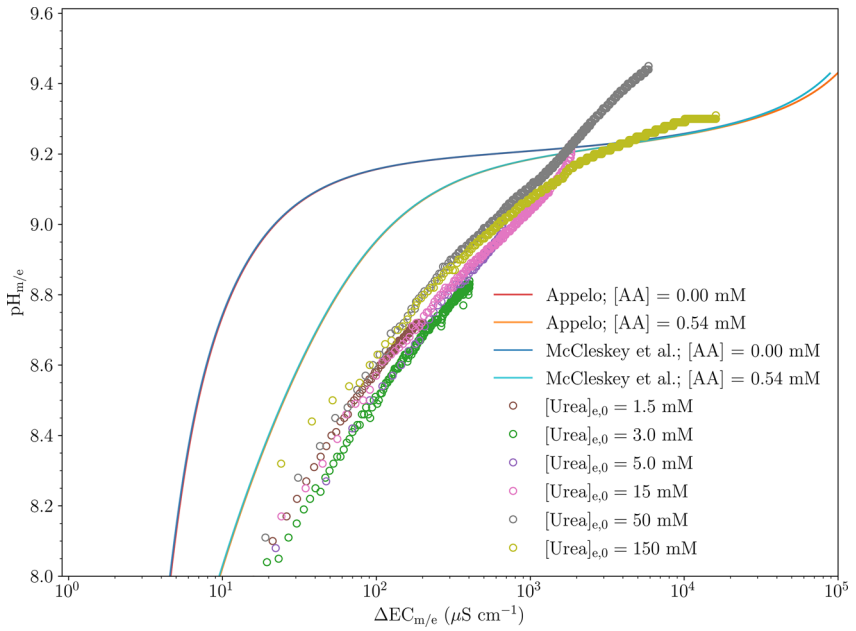


Figure 3. pH vs ΔEC from PHREEQC model output (denoted by subscript m) and experiments (denoted by subscript e). Notation: [AA]: amino acid concentration; $[\text{Urea}]_{e,0}$: initial urea concentration in experiments. At low ΔEC_m ($<10^4$ μS cm^{-1}), the PHREEQC model output curves with the same [AA] are coincident (with the curves with higher [AA] shifted to the right). At higher ΔEC_m ($>10^4$ μS cm^{-1}), the PHREEQC model output curves computed using the same method (i.e., Appelo or McCleskey et al.) are coincident regardless of [AA] (with the curves computed using McCleskey et al. shifted slightly above the curves computed using Appelo).

illustrated in a Microsoft Excel worksheet that is included in the Supporting Information.

RESULTS

For each experiment, the initial concentration of urea, the concentration of fully hydrolyzed urea, and the measured EC at the "end" of urea hydrolysis (i.e., when the EC stopped increasing) are shown in Table 1. In Figure 1, ΔEC_m vs $[\text{hydrolyzed urea}]_m$ is plotted based on the results of the PHREEQC model. In Figure 2, the ratio of $[\text{hydrolyzed urea}]_m$ to ΔEC_m is plotted as a function of $[\text{hydrolyzed urea}]_m$. The experimentally observed values of this ratio (i.e., $[\text{hydrolyzed urea}]_{e,t\to\infty}/\Delta EC_{e,end}$) are shown in Table 1 along with the values predicted by the PHREEQC model. The experimental and predicted values of the ratio are in good agreement at low concentrations of hydrolyzed urea. At higher concentrations, the experimentally observed ratio is greater than the predicted ratio. However, for all concentrations of hydrolyzed urea, the experimental and predicted ratios are less than the value of 11.1 reported by Whiffin.

