UREASE IMMOBILIZATION FOR ADVANCING ENZYME-INDUCED CALCIUM CARBONATE PRECIPITATION APPLICATIONS

by

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TABLE OF CONTENTS

1. INTRODUCTION .......................................................................................................................... 1
   Background ................................................................................................................................. 1
   Thesis Overview ......................................................................................................................... 3

2. IMMOBILIZATION OF UREASE IN LUDOX® GEL AND ON A POROUS CERAMIC PROPPANT TO INCREASE ITS THERMAL STABILITY ........................................ 5
   Introduction ............................................................................................................................... 5
   Material and Methods ............................................................................................................... 10
      Materials ................................................................................................................................. 10
      Microorganism and Growth Conditions ................................................................................. 10
      Immobilization of S. pasteurii in Ludox® Gel ....................................................................... 11
      Jack Bean Meal Suspension Preparation .............................................................................. 11
      Urease Immobilization on Ceramic Proppant ....................................................................... 11
      Temperature Exposure: Thermal Inactivation Study ............................................................... 12
      Determination of Urease Activity ........................................................................................... 12
      Negative Controls for the Immobilized Forms ....................................................................... 13
      First Order Thermal Inactivation Rate Coefficient Analysis .................................................. 14
      Thin Cross Section of the Ceramic Proppant ....................................................................... 15
      Microscopy Imaging of the Ceramic Proppant ..................................................................... 16
   Results and Discussion ............................................................................................................ 16
      Residual Activity of the Immobilized, Thermally Exposed Samples ....................................... 16
      Thermal Stability of the Immobilized Forms ....................................................................... 18
   Conclusions ............................................................................................................................... 25

3. COLUMN STUDIES USING UREASEIMMOBILIZED ON A POROUS CERAMIC PROPPANT TO INDUCE CALCIUM CARBONATE PRECIPITATION ............................................. 27
   Introduction ............................................................................................................................... 27
   Materials and Methods ............................................................................................................ 31
      Materials ................................................................................................................................. 31
      Jack Bean Suspension Preparation ....................................................................................... 32
      Urease Immobilization on the Ceramic Proppant .................................................................. 32
      Storage Stability Analysis of Immobilized Urease ................................................................. 32
      Room Temperature (22°C) Column Construction ................................................................. 33
      60°C Column Construction .................................................................................................... 34
      Approximate Pore Volume Calculation .................................................................................. 36
      Mineralization Procedure ..................................................................................................... 36
      Room Temperature Column Sampling Procedure ............................................................... 37
TABLE OF CONTENTS CONTINUED

60°C Column Sampling Procedure ................................................................. 37
Stereoscope Imaging ......................................................................................... 38
Thin Cross Section and Elemental Analysis ...................................................... 38
Raman Spectroscopy Analysis .......................................................................... 39
X-ray Computed Micro-Tomography Scan Analysis for Approximate Porosity ................................................................................................. 39
Calcium Digest of the Mineralized Columns ................................................. 40
Results and Discussion ...................................................................................... 41
Storage Stability of the Immobilized Urease ................................................... 41
Urea Concentration and pH Data for the Room Temperature Column ........... 42
Urea Concentration and pH Data for the 60°C Column ................................. 45
Post-Mineralization Analysis of the Room Temperature Column ................. 48
Thin Cross Section and Energy-Dispersive X-ray Analysis ............................ 54
Post-Mineralization Analysis of the 60°C Column ......................................... 56
Porosity Estimation Based on X-ray µ-CT Scan and Calcium Carbonate Quantification ................................................................. 59
Conclusions ....................................................................................................... 65

4. CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK ..................... 67

Conclusions ....................................................................................................... 67
Suggestions for Future Work ............................................................................ 68

APPENDICIES .................................................................................................. 70

APPENDIX A: Spectrophotometric Assay for Quantifying Calcium Concentration ................................................................. 71
APPENDIX B: Inhibition Studies on JBM Urease with Differing Concentrations of Divalent Heavy Metal Cations and Sodium Chloride ......................................................................................... 81
APPENDIX C: Supplemental Urea and pH Data for the Proppant Columns ...... 81
APPENDIX D: Determination of Urease Source for Ceramic Proppant Experiments ......................................................................................... 90
APPENDIX E: Supplemental X-ray Computed Micro-Tomography Scans of the 60°C Columns ................................................................................................. 93

REFERENCES CITED ....................................................................................... 95
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>P-values for unequal variance t-tests between the values of the first order thermal inactivation coefficient of immobilized and suspended urease</td>
<td>20</td>
</tr>
<tr>
<td>2.2</td>
<td>Half-lives for each form of the urease enzyme tested at various temperatures (60°C-90°C)</td>
<td>22</td>
</tr>
<tr>
<td>3.1</td>
<td>P-values for equal variance t-tests comparing pieces of the room temperature column</td>
<td>63</td>
</tr>
<tr>
<td>3.2</td>
<td>P-values for equal variance t-tests comparing pieces of the 60°C column</td>
<td>63</td>
</tr>
<tr>
<td>A.1</td>
<td>Calcium concentrations measured with the calcium assay and ICP-MS and compared</td>
<td>72</td>
</tr>
<tr>
<td>A.2</td>
<td>Calcium concentrations measured with the calcium assay to test the inhibition of certain compounds on the calcium assay</td>
<td>74</td>
</tr>
<tr>
<td>B.1</td>
<td>The fraction of initial activity of the JBM when subjected to each concentration of each inhibitor as compared to the initial, noninhibited urease activity</td>
<td>82</td>
</tr>
<tr>
<td>B.2</td>
<td>pH of the solutions containing the JBM and the specific metal inhibitor at the specific concentration</td>
<td>83</td>
</tr>
<tr>
<td>C.1</td>
<td>Urea concentration data for the first room temperature column</td>
<td>85</td>
</tr>
<tr>
<td>C.2</td>
<td>pH data for the first room temperature column</td>
<td>85</td>
</tr>
<tr>
<td>C.3</td>
<td>Urea concentration data for the second room temperature column</td>
<td>86</td>
</tr>
<tr>
<td>C.4</td>
<td>pH data for the second room temperature column</td>
<td>86</td>
</tr>
<tr>
<td>C.5</td>
<td>Urea concentration data for the first 60°C column</td>
<td>87</td>
</tr>
<tr>
<td>C.6</td>
<td>pH data for the first 60°C column</td>
<td>87</td>
</tr>
<tr>
<td>C.7</td>
<td>Urea concentration data for the second 60°C column</td>
<td>88</td>
</tr>
</tbody>
</table>
### LIST OF TABLES CONTINUED

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.8</td>
<td>pH data for the second 60°C column</td>
</tr>
<tr>
<td>D.1</td>
<td>First order thermal inactivation coefficients for different urease forms</td>
</tr>
<tr>
<td>D.2</td>
<td>Ureolytic activity of different urease forms</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Graphic illustrating the temperature gap currently hampering some UICP applications</td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>Residual activity of urease immobilized in Ludox® gel when exposed to elevated temperatures</td>
<td>17</td>
</tr>
<tr>
<td>2.3</td>
<td>Residual activity of urease immobilized on ceramic proppant when exposed to elevated temperatures</td>
<td>17</td>
</tr>
<tr>
<td>2.4</td>
<td>Arrhenius plot of the comparison of the first order thermal inactivation coefficients for urease immobilized in Ludox® gel and suspended</td>
<td>18</td>
</tr>
<tr>
<td>2.5</td>
<td>Arrhenius plot of the comparison of the first order thermal inactivation coefficients for urease immobilized on the ceramic proppant and suspended</td>
<td>19</td>
</tr>
<tr>
<td>2.6</td>
<td>FE-SEM image of the ceramic proppant</td>
<td>24</td>
</tr>
<tr>
<td>2.7</td>
<td>FE-SEM image of the thin cross sectioned ceramic proppant</td>
<td>24</td>
</tr>
<tr>
<td>3.1</td>
<td>Experimental setup for the room temperature column</td>
<td>34</td>
</tr>
<tr>
<td>3.2</td>
<td>Experimental setup for the 60°C column</td>
<td>35</td>
</tr>
<tr>
<td>3.3</td>
<td>Fraction of initial of immobilized urease compared to suspended urease when subjected to various storage durations</td>
<td>41</td>
</tr>
<tr>
<td>3.4</td>
<td>Urea concentrations for the room temperature column</td>
<td>42</td>
</tr>
<tr>
<td>3.5</td>
<td>pH values for the room temperature column</td>
<td>42</td>
</tr>
<tr>
<td>3.6</td>
<td>Urea concentrations for the 60°C column</td>
<td>46</td>
</tr>
<tr>
<td>3.7</td>
<td>pH values for the 60°C column</td>
<td>46</td>
</tr>
<tr>
<td>3.8</td>
<td>The extracted, mineralized room temperature column</td>
<td>48</td>
</tr>
<tr>
<td>3.9</td>
<td>Raman spectra for the mineralized proppant particles extracted from the room temperature column</td>
<td>49</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES CONTINUED

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.10</td>
<td>Stereoscope images of the ceramic proppant before mineralization. ..........50</td>
</tr>
<tr>
<td>3.11</td>
<td>Stereoscope images of the mineralized proppant extracted from the room temperature column ........................................50</td>
</tr>
<tr>
<td>3.12</td>
<td>FE-SEM images of the non-mineralized, ceramic proppant ..................51</td>
</tr>
<tr>
<td>3.13</td>
<td>FE-SEM image of the mineralized proppant from the room temperature column ..........................................................51</td>
</tr>
<tr>
<td>3.14</td>
<td>FE-SEM image of three proppant particles bonded to one another due to calcium carbonate ........................................52</td>
</tr>
<tr>
<td>3.15</td>
<td>A magnified FE-SEM image of an interface between two proppant particles bonded by calcium carbonate ........................................53</td>
</tr>
<tr>
<td>3.16</td>
<td>FE-SEM image of the thin cross sectioned non-mineralized proppant ......54</td>
</tr>
<tr>
<td>3.17</td>
<td>FE-SEM images of representative pores that are found in the ceramic proppant for both non-mineralized and mineralized proppant ..........................................................54</td>
</tr>
<tr>
<td>3.18</td>
<td>EDX maps for the elements present in the non-mineralized proppant ......55</td>
</tr>
<tr>
<td>3.19</td>
<td>EDX maps for the elements present in the mineralized proppant extracted from the room temperature column ..........................55</td>
</tr>
<tr>
<td>3.20</td>
<td>The extracted, mineralized 60°C column .......................................................57</td>
</tr>
<tr>
<td>3.21</td>
<td>Raman spectra received from the mineralized proppant extracted from the 60°C column .....................................................57</td>
</tr>
<tr>
<td>3.22</td>
<td>FE-SEM images of the mineralized proppant particles extracted from the 60°C column ..........................................................58</td>
</tr>
<tr>
<td>3.23</td>
<td>μ-CT scan data for porosity measurements of the room temperature, 60°C, and control columns. ........................................59</td>
</tr>
<tr>
<td>3.24</td>
<td>Calcium digest data for the room temperature column ...............................62</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES CONTINUED