In Figure 3, pH vs ΔEC during urea hydrolysis is plotted using data collected during the experiments and from the

https://doi.org/10.1021/acsomega.2c08152
ACS Omega 2023, 8, 13791−13798

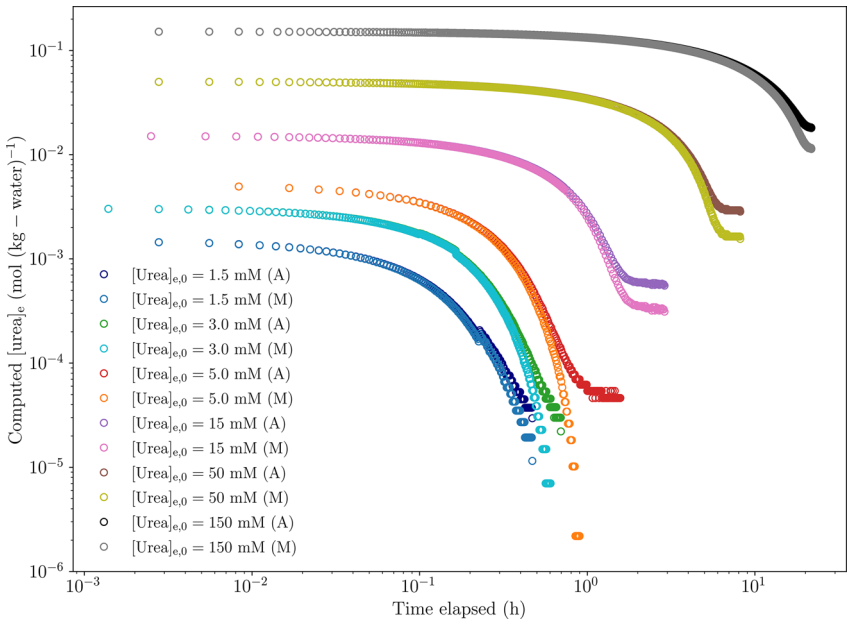


Figure 4. [urea]$_e$ vs time ([Urea]$_{e,0}$: initial urea concentration in experiments). (A) and (M) denote [urea]$_e$ derived from the Appelo and McCleskey et al. PHREEQC models, respectively.

PHREEQC model output. It should be noted that due to nonzero response times of the pH and EC probes, the real-time experimental readings of pH and EC are close to, but not equal to, the true values (i.e., are lesser than the true values). As the experimental data plotted in Figure 3 are the real-time readings (and not the true values), they do not necessarily align with the PHREEQC model results. The difference between the real-time readings and the true values is particularly significant during the initial stages of the ureolysis reaction when the measured pH and EC increase at a higher rate. The rated response time of the EC probe used in this experiment was 98% full-scale reading in 5 s. The response time of the pH probe was not provided by the manufacturer. In this study, the datasets of ΔEC_e values for evaluating urease activity are obtained from the real-time EC readings and not the true values. Further research is required to evaluate the difference between the real-time readings and the true values for the pH and EC probes used in the urea hydrolysis experiments.

Besides the effect of the response times, the measured pH at low ionic strength (i.e., low EC) may be somewhat inaccurate also due to the development of anomalous liquid junction potentials (LJP). LJP refers to the junction potential between the sample solution and the reference electrolyte of the pH electrode (e.g., KCl). In pH measurements, it is implicitly assumed that the LJP of the solution being measured equals the LJP of standard buffers used for calibrating the pH electrode. However, this assumption does not hold when the ionic strength of the solution being measured is much smaller than the ionic strength of the calibration buffers. The variation in LJP may result in inaccurate pH readings.

Figure 4 presents [urea]$_e$ vs time for each experiment by applying the relationship between ΔEC_m and [hydrolyzed urea]$_m$ to the dataset of measured ΔEC_e values. The computed [urea]$_e$ at the "end" of hydrolysis (i.e., when the EC stopped increasing) is not necessarily equal to zero. Small concentrations of unhydrolyzed urea may remain in the reactor, likely due to either (i) noncompetitive inhibition of the urease by ammonium produced during urea hydrolysis or (ii) reduction in urease activity resulting from the increased pH.[12] The remnant unhydrolyzed urea is particularly significant in the experiments with higher initial urea concentrations. Put another way, [hydrolyzed urea]$_{e,end}$ < [hydrolyzed urea]$_{e,t\to\infty}$. For the reaction conditions (ion concentrations and reactor temperature) in this study, the discrepancy between the calculated and measured values of the molal ionic conductivity of NH_4^+ and HCO_3^- reported by McCleskey et al. is negligible.[8] Therefore, the best estimate of [hydrolyzed urea]$_{e,end}$ (and [urea]$_{e,end}$) is obtained using the relationship between ΔEC_m and [hydrolyzed urea]$_m$ derived using McCleskey et al. In comparison, the equations by Appelo overpredict the molal ionic conductivity of NH_4^+ and HCO_3^-, particularly at higher concentrations of hydrolyzed urea. Therefore, when the relationship between ΔEC_m and [hydrolyzed urea]$_m$ derived using Appelo is applied to the measured ΔEC_e at the "end" of urea hydrolysis, [hydrolyzed urea]$_{e,end}$ is underpredicted (and [urea]$_{e,end}$ is overpredicted).