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.25</td>
<td>Calcium digest data for the 60°C column</td>
<td>62</td>
</tr>
<tr>
<td>C.1</td>
<td>Influent and effluent urea concentration data for the first room temperature column</td>
<td>86</td>
</tr>
<tr>
<td>C.2</td>
<td>Influent and effluent pH data for the first room temperature column</td>
<td>86</td>
</tr>
<tr>
<td>C.3</td>
<td>Influent and effluent urea concentration data for the second room temperature column</td>
<td>87</td>
</tr>
<tr>
<td>C.4</td>
<td>Influent and effluent pH data for the second room temperature column</td>
<td>87</td>
</tr>
<tr>
<td>C.5</td>
<td>Influent and effluent urea concentration data for the first 60°C column</td>
<td>88</td>
</tr>
<tr>
<td>C.6</td>
<td>Influent and effluent pH data for the first 60°C column</td>
<td>88</td>
</tr>
<tr>
<td>C.7</td>
<td>Influent and effluent urea concentration data for the second 60°C column</td>
<td>89</td>
</tr>
<tr>
<td>C.8</td>
<td>Influent and effluent pH data for the second 60°C column</td>
<td>89</td>
</tr>
<tr>
<td>E.1</td>
<td>Supplemental µ-CT scans of the 60°C columns</td>
<td>94</td>
</tr>
</tbody>
</table>
Microbially induced calcium carbonate precipitation (MICP) is a widely studied field of research exploiting bacterial activity to form a calcium carbonate precipitate that has been used to modify porous media. MICP is an enzymatically driven process and uses the enzyme urease to change solution chemistry to favor calcium carbonate precipitation. An enzyme slurry can be used in lieu of microbial growth and can be applied in a similar fashion and is commonly referred to as enzymatically induced calcium carbonate precipitation (EICP). For some applications temperature can stunt microbial growth and EICP may be the preferred method. However, as the temperature increases further the urease enzyme is thermally inactivated inhibiting calcium carbonate precipitation. Thermal inactivation limits the potential use of EICP in higher temperature environments. To combat thermal inactivation, immobilization of the urease enzyme through entrapment in silica gel and adsorption on an internally porous ceramic proppant was evaluated, and the first order inactivation coefficient (k_d) was determined for temperatures between 60°C and 90°C. It was found that immobilization of the urease enzyme drastically reduced the apparent k_d when compared to the free, non-immobilized form. Column experiments were performed using the urease immobilized on the ceramic proppant at room temperature (~23°C) and at 60°C. It was found that the immobilized urease retained high activity for the duration of the experiments even when subjected to the elevated temperature condition. The immobilized form of the urease enzyme was indeed protected from thermal degradation. It also seemed that the immobilized form of the urease enzyme was shielded from inactivation from active calcium carbonate precipitation, as observed in previous EICP and MICP experiments, in which ureolytic activity decreased rapidly as calcium carbonate precipitated. As a result, the immobilized form of the urease enzyme showed promise for advancing EICP applications.
CHAPTER ONE

INTRODUCTION

Background

The experiments presented in this thesis focus primarily on advancing urea-hydrolysis (ureolysis) induced calcium carbonate precipitation (UICP). UICP occurs through three main mechanisms: (1) microbially-induced calcium carbonate precipitation (MICP), (2) enzyme-induced calcium carbonate precipitation (EICP), and (3) thermally induced calcium carbonate precipitation (TICP). The MICP and EICP mechanisms both utilize the enzyme urease, an enzyme commonly found in some bacteria, plants and fungi, whereas TICP utilizes thermal energy to catalyze the ureolysis reaction (Stocks-Fischer, Galinat and Bang 1999, Abdel-Rahman et al. 1992, Mahmoud, El-Shanawany and Omar 1996, Phillips 2013, Kavazanjian and Hamdan 2015, Koebel and Strutz 2003). Further development and analysis of TICP was not considered in this thesis, as the enzyme urease was the main focus. Each mechanism of urea hydrolysis (Equation 1.1) ultimately produces ammonia (NH$_3$) and carbonic acid (H$_2$CO$_3$) raising the overall pH of the system through the ammonia acting as a Brønsted-Lowry base by accepting a proton to produce ammonium (NH$_4^+$) and a hydroxide ion in aqueous solution (Equation 1.4). Then through a series of additional acid-base reactions carbonate (CO$_3^{2-}$) is formed (Equations 1.2-1.5)(Dick et al. 2006, Mitchell et al. 2010). If calcium ions (Ca$^{2+}$) are present in sufficiently high concentrations calcium carbonate may precipitate creating a solid mineral and lowering the pH (Equation 1.6).
\[ CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} NH_2COOH + NH_3 \]  
Equation. 1.1

\[ NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \]  
Equation. 1.2

\[ H_2CO_3 \leftrightarrow HCO_3^- + H^+ \]  
Equation. 1.3

\[ 2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \]  
Equation. 1.4

\[ HCO_3^- + H^+ + 2OH^- \leftrightarrow CO_3^{2-} + 2H_2O \]  
Equation. 1.5

\[ CO_3^{2-} + Ca^{2+} \leftrightarrow CaCO_3 (s) \]  
Equation. 1.6

Calcium carbonate precipitation has been suggested for a wide variety of engineering applications such as restricting fluid flow in the subsurface, dust suppression, remediation of heavy metals, enhanced oil recovery and many more (Cunningham et al. 2014, Hamdan and Kavazanjian Jr 2016, Kang and So 2016, Phillips et al. 2016, Cunningham et al. 2011, Phillips et al. 2013, DeJong et al. 2010, Kumari et al. 2016, Cuthbert et al. 2013). Studies have even used this technology to develop a self-healing concrete highlighting the flexibility of this technology (Bang et al. 2010, Jonkers et al. 2010). The broad spectrum of engineering applications makes UICP an attractive topic of research and development.

One challenge of MICP and EICP is that they are dependent on microbial growth and/or the enzyme urease consequently limiting the environmental conditions where the technologies may be applied. Microbial and enzyme activity may be hampered by several different conditions including temperature, pH, and the presence of inhibitors (Amtul, Siddiqui and Choudhary 2002, Aymard and Belarbi 2000, Singh and Nye 1984).
Appendix B highlights several solutes that inhibit urease activity. Therefore, MICP and EICP are currently only able to be applied under certain conditions decreasing the range of engineering applications available. For subsurface applications temperature can increase with depth creating temperatures that exceed the operating threshold of urease. Temperatures of 55°C and above have been observed to decrease enzyme activity as the enzyme is thermally inactivated (Illeová et al. 2003). The thermal inactivation of urease as a result of this increased temperature can be a challenge to using MICP and EICP in some subsurface applications. However, the immobilization of enzymes is known to increase their overall stability when subjected to inactivating conditions (Sheldon 2007). This thesis describes work that, through the immobilization of urease, may increase the viability of MICP and EICP at higher temperatures to create new areas where the technology may be applied.

**Thesis Overview**

The first study reported in this thesis was to immobilize the urease enzyme both in a colloidal silica gel (Ludox®) and on a porous ceramic proppant to understand how thermally stable the immobilized form was compared to the free (i.e. suspended) form of urease. This study (Chapter 2) aimed to show that the rate of thermal inactivation of urease decreases when in immobilized form. A decrease in thermal inactivation rate would allow for a greater thermal range that MICP and EICP may be used in the future.

The second study (Chapter 3) describes several column experiments that used urease immobilized on ceramic proppant for calcium carbonate precipitation. The goal of
this study was to determine how well the immobilized form was able to induce mineralization. The immobilized form of urease was constrained on a carrier and therefore its ability to induce calcium carbonate precipitation may have been different than for the free form. Kinetic parameters of enzymes have been shown to change with immobilization, and this may have influenced the ability of the immobilized urease to induce mineralization (Lai and Tabatabai 1992, Krajewska, Leszko and Zaborska 1990, Krajewska 2009b). A column operated at room temperature, 22°C, first investigated the mineralization of immobilized urease. A second column operated at 60°C considered the effectiveness of mineralization at an elevated temperature where thermal inactivation of the enzyme could play a role in the dynamics of the system. The temperature condition of 60°C is approximately the threshold at which EICP becomes no longer feasible. Therefore, if the immobilized urease was able to efficiently induce mineralization at this temperature then the thermal range of EICP applications may be expanded.

Chapter 4 summarizes the key results of Chapter 2 and Chapter 3 while also recommending future work to be performed in advancing calcium carbonate precipitation technologies. Several key appendices highlight studies to develop a spectrophotometric assay for quantifying calcium concentration in dilute solutions (Appendix A), and enzyme inhibition by divalent cations: copper (Cu$^{2+}$), zinc (Zn$^{2+}$), and cobalt (Co$^{2+}$) and sodium chloride (Appendix B).
CHAPTER TWO

IMMOBILIZATION OF UREASE IN LUDOX® GEL AND ON A POROUS CERAMIC PROPPANT TO INCREASE ITS THERMAL STABILITY

Introduction

Ureolysis-induced calcium carbonate precipitation (UICP) is a process that results in the formation of calcium carbonate precipitates through the hydrolysis of urea. Hydrolysis can occur because of the enzymatic activity of urease that can be found in some microbes (microbially-induced calcium carbonate precipitation) and plant-based or extracted enzyme (enzyme-induced calcium carbonate precipitation) (Stocks-Fischer et al. 1999, Kavazanjian and Hamdan 2015, Phillips 2013). Urease catalyzes the hydrolysis of urea to ultimately yield ammonium (NH₄⁺) and carbonate ions (CO₃²⁻), resulting in a local rise in pH and alkalinity. If calcium is present in sufficiently saturated conditions, then calcium carbonate may precipitate out of solution (Equation 2.1).

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

Equation 2.1

UICP technology has been exploited for many novel engineering applications including carbon dioxide sequestration, heavy metal removal for environmental remediation, and reduction of permeability to mitigate wellbore leakage or enhanced oil recovery (Phillips et al. 2013, Cunningham et al. 2011, Phillips et al. 2016, Reddy 2013, Kumari et al. 2016). Microbially-induced calcium carbonate precipitation (MICP) has been successfully implemented in permeability modification field studies (Phillips et al.
The bacterium used, *Sporosarcina pasteurii*, grows and acts not only as a source of the urease enzyme needed for biomineralization, but can also act as possible nucleation point for the calcium carbonate to develop (Stocks-Fischer et al. 1999, De Muynck et al. 2008). The bacteria secrete extracellular polymeric substances (EPS) creating a biofilm that may change the mechanical properties of the biomineral relative to abiotic forms making it highly desirable for subsurface applications (Besar 2018). However, the conditions of the engineering applications of UICP may be less than favorable for bacterial growth. The presence of certain inhibitory solutes, such as a high concentration of sodium chloride, low or high pH (<3 or >9), or high temperatures (>40°C) may cause little to no bacterial growth.

The urease enzyme used in enzyme-induced calcium carbonate precipitation (EICP) can be extracted from plants such as jack bean or soybean but could also be harvested from bacteria (Krajewska 2009a). Calcium carbonate precipitation technologies can then be applied where MICP may not be a viable option due to harsh environmental conditions limiting microbial growth. A major concern that possibly limits the use of MICP in some environments is due to increasing temperatures often found in deeper subsurface applications that might hinder growth. EICP can be implemented in higher temperature environments without the need for microbial growth and propagation making it a viable alternative. However, the extracted enzyme may also become inactivated at sufficiently high temperatures.
The urease enzyme may become inactivated as temperature increases resulting in decreased activity or complete inactivation of the enzyme (Tomazic and Klibanov 1988, Aymard and Belarbi 2000). Therefore, a limitation is imposed on the temperature range where the urease enzyme may be used in engineering applications. In the subsurface where temperatures can increase greatly with depth, the inactivation threshold of the urease enzyme may be exceeded. In previous work, it was shown that as temperature increases the activity of the urease enzyme also increases, so there is a tradeoff between increasing ureolysis rates and enzyme inactivation (Morasko 2018). Above 60°C, the urease enzyme becomes thermally inactivated reducing ureolysis rates and calcium carbonate precipitation can be significantly limited. Spontaneous thermally-induced calcium carbonate precipitation (TICP) occurs reliably if the temperature is high enough (i.e. >110°C). This leaves a temperature zone between 60°C and 110°C where calcium carbonate precipitation does not dependably occur, and UICP technology cannot currently be exploited consistently (Figure 2.1).

Figure 2.1: Graphic highlighting the temperature gap in which UICP techniques are currently hampered through thermal inactivation of the enzyme or not enough thermal energy to induce spontaneous ureolysis. Immobilization of the urease enzyme may be a key in bridging this gap as it may protect the enzyme from thermal inactivation enabling EICP techniques to be operated at elevated temperature conditions.
Expanding the workable temperature zone would allow calcium carbonate precipitation technologies to be utilized in situations that are presently more difficult to access. One way proposed to overcome the thermal inactivation of urease and improve its thermal stability is through immobilization (Sheldon 2007).

Immobilization is a process in which an enzyme is adsorbed, covalently bound, or otherwise attached to a carrier that may serve to protect the enzyme (Garcia-Galan et al. 2011). Immobilization has also been performed by entrapping the enzyme in a gel or other polymer matrix (Reetz, Zonta and Simpelkamp 1996, Smidsrød and Skja 1990, Kato et al. 2009). The immobilized enzyme may then be protected from thermal degradation, pH influence, or inactivation by inhibitors such as heavy metals (Mateo et al. 2007, Klibanov 1979). Immobilized enzymes have been shown to exhibit increased storage stability in addition to the described protection abilities (Poźniak, Krajewska and Trochimczuk 1995, Krajewska 2009b). Equally important is that immobilization allows for continuous or repeated use of the enzyme as the enzyme is retained in a specific location rather than being washed out for a given application (Bornscheuer 2003). This allows for the enzyme to be preserved within the reactor creating the possibility of unconventional reactor designs (Bickerstaff 1997, Chibata, Tosa and Sato 1986). Compared to the dissolved or freely suspended enzyme, enzymes immobilized on a surface can create a localized active zone that can be used repeatedly. Therefore, immobilization has been shown to provide a way of addressing some previous biochemical barriers and has allowed for innovative ideas within many research fields. Ureases from a variety of sources have been the subject of much immobilization work
and are well characterized for many engineered applications such as urea biosensors (Krajewska 2009b).

Although there are numerous reports on immobilizing urease, many immobilization procedures of ureases have not been studied in detail to determine the thermal inactivation of immobilized urease at several temperatures. Studies focus on one or a small range of temperature conditions to test the efficacy of thermal protection offered by immobilization (Bayramoğlu et al. 2003, Rejikumar and Devi 1998). A thermal inactivation rate coefficient at multiple temperatures is often not calculated for the immobilized forms of urease, but these coefficients may be essential for associated modelling. Modelling can meaningfully help in applying and understanding UICP technology (Cunningham et al. 2018). As a result, it may be necessary to obtain these coefficients to advance calcium carbonate precipitation technologies, and more effectively apply EICP. The work presented here is devoted to understanding the rates at which the urease enzyme (from *S. pasteurii* and jack bean (*Canavalia ensiformis*)) is thermally inactivated at different temperatures when it is immobilized through entrapment in a silica gel and adsorbed on a porous ceramic proppant. The inactivation rates were also compared to those of the free, suspended enzyme. First order thermal inactivation rate coefficients were calculated for both immobilized forms of urease for a range of temperatures (60°C-90°C) that may aid in understanding how the immobilized urease could be best employed in UICP applications.
Materials and Methods

Materials

Ludox® gel (420840, Sigma-Aldrich, St. Louis, MO) was used to encapsulate bacterial cells containing the urease enzyme. Also, a proprietary, porous, aluminosilicate ceramic proppant was utilized as a carrier material for immobilization of urease. Jack bean meal (JBM) with an activity specified as ≥ 1500 units/gram (J0125, Sigma-Aldrich, St. Louis, MO) was utilized as a source of urease for immobilization experiments using the ceramic proppant.

Microorganism and Growth Conditions

A 1 mL vial of thawed frozen stock of the bacteria *Sporosarcina pasteurii* (ATCC 11859) was added to 100ml of 37 g*L⁻¹* brain heart infusion (BHI) broth amended with 20g*L⁻¹* urea (Fisher) that had been previously filter sterilized (0.2 µm Nalgene). The culture grew overnight on an orbital shaker at 150 rpm at room temperature (~22°C). After the overnight growth period, 1 mL of the culture was added to 300 mL of 37 g*L⁻¹* BHI amended with 20 g*L⁻¹* urea and grew for an additional 16 hours at room temperature. After the growth period, the optical density of the culture was measured by adding 200 µL to a 96 well plate, and the culture was diluted using a solution of 37 g*L⁻¹* BHI to a final optical density reading of 0.86 at 600 nm (Biotek plate reader).
Immobilization of S. pasteurii in Ludox® gel

20 ml samples of the OD adjusted culture were placed in 50 ml conical centrifuge tubes. These samples were centrifuged at 10,480 x g for 15 minutes at 22°C to create a pellet of cells. The supernatant was decanted and 10ml of 30 wt% Ludox® was added. After addition of the Ludox®, each sample was vortexed for approximately 15 seconds to resuspend the cells. To induce gelling 2.41 ml of a 10 wt% NaCl (Fisher) solution was added to each sample resulting in a final concentration of 1 wt% NaCl. Samples were incubated at room temperature for two hours to initiate gelling and immobilize the cells.

Jack Bean Meal Suspension Preparation

A urease enzyme suspension was generated by adding 2.5 grams of finely powdered JBM to 500 mL of deionized (DI) water for a final concentration of 5 g*L⁻¹. This suspension was stirred on a magnetic stir plate at room temperature (~22°C) for 16 hours.

Urease Immobilization on Ceramic Proppant

After stirring for 16 hours, 25 ml of the unfiltered JBM suspension was added to 6 grams of ceramic proppant in a 125 mL Erlenmeyer flask. Prior to mixing with the JBM, the ceramic proppant was washed thoroughly with DI water and dried at 80 °C for 24 hours. The mixture of ceramic proppant and JBM suspension was mixed for 24 hours on an orbital shaker at 150 rpm at room temperature. The liquid was decanted, retaining the ceramic proppant in the flask. Phosphate buffered saline solution (PBS, 25 mL, 8.5 g*L⁻¹ sodium chloride, 0.61 g*L⁻¹ monopotassium phosphate, and 0.96 g*L⁻¹ dipotassium...
phosphate adjusted to pH 7.0, Fisher) was added to the ceramic proppant before being decanted again. This rinse procedure was repeated twice to remove residual suspended enzyme. JBM was utilized as another urease source for experiments using ceramic proppant due to factors outlined in Appendix D.

Temperature Exposure: Thermal Inactivation Study

Immediately after immobilization, samples, entrapped in Ludox® or adsorbed on ceramic proppant, were placed in a water bath maintained at the target temperature (60-90°C). Samples were exposed to the target temperature for 5 minutes to 8 hours before immediately being placed in an ice bath. Samples were tested right away to determine residual activity of the immobilized urease.

Determination of Urease Activity

10 mL of a solution of 20 g*L⁻¹ urea (Fisher) was added to the immobilized, temperature exposed samples and they were incubated at 40°C for 2.5 hours in a shaking water bath operated at 135 rpm. Every 30 minutes 60 µL-aliquots were collected, diluted in 1140 µL of 0.635M sulfuric acid (Fisher) for a final dilution of 1:20 in preparation for the Jung Assay to determine the urea concentration at each time point (as presented in Phillips 2013, modified from Jung et al. (1975)). The time-averaged urea hydrolysis rate, or activity A, was calculated by taking the difference between the initial urea concentration, $U_0$, and the urea concentration at the two-hour time point, $U_t$, divided by the time elapsed, $\Delta t$, for each triplicate specimen (Equation 2.2).

$$A = \frac{U_0 - U_t}{\Delta t}$$  

Equation 2.2
The initial activity, $A_0$, was determined for immobilized, non-temperature exposed samples using the procedure described above to obtain the kinetic activity without thermal inactivation as the positive control.

**Negative Controls for the Immobilized Forms**

The immobilized cell samples did not initially contain urea, and as a result urea diffused into the Ludox® gel. Due to this, aliquots taken from the fluid on top of the gel steadily decreased in urea concentration even if no urea hydrolysis occurred. To reconcile this, 20 mL of the microbial culture was placed in three 50 mL conical tubes and positioned in a water bath at 80°C for five hours to completely inactivate the enzymes. Samples were centrifuged and entrapped in the silica gel following the immobilization procedure described above to obtain comparable results. The urea solution was added to the top of the gel and samples were collected and analyzed as described above to determine the activity resulting from the diffusive loss of urea into the gel matrix. The activity resulting from diffusion was subtracted from the overall apparent activity of each immobilized cell sample to get the activity due to urea hydrolysis. The subsequent final activity was used to calculate the first order thermal inactivation rate coefficient for each temperature.

To determine whether the ceramic proppant had any innate ureolysis ability a control study was performed. As a control, the proppant was treated with DI water (without the addition of JBM), then washed twice with PBS, and exposed to the 60°C temperature condition in the same manner as the immobilized JBM samples. The same activity protocol was followed, and the activity of the ceramic proppant was determined.
First Order Thermal Inactivation Rate Coefficient Analysis

The thermal inactivation of urease has been adequately described using a first order inactivation model and as a result no higher order models were explored (Equation 2.3) (Henley and Sadana 1984, Sadana 1991). A first order model usually describes a single-step, irreversible process in which the native form of the urease enzyme is denatured to an inactive form.

\[
\frac{dA}{dt} = k_d [A]
\]

Equation 2.3

The first order thermal inactivation rate coefficient \((k_d)\) for each temperature was calculated by first determining the residual activity, \(A_R\) by dividing the activity of the temperature exposed sample, \(A_t\), by the activity of the non-exposed sample, or initial activity \(A_0\) (Equation 2.4).

\[
A_R = \frac{A_t}{A_0}
\]

Equation 2.4

To obtain a consistent baseline, this initial activity was determined each time a new study was performed. The natural log of the residual activity was calculated and plotted versus the thermal exposure time for each sample. A linear regression scheme (Excel) was used to determine the slope of this line, which represented an estimate of \(k_d\). The same procedure was performed for all temperatures to determine the \(k_d\) value at different temperatures (60-90°C). These \(k_d\) data were compared to data previously determined for that of the free, non-immobilized enzyme (Morasko 2018). Comparisons were conducted by relating the Arrhenius relationships for the free and immobilized urease enzyme (Equation 2.5).
\[ k_d = A \times \exp\left(\frac{-E_a}{RT}\right) \]  

Where \( A \) is the preexponential factor, \( E_a \) is the activation energy of inactivation, and \( R \) is the universal gas constant with appropriate units (\( J*K^{-1}*mol^{-1} \)). The data were fitted using an exponential regression and these Arrhenius parameters could be calculated for each model. Differing parameters highlighted whether the immobilized and free forms of the enzyme were thermally inactivated at dissimilar kinetic rates.