In Table 1, at higher concentrations of hydrolyzed urea, we observed that the ratio [hydrolyzed urea]$_{e,t\to\infty}/\Delta EC_{e,\,end}$ was greater than [hydrolyzed urea]$_m/\Delta EC_m$. Here, we must emphasize that we have not computed [hydrolyzed urea]$_{e,end}/\Delta EC_{e,end}$, which will be less than [hydrolyzed urea]$_{e,t\to\infty}/\Delta EC_{e,end}$. Further investigation is required to experimentally determine [urea]$_{e,end}$ (and [hydrolyzed urea]$_{e,end}$) using analytical techniques independent of EC. Subsequently, a comparison can be made between the ratios [hydrolyzed urea]$_{e,end}/\Delta EC_{e,end}$ and the predicted [hydrolyzed urea]$_m/\Delta EC_m$ (i.e., when [hydrolyzed urea]$_m$ = [hydrolyzed urea]$_{e,end}$).

For urease activity measurement, the relevant data is collected at the beginning (≈ 3−5 min) of the reaction. The concentration of hydrolyzed urea during this period is low, and therefore, any error due to the methods of computing EC is negligible.

The computed decay constant of ureolysis for each experiment is presented in Table 1. The decay constant decreases with increasing concentration of urea. In Figure 5,

https://doi.org/10.1021/acsomega.2c08152
ACS Omega 2023, 8, 13791−13798

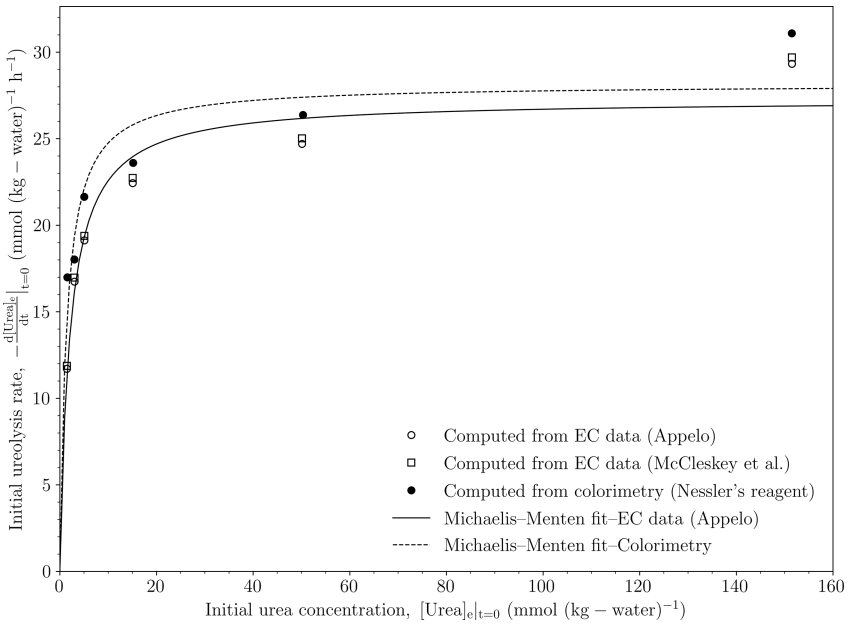


Figure 5. Estimated initial urea hydrolysis rate vs initial urea concentration along with the best-fitting Michaelis−Menten rate curves.