**Thin Cross Sectioning of the Ceramic Proppant**

Epoxy was prepared using a 10:3 by weight ratio of resin (#145-10025 EpoxyMount Resin, Allied High-Tech Products, Inc.) and hardener (#145-10030 EpoxyMount Hardener, Allied High Tech Products, Inc.). After mixing the epoxy in a beaker, it was poured into a plastic cylindrical mold approximately 2.5 centimeters by 3.8 centimeters. Ceramic proppant was poured over the epoxy and allowed to sink to the bottom of the mold. The resulting epoxy containing the proppant was left to cure at room temperature for 24 hours. After curing, the epoxy was removed from the mold. The base of the epoxy cylinder was polished using a Techprep polisher (Allied High-Tech Products, Inc.) and a 9 µm aluminum oxide lapping film disc. Polishing was complete when the top portion of the proppant particles had been removed. The bottom of the cylinder was cut off using a TechCut 4 precision saw to produce a thin cross section that could be imaged (Allied High-Tech Products, Inc.).
Microscopy Imaging of the Ceramic Proppant

The ceramic proppant particles and thin cross sections were mounted to a sample holder with carbon tape. They were coated with iridium at 20 mA for 60 seconds using a sputter coater (Emitech K575x). The proppant particles and thin cross sections were imaged with a Zeiss SUPRA 55VP Field Emission Scanning Microscope (FE-SEM) using the SE2 detector.

Results and Discussion

Residual Activity of the Immobilized, Thermally Exposed Samples

To estimate the thermal stability of the immobilized forms of urease the residual activity of the thermally exposed samples was plotted for each temperature and exposure time condition. The specific thermal exposure times differ with each temperature because the enzyme is inactivated at a different rate at each temperature (Ludox® entrapment (Figures 2.2) and ceramic proppant adsorption (Figure 2.3)). The residual activity at each temperature decreases as thermal exposure time increases indicating that the urease enzyme was thermally inactivated at each temperature measured. The slope of the linear model fitted to the residual activity data became more negative as temperature increased (-0.07 and -0.038 hr\(^{-1}\) at 60°C as compared to -10.4 and -10.3 hr\(^{-1}\) at 90°C for the Ludox and ceramic proppant immobilized samples respectively) demonstrating that the rate at which urease is inactivated increases as temperature increases.
Figure 2.2: A linear profile of the natural log of residual activity ($\ln(A_t/A_0^{-1})$) of Ludox® immobilized *S. pasteurii* exposed to various temperatures (60°C-90°C) for different lengths of time for each temperature condition. The negative slope of this linear profile was the first order thermal inactivation rate coefficient ($k_d$) for each temperature.

Figure 2.3: A linear profile of the natural log of residual activity ($\ln(A_t/A_0^{-1})$) of JBM urease immobilized on the ceramic proppant exposed to various temperatures (60°C-90°C) for different lengths of time for each temperature condition. The slope of this linear profile was the first order thermal inactivation rate coefficient ($k_d$) for each temperature.
Thermal Stability of the Immobilized Forms

The immobilized forms of urease were exposed to different elevated temperatures to determine whether the immobilization procedures protected the enzyme from thermal degradation. The protection offered by immobilization was quantified by comparing the $k_d$ values at each temperature for the free and immobilized forms. A comparison of the $k_d$ values at each temperature of the extracted *S. pasteurii* urease (Akyel, Gerlach and Phillips) to that of the Ludox® immobilized *S. pasteurii* urease was conducted (Figure 2.4).

Figure 2.4: Arrhenius plot of the first order thermal inactivation rate coefficients at each temperature tested for the Ludox® immobilized *S. pasteurii* urease as well as the free form of the *S. pasteurii* urease. The comparison between the exponential models shows that the $k_d$ values of the immobilized urease are consistently lower than those of the free phase.
The $k_d$ values at each temperature for the Ludox® immobilized urease are consistently lower than those of the free enzyme (at 80°C 2.43±0.24 and 9.76 hr$^{-1}$ respectively) indicating that the urease enzyme is more thermally stable when immobilized. A larger $k_d$ specifies that the thermal inactivation of the enzyme occurs at a faster rate. A comparison was repeated for the JBM urease immobilized on the ceramic proppant and free JBM urease (Figure 2.5).

Figure 2.5: Arrhenius plot of the first order thermal inactivation coefficients at each temperature tested for the JBM urease immobilized on the ceramic proppant as well as the free form of the JBM urease. The comparison between the exponential models shows that the $k_d$ values of the immobilized urease are consistently lower than those of the free phase.

Figure 2.5 shows that the $k_d$ value at each temperature is lower for the immobilized urease than for the suspended urease (at 80°C 2.26±0.38 and 9.45 hr$^{-1}$ respectively)
indicating increased thermal stability for the immobilized form. These $k_d$ values could be useful in modelling the thermal inactivation of urease in future UICP applications utilizing the immobilized enzyme. The ability to better predict how the urease enzyme acts in different environments could allow for improved applications of this technology.

The first order thermal inactivation coefficients for suspended urease at each temperature were statistically compared to the immobilized urease using unequal variance t-tests (Table 2.1). The null hypothesis was that there was no difference in the means, and a 95% confidence interval was applied.

Table 2.1: Statistical comparison between the suspended forms of urease and *S. pasteurii* urease entrapped in Ludox® and JBM urease adsorbed to the ceramic proppant.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>P-value for Comparison Between <em>S. pasteurii</em> Urease Immobilized in Ludox® and Suspended</th>
<th>P-value for Comparison Between JBM Urease Immobilized on Ceramic Proppant and Suspended</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>2.9*10^-3</td>
<td>5.5*10^-3</td>
</tr>
<tr>
<td>65</td>
<td>0.15</td>
<td>1.3*10^-4</td>
</tr>
<tr>
<td>70</td>
<td>0.10</td>
<td>6.0*10^-4</td>
</tr>
<tr>
<td>75</td>
<td>2.8*10^-3</td>
<td>1.3*10^-3</td>
</tr>
<tr>
<td>80</td>
<td>5.5*10^-5</td>
<td>1.4*10^-3</td>
</tr>
</tbody>
</table>

Table 2.1 shows that the value of the first order thermal inactivation coefficient at each temperature (60-80°C) for the JBM urease immobilized on ceramic proppant was not statistically similar to the value of the suspended JBM urease. Every p-value found was less than the 0.05 of the confidence intervals. Therefore, the null hypothesis can be
rejected and the values of the first order thermal inactivation coefficient for each form is statistically different. It can therefore be concluded that the immobilization of JBM urease on ceramic proppant changed the value of the first order thermal inactivation coefficient for each temperature as compared to suspended urease. For the urease immobilized in Ludox® the p-values of first order thermal inactivation coefficients at 65°C and 70°C (0.10 and 0.15 respectively) were greater than the confidence interval of 0.05. At these temperatures the null hypothesis cannot be rejected. The p-values at the other temperatures were less than 0.05, and at these temperatures the null hypothesis can be rejected. Therefore, the immobilization of urease in Ludox® did not seem to change the value of the first order thermal inactivation coefficient at every temperature when compared to suspended urease. Hence, immobilizing urease on ceramic proppant is more effective in influencing the first order thermal inactivation coefficient than immobilizing urease in Ludox®.

Additionally, the thermal inactivation data can be explained in half-lives by using Equation 2.6.

\[ t_{1/2} = \frac{\ln 2}{k_d} \]  

Equation 2.6

Where \( t_{1/2} \) is the half-life of the enzyme at a specific temperature. The \( t_{1/2} \) shows the thermal stability as it quantifies a lifespan for the urease enzyme at each temperature. A larger \( t_{1/2} \) means the enzyme remains active longer at a specific temperature as it appears to have a prolonged life. Table 2.2 displays that the half-lives of the immobilized urease are always greater than those of the free enzyme for each temperature. The half-lives of
the Ludox® and ceramic proppant immobilized urease at 75°C were 24.9±1.8 and 72.5±14.1 minutes respectively and were greater than the half-lives of the corresponding suspended urease of 12.8 and 19.5 minutes. The half-lives of the suspended urease were not able to be calculated by experimentation at the temperatures of 85°C and 90°C. The thermal inactivation of urease happened so quickly at these temperatures that meaningful data was unable to be collected. For this reason, 80°C was identified as the upper limit of data collection for the free urease. However, as the immobilization increased the thermal stability of urease data were able to be obtained for the immobilized forms at these increased temperatures.

Table 2.2: Half-lives for each form of the urease enzyme tested at various temperatures (60°C-90°C). The half-lives of the immobilized forms were consistently greater than those of the suspended forms showing that the immobilized phases had longer life-spans than their free counterparts at each temperature.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Half-Life of Free <em>S. pasteurii</em> Urease (min)</th>
<th>Half-Life of Ludox® Immobilized <em>S. pasteurii</em> Urease (min)</th>
<th>Half-Life of Free JBM Urease (min)</th>
<th>Half-Life of Proppant Immobilized JBM Urease (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>310</td>
<td>600 ± 54</td>
<td>416</td>
<td>1100 ± 270</td>
</tr>
<tr>
<td>65</td>
<td>231</td>
<td>270 ± 34</td>
<td>189</td>
<td>455 ± 13</td>
</tr>
<tr>
<td>70</td>
<td>62.1</td>
<td>70.1 ± 5.4</td>
<td>67.1</td>
<td>194 ± 15</td>
</tr>
<tr>
<td>75</td>
<td>12.8</td>
<td>24.9 ± 1.8</td>
<td>19.5</td>
<td>72.5 ± 14.1</td>
</tr>
<tr>
<td>80</td>
<td>4.26</td>
<td>17.1 ± 1.8</td>
<td>4.40</td>
<td>18.4 ± 4.03</td>
</tr>
<tr>
<td>85</td>
<td>N/A</td>
<td>9.05 ± 1.46</td>
<td>N/A</td>
<td>8.79 ± 0.91</td>
</tr>
<tr>
<td>90</td>
<td>N/A</td>
<td>4.00 ± 0.19</td>
<td>N/A</td>
<td>4.05 ± 0.66</td>
</tr>
</tbody>
</table>
The results from Table 2.2 highlight that the immobilization procedures tested seem to protect urease from thermal inactivation. The observed protection may allow for higher temperature applications of the EICP process as it seems to increase the enzyme’s ability to work at elevated temperatures. The immobilization carrier may need to be customized specifically for each application, but these results suggest that the temperature barrier of EICP may not be indelible. With the variety of immobilization procedures and carriers that exist, EICP technology may be advanced greatly using immobilization. The process that thermally protects the enzyme is unknown at this time but may be due to the polymer matrix of the Ludox® or the confinement of urease within the pores of the ceramic proppant. The silicon dioxide particles within the Ludox® polymerize creating a gel that entraps the cells containing the enzyme. There is evidence in the literature demonstrating that enzymes entrapped by polymers are prevented from inactivation through this entrapment (Kim, Grate and Wang 2006, Patil, Muthusamy and Mann 2004). These studies have been performed on very small scales (single enzymes) but may be pertinent to understanding how the Ludox® immobilization thermally stabilizes the urease enzyme. The enzyme and cells may associate with the polymer matrix in a way that constrains the enzyme and inhibits thermal degradation.

The ceramic proppant used as the carrier material can be seen in Figure 2.6 and exhibits uniform shape, however large pore entrances are not obviously visible. When the ceramic proppant is viewed after thin cross section internal pores can be clearly seen (Figure 2.7).
Figure 2.6: Field Emission Scanning Electron Microscopy (FE-SEM) image of the ceramic proppant used as the carrier material. The proppant seems to have a relatively uniform spherical shape. Notice there are no obvious, large pores for the urease enzyme to enter the proppant.

Figure 2.7: FE-SEM image of a thin cross section of the ceramic proppant embedded in epoxy. The arrow points to a single ceramic proppant particle, and the internal porosity of the ceramic proppant is evident as the dark holes in each proppant particle.

The internal pores seen in Figure 2.7 may act as a constraining network that restricts the urease limiting denaturation as compared to the suspended enzyme. This would account for the thermal stability that urease seems to exhibit when adsorbed to the proppant.

There is ample evidence in the literature showing that protein confinement within small
pores inhibits denaturation and increases the thermal stability (Ping et al. 2003, Ping et al. 2004, Ravindra et al. 2004).

A main problem with adsorption is that the enzyme may eventually wash out of the carrier material causing a loss in enzymatic activity, but it has been shown that pore sizes closer to the enzyme’s size cause more efficient enzyme retention (Yiu, Wright and Botting 2001, Diaz and Balkus Jr 1996, Vinu et al. 2004). As a result, the ceramic proppant has the possibility to be engineered to become a better carrier material. The ceramic proppant could be engineered to have smaller pores that may retain the urease better. Also, the ceramic proppant could be tailored specifically to thermally protect the enzyme more efficiently by engineering the pores to constrain the enzyme more effectively. The smaller pores may cause some reaction-diffusion problems due to smaller pores decreasing the transport rate of reactants. The trade-off between reduced reaction-diffusion and increased thermal protection may be worthwhile for some higher temperature applications. More research could be devoted to optimizing the properties of the ceramic proppant as a carrier material of urease.

Conclusions

The presented study highlights that the immobilization of urease through entrapment in Ludox® silica gel or adsorption on a ceramic proppant may indeed protect the enzyme from thermal degradation. The data show that the first order thermal inactivation rate coefficient of the immobilized forms of urease are consistently lower than those of the free enzyme at the range of temperatures tested, 60°C-90°C, meaning
the enzyme is more thermally stable in the immobilized form. These results show promise that the thermal barrier currently restricting some UICP applications can be extended and the technology can be applied in previously unavailable environments. The ceramic proppant was shown to be a promising carrier material for urease. Applications using urease that may be stifled by elevated temperature conditions may be enhanced through immobilization.
CHAPTER THREE

COLUMN STUDIES USING UREASE IMMOBILIZED ON A POROUS CERAMIC PROPPANT TO INDUCE CALCIUM CARBONATE PRECIPITATION

Introduction

Enzymes are biological catalysts that have a wide variety of uses in the modern world attributable to their high selectivity, specificity, and activity under moderate conditions (Pollard and Woodley 2007, Schmid et al. 2001). The wide use of enzymes is driven in part by the desire and incentive to become more ecologically conscious as traditional chemical catalysts may damage the environment (Daughton 2005). Therefore, enzymes may be seen as a more attractive alternative in many applications consequently driving intense research (Haki and Rakshit 2003, Vellard 2003, Hasunuma et al. 2013). However, enzymes are likely not more pervasive in industrial applications in part due to biochemical restrictions as they can be extremely fragile in many conditions (Leresche and Meyer 2006, Schoemaker, Mink and Wubbolts 2003). Temperature, pH, the presence of inhibitors, and the presence of proteases from microbial activity can all cause enzyme inactivation (Ahern and Klibanov 1985, Levine et al. 1981, Piper and Fenton 1965). The inherent fragility of enzymes has motivated research on stabilizing them, and one widely researched way to achieve this goal appears to be through immobilization.

Immobilization is the process in which an enzyme is restrained by a carrier through encapsulation, adsorption, covalent bonding, or other mechanisms and can be
achieved through a variety of physical and chemical methods (Garcia-Galan et al. 2011, Sirisha, Jain and Jain 2016). Immobilization can decrease the solubility of the enzyme as it may become associated with a surface, and this association has been shown to solve some of the stability problems of enzymes as compared to the suspended enzyme (Katchalski-Katzir 1993). Immobilized enzymes have been shown to have increased thermal stability, increased resistance to inactivation from the excess or lack of protons (low or high pH), improved storage stability, decreased sensitivity to inhibitors such as heavy metals, and enhanced durability in the presence of protease activity (Marzadori et al. 1998, Krajewska 1991, Pandya et al. 2005). Immobilization can enable different reactor configurations as the enzyme becomes localized on or in the immobilization support (Hartmeier 1985). Different immobilization techniques such as covalent linkages and adsorption do not appear to have the same efficacy in stabilizing different enzymes and seem to exhibit different characteristics regarding how the enzyme functions as a result of immobilization (Datta, Christena and Rajaram 2013). Therefore, immobilization can be tailored for precise applications making immobilization extremely valuable and adaptable. One simple way an enzyme can be immobilized is through adsorption.

**Adsorption is the physical interaction between the suspended enzyme and the carrier material in some way that enables the enzyme to become attached to the surface.** Adsorption typically involves the simple mixing of a carrier material and an enzyme suspension. As a result, adsorption bypasses using additional chemicals that other immobilization techniques, such as covalent enzyme-carrier linkages, require making adsorption an inherently simpler process. Successful adsorption requires adequate carrier-
enzyme interactions attributable to specific functional groups on the immobilization carrier (Jesionowski, Zdarta and Krajewska 2014). Consequently, some carriers are more effective in adsorbing enzymes to their surface as they contain strong functional groups. Surface properties of the carrier can be modified using different chemicals to induce better adsorption properties. However, additional steps, cost, and potentially harmful chemicals would be added to the immobilization procedure that may reduce some of the simplicity and appeal of adsorption. Another attractive feature of adsorption is that the immobilization procedure might result in only minimal disruption or distortion of the active site of the enzyme. In some other immobilization techniques, the active site of individual enzymes may become blocked resulting in reduced activity and efficiency (Hwang and Gu 2013, Hernandez and Fernandez-Lafuente 2011). The greatest disadvantage of adsorption is that it relies on a relatively weak enzyme-carrier attachment compared to other immobilization techniques. The study presented here utilizes adsorption to immobilize the enzyme urease because of its intrinsic ease and simplicity.

Urease was the first protein ever crystalized (Sumner 1926) and as a result has been the subject of many studies (Phillips et al. 2013, Reddy 2013, Qin and Cabral 2002). The urease enzyme can be found in many microorganisms, such as the bacterium *Sporosarcina pasteurii*, or extracted from some plants such as the seeds of jack bean (*Canavalia ensiformis*) (Stocks-Fischer et al. 1999, Blakeley and Zerner 1984). Urease catalyzes the hydrolysis of urea into ammonia (NH₃) and carbonic acid (H₂CO₃) changing the solution chemistry. Alkalinity is increased and has been exploited to promote precipitation of calcium carbonate (CaCO₃) if calcium (Ca²⁺) is present at high enough
concentrations (Dick et al. 2006, Mitchell et al. 2010). The overall balanced chemical equation of this precipitation reaction is seen in Equation 3.1.

\[(NH_2)_2 CO + 2H_2O + Ca^{2+} \rightarrow 2NH_4^+ + CaCO_3\]  

Equation 3.1

Calcium carbonate mineralization has been engineered for many applications such as permeability modification in the subsurface to mitigate fluid leakage, enhanced oil recovery, and the remediation of heavy metals, but many more applications are being developed and researched today (Cunningham et al. 2011, Cunningham et al. 2014, Phillips et al. 2013, Lauchnor et al. 2013). Immobilization may help advance some of these applications as they can be hindered by the fragility of the native urease enzyme as it readily becomes inactivated at temperatures of 60°C and above. Urease has been immobilized many ways for a variety of different applications and has been shown to readily adsorb to many organic and non-organic carriers (Krajewska 2009b).

Immobilized urease has been used to create active biosensors that can detect some heavy metals or can be used to measure urea concentration in blood serum for medical purposes (Lee and Lee 2002, Zhylyak et al. 1995, Mascini and Guilbault 1977). Nevertheless, how well many of these immobilization techniques induce calcium carbonate precipitation has yet to be understood. Many applications that utilize urease to catalyze calcium carbonate mineralization may be greatly enhanced with an optimized immobilization technique. Therefore, urease immobilization coupled with active mineralization may need to be studied in more depth to expand the usefulness of calcium carbonate mineralization applications. In the study presented here crude urease sourced from jack bean meal was adsorbed onto a porous ceramic proppant, and column studies at two different
temperature conditions (22°C and 60°C) were performed. In the column studies, mineralization was induced to understand how effective the immobilized urease was in producing calcium carbonate. The elevated temperature condition of 60°C was chosen to understand how the urease immobilized on ceramic proppant performed at a temperature which has been shown to inactivate the suspended enzyme. How well the urease, immobilized on ceramic proppant, retained activity over a long period of storage at 4°C compared to the suspended form was also investigated. Suspended urease loses activity quickly when stored, and if the immobilized form exhibits increased storage stability it would offer an advantage in some UICP applications. In large scale applications a source of urease that remains stable over a long period of time would allow easier use of the UICP technology as it would negate the need to constantly mix enzyme suspensions. The column studies presented here elucidated how immobilized urease on a porous ceramic proppant may be used in future mineralization applications and in enhancing current ones.

Materials and Methods

Materials

A proprietary, porous, aluminosilicate ceramic proppant was utilized as a carrier material. Jack bean meal (JBM) with an activity specified as ≥ 1500 units/gram (J0125, Sigma-Aldrich, St. Louis, MO) was employed as a crude source of urease.
Jack Bean Meal Suspension Preparation

A urease enzyme suspension was generated by adding 2.5 grams of finely powdered JBM to 500 mL of deionized (DI) water for a final concentration of 5 g*L⁻¹. The enzyme suspension was stirred on a magnetic stir plate at room temperature (~22°C) for 16 hours.

Urease Immobilization on the Ceramic Proppant

After mixing for 16 hours, 50 mL of the unfiltered JBM solution were added to 12 grams of ceramic proppant in a 250 mL Erlenmeyer flask. Previously, the ceramic proppant had been washed with DI water to remove any residual dust and then dried at 80°C for 24 hours. The mixture of ceramic proppant and JBM suspension was mixed for 24 hours on an orbital shaker at 150 rpm at room temperature. The JBM suspension was carefully decanted while ensuring that all the ceramic proppant was retained within the flask. After enzyme sorption, the ceramic proppant was washed twice with 50 mL of phosphate buffered saline solution (PBS, 8.5 g*L⁻¹ sodium chloride, 0.61 g*L⁻¹ monopotassium phosphate, and 0.96 g*L⁻¹ dipotassium phosphate adjusted to pH 7.0, Fisher) to remove residual suspended urease. It was assumed that the remaining urease was immobilized on the ceramic proppant.

Storage Stability Analysis of Immobilized Urease

JBM urease was immobilized on the ceramic proppant following the previously outlined procedure, but instead 6 grams of ceramic proppant was mixed with 25 mL of JBM. The samples prepared in 125 mL Erlenmeyer flasks were washed twice with 25 mL
of PBS and stored at 4°C for a range of times (1 day to 28 days). The initial activity, $A_0$, of the immobilized urease was assessed as previously described in Chapter 2 to obtain a baseline activity for stability comparisons. After pre-determined times the remaining enzyme activity, $A_S$, was estimated as described in Chapter 2, and this activity was compared to the initial activity (Equation 3.2).

$$A_R = \frac{A_S}{A_0} \quad \text{Equation 3.2}$$

Where $A_R$ was the calculated residual activity of the stored sample. Activity of the suspended enzyme was calculated in the same way to obtain a comparison of the immobilized and suspended form of urease. For the non-immobilized samples, 5 g*L$^{-1}$ JBM solution was passed through a 0.2 µm Nalgene filter. Then, 10 mL of the filtered suspension was added to a 25 mL vial in a laminar flow hood to mitigate microbial contamination. The same storage and stability testing procedures were employed on these samples to determine the storage stability of the suspended enzyme. In testing the activity of the free enzyme samples 10 mL of a solution containing 40 g*L$^{-1}$ urea was added to the filtered enzyme. A starting urea concentration of 20 g*L$^{-1}$ was obtained.