$-d[urea]_e/dt|_{t=0}$ and $[urea]_{e,t=0}$ computed from the experiments is plotted along with the best fit Michaelis−Menten rate curves. The values of V_{max} estimated based on the EC and OD_{412} data are 27.3 and 28.1 mmol urea/kg−water/h, respectively. The urease activity estimated from the EC data is

$$\frac{27.3\frac{\text{mmol}-\text{urea}}{\text{kg}-\text{water}\times\text{h}}\times 10^3\frac{\mu\text{mol}-\text{urea}}{\text{mmol}-\text{urea}}\times 2.0\frac{\mu\text{mol}-(NH_4^+ + NH_3)}{\mu\text{mol}-\text{urea}}}{0.30\frac{\text{g}-\text{enzyme}}{\text{kg}-\text{water}}\times 60\frac{\text{min}}{\text{h}}} = 3030\frac{\mu\text{mol}-(NH_4^+ + NH_3)}{\text{min}\times\text{g}-\text{enzyme}} \quad (13)$$

Similarly, the urease activity estimated from the OD_{412} data is 3130 μmol of $(NH_4^+ + NH_3)$/min/g of enzyme (see the Supporting Information for experimental data and computations). The values of K_m estimated based on the EC and OD_{412} data are 2.1 mmol urea/kg of water and 1.4 mmol urea/kg of water, respectively. The rate constants (V_{max} and K_m) estimated from the EC data and the OD_{412} data compare well, thereby substantiating the viability of using EC measurements in conjunction with chemical equilibrium modeling for estimating the activity of urease enzymes. The estimated urease activity is consistent with the activity of the same urease enzyme product reported by Khodadadi Tirkolaei et al., namely, 4204 U/g.[11]

DISCUSSION

The EC-based method for evaluating urease activity presented herein involves several simplifying assumptions. First, the contribution of proteins and ions in the urease source to the EC is not modeled in PHREEQC. In the experiments, this contribution is assumed constant for evaluating ΔEC_e. Second, complexation and ion pairing reactions involving proteins or ions in the urease source are not modeled in PHREEQC. Only the protonation/deprotonation (i.e., buffering capacity) of the amine groups in the proteins is modeled via a simplified approach. These assumptions are generally acceptable provided the amount of urease added to the reactor is small. Compared to common colorimetric assays, an EC-based method of determining urease activity uses more data points and is therefore likely to provide a more precise estimate of the initial rate of hydrolysis.

The urea hydrolysis experiments described in this study do not include the addition of a buffer (other than the proteins in the urease source), and the urease activity estimated corresponds to a pH of 8.0 to 9.0. As such, buffers can be considered in the PHREEQC model by specifying the concentration of the buffer and the pK_a of protonation (or deprotonation) reactions. Possible complexation reactions and ion pairing between ions in the buffer and the products of urea hydrolysis will also need to be specified in the PHREEQC model. Therefore, it is preferable to use a low concentration of buffer (relative to the initial urea concentration) to minimize errors in PHREEQC modeling as well as in the determination of ΔEC_e.

The EC-based method presented herein is viable for measuring the activity of urease enzymes over a diverse range of forms, including highly purified pharmaceutical grade urease, less purified commercial urease, and crude urease extracts. This capability is especially useful in field applications where the availability of standard laboratory equipment may be limited.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c08152.

(i) Experimental details, materials and methods, including photograph of experimental setup (one Microsoft Word file), (ii) PHREEQC codes (one text file), and (iii) worksheet comprising experimental datasets and computations (one Microsoft Excel file) (ZIP)

https://doi.org/10.1021/acsomega.2c08152
ACS Omega 2023, 8, 13791−13798

AUTHOR INFORMATION

Corresponding Author

Vinay Krishnan − *School of Sustainable Engineering and the Built Environment, Arizona State University, Tempe, Arizona 85287-3005, United States;* orcid.org/0000-0002-5481-4208; Email: vkrishnan@asu.edu

Authors

Hamed Khodadadi Tirkolaei − *School of Sustainable Engineering and the Built Environment, Arizona State University, Tempe, Arizona 85287-3005, United States*

Edward Kavazanjian, Jr. − *School of Sustainable Engineering and the Built Environment, Arizona State University, Tempe, Arizona 85287-3005, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c08152

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The material in this research study is based upon work primarily supported by the National Science Foundation (NSF) under NSF Cooperative Agreement No. EEC-1449501. The authors are grateful for the NSF support. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect those of the NSF.