**Room Temperature (22°C) Column Construction**

A 30 mL sterile syringe was used as a column by discarding the plunger and adding a nylon filter to the bottom prior to connecting tubing (Masterflex® L/S C-Flex, 1/3-centimeter inner diameter) to the bottom of the syringe (influent). The inlet tubing was placed in a peristaltic pump head (Masterflex® Easy Load, 7518-00) operated at a flow rate of 10 mL*min$^{-1}$ throughout the entire experiment by a pump (Cole-Palmer®, 6-
600 RPM, 7553-20). The syringe and inlet tubing were filled halfway with DI water, and the washed proppant containing the immobilized urease enzyme was added to the syringe using a small metal spatula. The syringe was lightly tapped to prevent the creation of air bubbles in the column. Once the column was filled to the top with the proppant, a rubber stopper with a hole containing the effluent tubing and plastic filter was affixed to the top of the column using a 5-minute epoxy resin. The epoxy resin was given one hour to fully cure before the experiment commenced. A second column was also constructed. The column setup can be seen in Figure 3.1.

![Figure 3.1: The experimental setup for the room temperature proppant column.](image)

60°C Column Construction

A column was constructed using 10 centimeters of high temperature resistant CPVC (up to 90°C), along with CPVC pipe fittings. A CPVC fitting with a nylon filter and inlet tubing (Masterflex® L/S C-Flex, 1/3-centimeter inner diameter) was attached to
the bottom of the column and held in place using high temperature resistant silicone sealant. The inlet tubing was then placed in a peristaltic pump head (Masterflex® Easy Load, 7518-00) operated at a flow rate of 10 mL min\(^{-1}\) throughout the entire experiment by a pump (Cole-Palmer®, 6-600 RPM, 7553-20). The column and inlet tubing were filled halfway with DI water. The setup was placed in an oven continuously operated at 60°C. The washed proppant containing the immobilized urease enzyme was added to the column quickly using a small metal spatula to minimize thermal inactivation of the enzyme before the experiment commenced. The column was periodically, lightly tapped to discourage the creation of air bubbles in the column during this process. Once the column was filled with the proppant a CPVC fitting with a nylon filter and effluent tubing was affixed to the top of the column using a high temperature resistant silicon sealant. The column was given 30 minutes to equilibrate to the desired temperature (60°C) before the experiment commenced. This was repeated to create a second column used later. The column setup can be visualized in Figure 3.2.

Figure 3.2: The CPVC column containing the ceramic proppant with immobilized urease placed in the oven operated at 60°C.
Approximate Pore Volume Calculation

The pore volume of each column was estimated before the experiment for each temperature condition. The column was plugged at the bottom to ensure no material could escape, and then dry proppant was added until the level reached the top of the syringe or CPVC column. This setup was placed on a balance and the mass measurement was zeroed. DI water was slowly added to the top of the syringe while periodically tapping to prevent the trapping of air bubbles. The setup was weighed again to determine the mass of water added and assuming the density of DI water to be 1 g*mL\(^{-1}\) the pore volume of the column was estimated.

Mineralization Procedure

A mineralization promoting solution was prepared by mixing 20 grams of urea (Fisher) and 49 grams of calcium chloride dihydrate (Fisher) to approximately 750 mL of DI water. Once the solids were completely dissolved this solution was raised to a final total volume of one liter using DI water. A washing solution of 10 g*L\(^{-1}\) ammonium chloride (Fisher) was also prepared before the experiment. To begin the experiment 1.5 pore volumes of a 20 g*L\(^{-1}\) urea and 49 g*L\(^{-1}\) calcium chloride dihydrate solution were pumped through the column. Immediately after, 4 mL of the 10 g*L\(^{-1}\) ammonium chloride solution was pumped to clear the influent tubing of the urea/calcium chloride dihydrate solution. After injection, flow was stopped, and a reaction batch period was initiated. For the column operated at room temperature two hours were used as the reaction batch period, and for the column operated at an elevated temperature a reaction batch period of one hour was used. After the reaction batch period had completed, 0.5
pore volumes of the 10 g*L⁻¹ ammonium chloride solution were pumped through the column, and the entire procedure, referred to as a pulse, was repeated. For the column operated at an elevated temperature the solutions were placed in the oven to remain near 60°C constantly. This was performed for a total of 16 pulses for each column, and after the final pulse each column was washed with ten pore volumes of DI water. The tubing and fittings were removed, and each column was dried at 37°C for two days. Each column experiment was performed in duplicate (at different times).

**Room Temperature Column Sampling Procedure**

The experiment was performed on the benchtop at room temperature (22°C). A 1 mL sample of the inlet solution was placed into a 1.5 mL microcentrifuge tube before every pulse, and 60 µL of this sample was diluted in 1140 µL of 0.625 M sulfuric acid. The urea concentration was measured using the Jung Assay (as presented in Phillips 2013, modified from Jung et al. (1975)). The pH of the solution in the 1.5 mL microcentrifuge tube was also measured. Effluent samples were collected at the end of each reaction batch period, and the same urea concentration and pH analysis was performed. Four pulses were completed every day for four days, and the final pulse of each day was left to sit in the column overnight before the procedure was repeated the following day.

**60°C Column Sampling Procedure**

The experiment was performed in an oven operated at 60°C constantly. The oven was turned off at night and the column decreased in temperature overnight ensuring
thermal inactivation did not occur when the experiment was not actively conducted. The next day the oven was turned back on and allowed 30 minutes for the column to increase to the desired temperature before the experiment was started again. A total number of eight pulses were completed each day and the final pulse of the first day was left to sit in the column overnight. The experiment was then terminated on the second day. The same sampling and analysis procedures were repeated for the elevated temperature column as were performed for the room temperature column. Inlet and effluent urea concentrations and pH values were obtained.

**Stereoscope Imaging**

Images were taken with a Leica M205 FA Stereomicroscope using two spotlight illuminators (SLI) at 100% intensity. The microscope gain was set to 3.1 and the exposure time was 12.20 milliseconds.

**Thin Cross Section and Elemental Analysis**

After the mineralization experiment was completed approximately two grams of the proppant particles mineralized with calcium carbonate were thinly sliced and analyzed using a Field Emission Scanning Electron Microscope (FE-SEM, Zeiss SUPRA 55VP) as previously outlined in Chapter 2. The procedure was repeated for the control, non-mineralized proppant. Elemental mapping was also conducted using a SEM by using Energy Dispersive X-ray analysis (EDX) to identify which elements were present and where they were present within the samples.
Raman Spectroscopy Analysis

Samples to be analyzed were first dried at 30°C for two days to remove all moisture. Then analyzed using a LabRAM HR Evolution Raman microscope (Horiba Scientific, France). The samples were analyzed at 20X magnification with a 532 nm laser (50 mW). The spectra were collected from 200-2000 cm\(^{-1}\) using a 1800 g*mm\(^{-1}\) grating. Peak identification was supported using the KnowItAll Raman spectra library (Bio-Rad, Hercules, USA).

X-ray Computed Micro-tomography (μ-CT) Scan Analysis for Approximate Porosity

After the mineralization experiments concluded, the bonded proppant particles were extracted as cohesive columns (Figure 3.8 and Figure 3.20). Images were taken with a SkyScan 2273 μ-CT system in three rounds: (1) room temperature mineralized column (2) 60°C mineralized column and (3) control non-mineralized column in a 30 mL syringe. Two-dimensional images were taken as the core rotated around its vertical axis every 0.5°. Scans were performed at an X-ray production voltage of 130 kV and a current of 40 μA with a 0.25 mm brass filter. The approximate resolution was 50.0 μm. From the raw vertical images, two-dimensional horizontal stacks of projection radiographs were reconstructed using NRecon software using the Feldkamp algorithm (Feldkamp, Davis and Kress 1984). The reconstructed images were made up of pixels, each assigned a linear attenuation coefficient corresponding to the X-ray signal intensity received at that location. The X-ray signal intensity is a function the material properties such as density (Wildenschild and Sheppard 2013). The porosity was estimated from binary images created in ImageJ by setting an attenuation threshold using the Otsu algorithm (Otsu
1979). Above the threshold all material was considered to be in the solid phase and below, everything was assumed to be open pore space. The percent of each image that was below the attenuation threshold was assumed to represent the porosity of the core. The porosity measurements along the length of each column were then compared to obtain a qualitative representation of how the porosity differed in each experiment.

**Calcium Digest of the Mineralized Columns**

The extracted columns were broken into four 2.5-centimeter pieces using a saw. Each piece was crushed using a mortar and pestle to break the calcium carbonate bonds between the proppant particles. Approximately one gram of each crushed piece was placed in a 15 mL conical tube (performed in triplicate). To dissolve the calcium carbonate, 5 mL of 10% trace metal grade nitric acid was added to each conical tube, and then each tube was vortexed for roughly 15 seconds. The tubes were incubated at room temperature for 24 hours to allow complete dissolution of the calcium carbonate. After the 24-hour, incubation, 2 mL of the liquid in each conical tube was placed in another 15 mL conical tube containing 2 mL of Milli-Q® water. Further dilutions were performed using 5% nitric acid for a final 1:400 dilution of the supernatant in the original conical tubes. The calcium concentration was estimated for the final 1:400 dilutions of each sample using the calcium assay outlined in Appendix A. The calcium concentration of each tube was then used to estimate the amount of calcium carbonate per gram of proppant in the crushed column pieces.
Results and Discussion

Storage Stability of the Immobilized Urease

Suspended urease has a limited lifespan as its activity decreases with time even when stored at 4°C, and it was tested whether the immobilization of the enzyme on the ceramic proppant improved storage stability. Figure 3.3 shows the fraction of initial activity of samples after specific storage times and was used to compare the immobilized and suspended urease. The fraction of initial activity multiplied by one-hundred gives a percent activity remaining in the sample relative to the initial activity. It was found that the stability of the enzyme greatly increased when immobilized as compared to when suspended as after 27 days of storage the immobilized urease had 90% of the initial activity whereas suspended urease had 60% activity (Figure 3.3). The immobilization of urease on ceramic proppant seems to protect the enzyme from natural inactivation while in storage significantly increasing its value in some EICP applications.

![Figure 3.3: Fraction of the initial activity of the JBM urease immobilized on the ceramic proppant (■) compared to the free JBM urease (●) for various storage times at 4°C. The immobilization of the urease enzyme increases its storage stability as the fraction of initial activity was greater in the immobilized phase than the free phase for each of the storage times tested.](image-url)
The greater activity retained through enzyme immobilization as compared to the suspended enzyme could benefit some UICP applications where large amounts of urease may be necessary. The immobilized urease could be prepared well in advance without a significant loss in activity, and therefore could streamline some UICP applications.

Growing of ureolytic bacteria or mixing of suspended enzyme takes time, and immobilization could mitigate this time. The enhanced storage stability of immobilized urease offers a way to enhance the efficiency of some UICP applications.