NOTATION LIST

A, Debye−Hückel parameter A (equals 0.5100 $mol^{-0.5}$ $kg^{0.5}$ at 25 °C)
B, Debye−Hückel parameter B (equals 0.3285 nm^{-1} $mol^{-0.5}$ $kg^{0.5}$ at 25 °C)
A_i' and B_i', empirical coefficients for ion i used in McCleskey et al.
$D_{w,i}$, diffusion coefficient of ion i at infinite dilution (m^2 s^{-1})
F, Faraday's constant (96,485.3 C mol^{-1})
I, ionic strength (mol kg^{-1})
R, gas constant (8.31446 J K^{-1} mol^{-1})
T, temperature (K)
a_{1i} and a_{2i}, empirical coefficients used in Appelo
m_i, molality of ion i (mol kg^{-1})
z_i, charge of ion i
$\Lambda_{m,i}^{0}$, molar ionic conductivity of ion i at infinite dilution used in Appelo (S m^2 mol^{-1})
$\gamma_{EC,i}$, factor correcting molar ionic conductivity of ion i for effects of ion concentration
$\lambda_{m,i}$, molal ionic conductivity of ion i used in McCleskey et al. (mS cm^{-1} mol^{-1} kg)
$\lambda_{m,i}^{0}$, molal ionic conductivity of ion i at infinite dilution used in McCleskey et al. (mS cm^{-1} mol^{-1} kg)

REFERENCES

(1) Mobley, H. L. T.; Hausinger, R. P. Microbial ureases: Significance, regulation, and molecular characterization. *Microbiol. Rev.* **1989**, *53*, 85−108.
(2) Krajewska, B.; Chudy, M.; Drozdek, M.; Brzozka, Z. Potentiometric study of urease kinetics over pH 5.36-8.21. *Electroanalysis* **2003**, *15*, 460−466.
(3) Bayliss, W. M. *The nature of enzyme action*, 2nd ed.; Longmans, Green, and Co.: London, 1911; pp. 39−40.
(4) Sastri, B. N.; Sreenivasaya, M. Conductometric determination of enzyme activity. *Ind. Eng. Chem., Anal. Ed.* **1936**, *8*, 458−459.
(5) Croston, C. B.; Smith, A. K.; Cowan, J. C. Measurement of urease activity in soybean oil meal. *J. Am. Oil Chem. Soc.* **1955**, *32*, 279−282.
(6) Chin, W.-T.; Kroontje, W. Conductivity method for determination of urea. *Anal. Chem.* **1961**, *33*, 1757−1760.
(7) Whiffin, V. S. Appendix B−Conversion of conductivity (mS) to urea hydrolysed (mM). In *Microbial CaCO3 precipitation for the production of biocement*; Ph.D. dissertation, Murdoch University, Perth, Australia, 2004; p. 147. https://researchrepository.murdoch.edu.au/id/eprint/399/.
(8) McCleskey, R. B.; Nordstrom, D. K.; Ryan, J. N.; Ball, J. W. A new method of calculating electrical conductivity with applications to natural waters. *Geochim. Cosmochim. Acta* **2012**, *77*, 369−382.
(9) Appelo, C. A. J. Solute transport solved with the Nernst-Planck equation for concrete pores with 'free' water and a double layer. *Cem. Concr. Res.* **2017**, *101*, 102−113.
(10) Parkhurst, D. L.; Appelo, C. A. J. *Description of input and examples for PHREEQC version 3—A computer program for speciation, batch-reaction, one-dimensional transport, and inverse geochemical calculations: U.S. Geological Survey Techniques and Methods, Book 6, Chapter A43*; U.S. Geological Survey: Denver, 2013. http://pubs.usgs.gov/tm/06/a43/.
(11) Khodadadi Tirkolaei, H.; Javadi, N.; Krishnan, V.; Hamdan, N.; Kavazanjian, E., Jr. Crude urease extract for biocementation. *J. Mater. Civ. Eng.* **2020**, *32*, 04020374.
(12) Krajewska, B. U. I. Functional, catalytic and kinetic properties: A review. *J. Mol. Catal. B: Enzym.* **2009**, *59*, 9−21.