**Urea Concentration and pH Data for the Room Temperature Column**

Replicate columns were performed identically at room temperature and were operated at different times. The urea and pH data for each pulse from the replicate columns were averaged to obtain a comprehensive data set. The urea and pH data for individual columns can be found in Appendix C. The plot of urea concentration for the columns operated at room temperature can be seen in Figure 3.4 and the plot of the pH values for the same columns can be seen in Figure 3.5.

![Figure 3.4](image_url)

**Figure 3.4:** The averaged inlet (●) and effluent (■) urea concentrations over the two columns operated at room temperature for each pulse. The error bars represent the standard deviation between the two trials performed at room temperature.
Figure 3.5: The averaged inlet (●) and effluent (■) pH values over the two columns ran at room temperature for each pulse. The error bars represent the standard deviation between the two trials performed at room temperature. The inlet pH values seemed to remain around approximately 6.2 and the effluent values seemed to be consistently near 7.6.

Examining Figure 3.4 shows that the immobilized urease enzyme on the ceramic proppant was able to hydrolyze nearly 20 g*L$^{-1}$ of urea in the allotted two-hour reaction batch period for each pulse. The hydrolysis of this much urea is indicative of active mineralization as it creates favorable conditions for calcium carbonate precipitation to occur. The data in Figure 3.5 provide further evidence that active mineralization occurred in the column as it shows the pH increase over the two-hour reaction batch period due to urea hydrolysis. However, pH did not continue to rise indefinitely as the precipitation of calcium carbonate buffered the pH through the consumption of carbonate (Equation 1.6). Therefore, the measured effluent pH values were around the expected values attributed with active mineralization.

It was hypothesized that flowing enzyme-free solutions through the columns would remove the adsorbed enzyme from the proppant thus decreasing the ureolytic
activity within the column. The ureolytic activity sustained over time within the column was a surprising result. Adsorption is known to result in a relatively weak form of immobilization, and it was suspected that urease would easily become desorbed. Desorption may have been occurring but did not have enough of an impact to significantly decrease the activity of the column. As a result, urease may have a much stronger affinity to the ceramic proppant used as the carrier material than previously suspected. The mechanism by which urease may be strongly bound to the ceramic proppant was not explored in further detail. Literature has suggested that Van der Waals forces, hydrogen bonding, and some ionic interactions between the adsorbed enzyme and the carrier material may result in the robust adhesion that was observed (Jesionowski et al. 2014). It seemed these bonding forces were indeed sufficient in binding the urease tightly to the ceramic proppant. The ceramic proppant may be an effective carrier material for enzymes as urease seemed to strongly adsorb to it.

More importantly, however, is that even during active calcium carbonate mineralization substantial ureolytic activity can be sustained in the column (Figure 3.4). This was intriguing because in previous mineralization studies using quartz sand, JBM, and S. pasteurii the overall activity of the columns decreased rapidly with subsequent pulses. To maintain sufficient urease activity repeated pulses of the urease source, either an enzyme slurry from JBM or fresh S. pasteurii culture, were necessary throughout the experiment.

Mineralization seems to decrease urease activity by entombing the microbes or enzymes in the calcium carbonate mineral (Cuthbert et al. 2012). However, the
immobilized urease column study performed exhibited no such decrease in ureolytic activity or need for a fresh urease source. Inactivation of urease by calcium carbonate mineralization appeared to have been alleviated through immobilization. It seems that urease adsorbed to the ceramic proppant can evade much of this entombment and the subsequent inactivation. It was clearly shown that the immobilized urease can retain a high ureolytic activity even when subjected to high shear flows within the column and active precipitation of calcium carbonate. As a result, the ceramic proppant may be an effective carrier material of the urease or other enzymes as it seems to significantly protect the adsorbed enzyme.

**Urea Concentration and pH Data for the 60°C Column**

Replicate columns were performed identically at 60°C and were operated at different times. The urea and pH data for each pulse from the replicate columns were averaged to obtain a comprehensive data set. The urea and pH data for individual columns can be found in Appendix C. The plots for the averaged inlet and effluent urea concentrations and pH values for the columns operated at 60°C can be seen in Figure 3.6 and 3.7 respectively. The values for the influent pH seem to increase with each pulse (Figure 3.7). Thermal ureolysis can occur at 60°C, and due to the solution being kept at 60°C throughout the experiment some urea was hydrolyzed resulting in the increase in pH that was visualized.
Figure 3.6: The averaged inlet (●) and effluent (■) urea concentrations over the two columns operated at 60°C for each pulse. The error bars represent the standard deviation between the two trials performed at 60°C. The inlet urea concentrations remain around 20 g*L⁻¹ while the effluent urea concentrations change throughout the experiment. The increase in effluent urea concentrations is hypothesized to be due to the thermal inactivation of the immobilized enzyme decreasing some of the ureolytic ability of the column itself.

Figure 3.7: The averaged influent (●) and effluent (■) pH data for the two columns operated at 60°C for each pulse. The error bars represent the standard deviation between the two trials performed at 60°C. The effluent pH remains around 7.6 which is indicative of mineralization occurring within the column. The influent pH increases as thermal ureolysis occurs.
The columns operated under the elevated temperature condition seem to emphasize the same high ureolytic activity seen in the previous columns performed at room temperature (Figure 3.6). The immobilized urease was able to hydrolyze greater than 14 g*L\(^{-1}\) of urea each pulse even as the elevated temperature caused thermal inactivation of the enzyme. The reaction batch period for the 60°C column was decreased to one hour from the two-hours performed in the room temperature column. This was changed in response to the increased activity of urease at 60°C compared to room temperature (22°C), and to minimize the time during which the enzyme encounters thermal inactivation. As a result, more pulses were conducted per day as compared to the room temperature column. Still, Figures 3.6 and 3.7 show that the 60°C column was able to reliably hydrolyze enough urea to create a favorable environment for the precipitation of calcium carbonate. The column was able to hydrolyze all 20 g*L\(^{-1}\) of the urea in the first five pulses, but as the column was continually exposed to 60°C some of the immobilized urease may have become thermally inactivated. A competition between the high ureolytic ability shown to be present within the column and the thermal inactivation of the enzyme develops. The general trend was that the amount of urea hydrolyzed seemed to decrease with each pulse as more urease might have become thermally inactivated within the column. However, the immobilized urease seems to be resilient even when exposed to both active calcium carbonate precipitation and thermal inactivation as the column retains enzymatic activity throughout the entire experiment. Elevated temperature conditions can currently be challenging in optimally utilizing urease as it becomes thermally inactivated and as a result can limit some applications of
EICP. The immobilization of urease on the ceramic proppant seemed to protect the enzyme sufficiently to allow EICP over prolonged periods. At elevated temperatures the rate of thermal inactivation of suspended urease can often quickly overcome its ureolytic ability. The suspended enzyme then becomes inactivated before hydrolyzing enough urea to facilitate calcium carbonate precipitation. This causes suboptimal amounts of calcium carbonate to be precipitated. The high ureolytic ability of immobilized urease present in the room temperature column was shown to be retained in the 60°C column for an extensive amount of time. Urease immobilized on the ceramic proppant has been shown to be a useful catalyst of calcium carbonate precipitation even under thermal conditions that have been shown to thermally inactivate suspended urease.

**Post-Mineralization Analysis of the Room Temperature Column**

The extracted column after the mineralization experiment for the room temperature condition can be seen in Figure 3.8. The proppant particles seem to be bound together tightly due to the mineralization process.

Figure 3.8: The extracted column operated at room temperature. Notice that the proppant particles are held together well due to the mineralization that occurred in the experiment. The bottom of the column was the influent and the top was the effluent of the column.
Raman Spectroscopy was performed on a small sample of the mineralized proppant to determine the chemical composition of the precipitate. The collected spectra from the sample seem to follow the same peaks of the known spectra of calcium carbonate providing evidence that the mineral formed was indeed calcium carbonate (Figure 3.9). This was unsurprising as the formation of calcium carbonate was an objective of the experiment but showed that favorable conditions for the precipitation of calcium carbonate were certainly induced through urea hydrolysis.

![Spectra](image)

Figure 3.9: The spectra received from the mineralized proppant particles (Sample 1, black) overlaid with the known spectra of calcium carbonate (red). The two spectra seem to be aligned showing that the mineral formed was most likely calcite.

Additional techniques to visualize how tightly the proppant particles of the column were bound together and to better understand how the calcium carbonate was associated with the proppant were used. Imaging of the post-mineralization experiment column under the stereoscope and FE-SEM was performed. The non-mineralized proppant analyzed under the stereoscope can be seen in Figure 3.10, and the mineralized proppant can be viewed in Figure 3.11.
Figure 3.10: Stereoscope images of the non-mineralized proppant. Notice the relatively uniform spherical shape and smooth surface.

Figure 3.11: Stereoscope images of the mineralized proppant. The images clearly show that calcium carbonate crystals are binding the proppant particles together. When compared to the control proppant the calcium carbonate is easily seen growing from the surface of the proppant particles as the roughness of the proppant has greatly increased.

The stereoscope images highlight how the mineralization experiment created many calcium carbonate crystals that appear to bind the previously loose proppant particles. The images show that the immobilized urease was indeed able to create favorable conditions for calcium carbonate precipitation. The quantity of calcium carbonate crystals seen Figure 3.11 indicates that the immobilized urease was able to facilitate the precipitation of significant amounts of calcium carbonate. The copious
amounts of calcium carbonate in Figure 3.11 seems to provide parallel evidence to the urea concentration data that implied that a large amount of calcium carbonate could have precipitated with each pulse. To obtain further clues to how the calcium carbonate may have formed on the ceramic proppant both non-mineralized and mineralized samples were analyzed under the FE-SEM. The non-mineralized proppant can be studied in Figure 3.12, and the mineralized proppant can be observed in Figure 3.13.

Figure 3.12: FE-SEM images of the control, non-mineralized proppant particles used for the all column experiments performed in this study.

Figure 3.13: FE-SEM image of the mineralized proppant obtained from the room temperature column experiment. Notice the abundant calcium carbonate minerals coating the surface of the proppant exhibiting the classic cubical shape of calcite as also indicated by Raman spectroscopy.
Comparing Figure 3.13 to the non-mineralized proppant seen in Figure 3.12 provides more evidence for significant calcium carbonate precipitation within the column. These images seem to indicate that calcium carbonate did indeed precipitate as calcite (Figure 3.13). Calcite was expected as it is the most stable form of calcium carbonate. Nevertheless, the clear cubic shapes, large overall size, and flat faces of the calcium carbonate crystals were somewhat surprising. No microbes or organic media were intentionally added to the column as all biotic material was immobilized on the ceramic proppant. The morphology of calcium carbonate seen in Figure 3.13 may have been due to the absence of extraneous organic material.

Junctions between several proppant particles can be observed in Figure 3.14 and a closer view of the attachment can be seen in Figure 3.15.

Figure 3.14: FE-SEM image of three proppant particles attached to one another. Calcium carbonate crystals seem to grow between the proppant particles affixing them together.
Figure 3.15: An increased magnification FE-SEM image of the interface between two proppant particles. The calcium carbonate crystals seem to grow from both proppant particles bonding them together. The proppant particles may stay together after the experiment due to these calcium carbonate bonds. The white arrow in the image points to a black substance that is seen on many of the FE-SEM images. It is assumed to be evaporites, such as calcium chloride, left in the column after the washing and drying procedures.

Figure 3.14 and Figure 3.15 show that the calcium carbonate created mineral bridges across adjacent proppant particles bonding them together. The mineral bridges ensured that the proppant particles remained attached to each other even after the proppant was extracted from the syringe. However, the calcium carbonate did not seem to create a complete seal across each interface between proppant particles leaving some pore space for fluid flow. One interesting characteristic to note is that some calcium carbonate crystals exhibit terraced growth patterns. These patterns could indicate that mineral growth occurred in stages rather than continuously. Each stage might have coincided with a fresh pulse of the mineralization fluid. The calcite crystals might have grown a step further with each new pulse.
Thin Cross Section and Energy-dispersive X-ray (EDX) Analysis

Calcium carbonate was shown to clearly form on the outside of the proppant, but another question was whether the mineral formed within the internal pores as well. The internal structure of the proppant before the experiments can be seen in Figure 3.16, and a magnified view of a pore is seen in Figure 3.17a. A pore of the mineralized proppant with similar dimensions to the pore seen in Figure 3.17a was imaged to obtain a comparison (Figure 3.17b).

Figure 3.16: FE-SEM image of the thin cross section of the non-mineralized proppant. The arrow points to an individual proppant particle. Notice the internal pores that appear as black dots within the proppant.

Figure 3.17: FE-SEM images of a representative pore of the non-mineralized proppant (a) and the mineralized proppant (b). The pore of the non-mineralized proppant is relatively open while the mineralized proppant seems to have an obvious occlusion that may be calcium carbonate limiting the transport within the pore.
The pore of mineralized proppant seemed to have a mineral blocking most of its pore space (Figure 3.17b). No obvious cubic calcite structures indicating calcium carbonate were imaged within the pores of the mineralized proppant and therefore elemental mapping was employed to gauge what elements occurred and where they were present (Figure 3.18 and Figure 3.19).

Figure 3.18: EDX maps for the main elements present in the non-mineralized proppant. Bright areas indicate the abundance of specific elements in each image. Note there was no calcium detected within the sample. Aluminum, silicon, and oxygen constituted the bulk of the proppant while the epoxy was composed primarily of carbon.

Figure 3.19: EDX maps of the main elements present in the mineralized proppant. Bright areas indicate the abundance of specific elements in each image. Calcium is clearly present in the sample. Calcium seems to appear on the inside of the proppant indicating some of the pores may contain calcium carbonate.
The EDX maps (Figures 3.18 and 3.19) show that calcium was present inside the mineralized proppant while no calcium was detected in the proppant prior to mineralization. It seemed that calcium was not co-located with chlorine (chloride), therefore it is unlikely that calcium chloride from the mineralization solution precipitated within the pores of the proppant. As a result, calcium carbonate may have been precipitated in some of the pores of the proppant. However, there was no evidence of calcium throughout the entirety of the inside of the proppant meaning that not every pore contained calcium carbonate. The internal pores not containing calcium may have not been affected by calcium carbonate precipitation. The pores without calcium may be a reason the immobilized urease sustained ureolytic activity throughout the experiment. Some urease may have been able to escape encapsulation in calcium carbonate. However, due to some pores avoiding blockage by calcium carbonate complete sealing using mineralization may not be able to be achieved. A weakness in the usefulness of immobilized urease may be the lack of complete sealing as many EICP applications strive for comprehensive sealing of pore spaces. Nevertheless, for some other applications of EICP that do not directly involve fluid leakage and where complete sealing may not be the main goal, such as groundwater remediation and enhanced oil recovery, this may not be a disadvantage.

**Post-Mineralization Analysis of the 60°C Column**

The mineralized proppant column extracted from the CPVC fittings can be visualized in Figure 3.20. Again, the proppant particles seemed to be bound together.
Figure 3.20: The extracted 60°C proppant column. The proppant particles are bound together and there is an obvious, white mineral covering the proppant particles. The bottom was the influent and the top was the effluent of the column. The red coloring is an artefact from the clay that was used to hold the column in place for the µ-CT scan.

Raman Spectroscopy was performed on a sample of the mineral to determine if it was indeed calcium carbonate as was seen in the room temperature column (Figure 3.21).

Figure 3.21: The spectra received from the mineralized proppant particles of the 60°C column (Sample 3, black) overlaid with the known spectra of calcite (red). The two spectra seem to be aligned showing that the mineral formed was most likely calcite.

The spectra in Figure 3.21 show that the mineral formed seemed to be calcite indicating that at 60°C calcium carbonate also precipitated as calcite. To understand whether the
morphology of the calcium carbonate was different than the calcite crystals seen in the room temperature column FE-SEM imaging was performed on mineralized samples from the 60°C column (Figure 3.22).

Figure 3.22: FE-SEM images of the mineralized proppant particles from the 60°C column experiment. The mineral appears to be the same characteristic cubic shape as the calcite found in the room temperature column as was proven by the Raman spectra. The white arrow in Figure 3.22b points to a flat section of calcium carbonate that was most likely attached to another proppant particle. The particles appear to be cemented together by calcium carbonate bridges like the proppant imaged from the room temperature column.

Figure 3.22 highlights that the calcium carbonate mineral formed at 60°C had a similar calcite structure as the mineral found in the room temperature column. The difference in temperature between experiments environments did not appear to affect that calcite was the most likely polymorph of calcium carbonate precipitated. The porous ceramic proppant was found to be an effective carrier material of urease as it facilitates calcium carbonate precipitation at elevated temperatures. Current UICP techniques utilizing enzyme slurries or microbial injections struggle with the thermal inactivation of urease that seems to be mitigated through immobilization on the ceramic proppant
Porosity Estimation Based on X-ray μ-CT and Calcium Carbonate Quantification

The extracted mineralized columns for each temperature along with a control column were analyzed with μ-CT tomography. Porosity measurements for the total length of each column was performed (Figure 3.23).

Figure 3.23: μ-CT scan data showing the porosity along the length of each column: control (orange), room temperature (blue), and 60°C (green). The mineralization procedure decreased the apparent porosity for the columns due to the formation of calcium carbonate.

The μ-CT scan indicates that the mineralization induced by the immobilized urease decreased the overall porosity within the columns at each temperature. The calcium carbonate crystals filled some of the pore space between the proppant particles decreasing the porosity. The ability of the immobilized urease to perform porosity modification increases its usefulness in some EICP applications. However, because of the limited resolution of the μ-CT scans, the internal porosity of the proppant could not be visualized or quantified. Hence, the porosity measurements represent overall estimates of interstitial porosity for each column. Also, varying the thresholding algorithms that analyzed the scans drastically impacted the final porosity measurements. Hence, limitations of the μ-
CT scan should not be dismissed, and therefore the data obtained through μ-CT scans should be taken in more qualitative terms rather than quantitative. The data gained can be an asset as they confirm that the immobilized urease was able to decrease the overall porosity within each column. The 60°C column was able to significantly alter the porosity within the column showing that immobilizing urease on the ceramic proppant may be useful in increasing the thermal stability of the enzyme in some EICP experiments and applications. The calculated porosity within the non-mineralized column was approximately 55% which seems to match the μ-CT scans. The calculated porosity of the room temperature column was 45% assuming all urea was hydrolyzed and that one mol of urea hydrolyzed equated to 1 mol of calcium carbonate precipitated. The calculated porosity of the 60°C column was 47% assuming the same conditions while using the specific urea hydrolysis data for the 60°C column (Figure 3.6). These values are slightly higher than the μ-CT scans indicated highlighting some of the error associated with the μ-CT scans. Each column seemed to have large jumps in porosity change at certain points with the 60°C column contained more obvious spikes. This can also be slightly seen in the control column and it is hypothesized to be due to irregularities in packing the columns with proppant. As the columns are filled with several “charges” (batches) of proppant there could have been differences in packing which may have resulted in the large changes in porosity visualized in Figure 3.23. Several μ-CT scans were performed to verify that these jumps did indeed occur, and they can be seen in Appendix E.
The amount of calcium carbonate per gram of proppant within each column was also measured by digesting pieces of the column in nitric acid. Each column was broken into four 2.5-centimeter pieces along the length of the flow path to visualize whether the amount of calcium carbonate was homogeneous or heterogeneous throughout the entire columns. The calcium carbonate distribution within the room temperature column seemed to be homogeneous as each piece of the column contained similar amounts of calcium carbonate per gram of proppant (Figure 3.24). The porosity measurements estimated from the μ-CT scan of the room temperature column also showed approximately homogeneous porosity throughout the length of the column. The p-values for all comparisons between the column pieces verified the homogeneity as well, as no p-value was less than the confidence interval of 0.05 (Table 3.1). The null hypothesis was therefore unable to be rejected and the means of each comparison were not statistically significantly different. The calcium carbonate distribution within the 60°C column seemed to be heterogeneous as the mass of calcium carbonate per gram of proppant decreased from influent to effluent (Figure 3.25).
Figure 3.24: Grams of calcium carbonate per gram of proppant for each of the pieces of the extracted room temperature column. Column piece one indicates the first 2.5 centimeters of the column (influent) and piece four is the last 2.5 centimeters of the column (effluent).

Figure 3.25: Grams of calcium carbonate per gram of proppant for each of the pieces of the extracted 60°C column. Column piece one indicates the first 2.5 centimeters of the column (influent) and piece four is the last 2.5 centimeters of the column (effluent).

Several equal variance t-tests were performed on each of the pieces for the room temperature column (Table 3.1) and the 60°C column (Table 3.2) to determine whether
the measured grams of calcium carbonate per gram of proppant were statistically significantly different between pieces. The null hypothesis was that there was no difference in the means, and the confidence interval was 95%.

Table 3.1: P-values for equal variance t-tests comparing pieces of the room temperature column to one another.

<table>
<thead>
<tr>
<th>Comparison Performed</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piece 1 to Piece 2</td>
<td>0.12</td>
</tr>
<tr>
<td>Piece 1 to Piece 3</td>
<td>0.51</td>
</tr>
<tr>
<td>Piece 1 to Piece 4</td>
<td>0.09</td>
</tr>
<tr>
<td>Piece 2 to Piece 3</td>
<td>0.42</td>
</tr>
<tr>
<td>Piece 2 to Piece 4</td>
<td>0.60</td>
</tr>
<tr>
<td>Piece 3 to Piece 4</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Table 3.2: P-values for equal variance t-tests comparing pieces of the 60°C column to one another.

<table>
<thead>
<tr>
<th>Comparison Performed</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piece 1 to Piece 2</td>
<td>0.87</td>
</tr>
<tr>
<td>Piece 1 to Piece 3</td>
<td>0.14</td>
</tr>
<tr>
<td>Piece 1 to Piece 4</td>
<td>0.01</td>
</tr>
<tr>
<td>Piece 2 to Piece 3</td>
<td>0.27</td>
</tr>
<tr>
<td>Piece 2 to Piece 4</td>
<td>0.07</td>
</tr>
<tr>
<td>Piece 3 to Piece 4</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Consequently, in the 60°C column more calcium carbonate was precipitated towards the influent as compared to the effluent. The heterogeneity of the 60°C column is also visualized in the $\mu$-CT porosity measurements as the porosity increased from influent to effluent. The $p$-value of the equal variance t-test comparing piece 1 to piece 4 was 0.01 which is less than the confidence interval 0.05. The grams of calcium carbonate per gram of proppant measured for each piece was not statistically similar and the null hypothesis could be rejected. Therefore, the heterogeneity seen in the $\mu$-CT scan and calcium digest of the 60°C column was statistically significant. The calcium digest and $\mu$-CT scan porosity data seem to align in highlighting where the calcium carbonate was present in the room temperature and 60°C columns. The difference between the homogeneity of the room temperature column and the heterogeneity of the 60°C column can most likely be explained by the increased ureolysis rate at the elevated temperature. The influent of the 60°C column was subjected to fresh mineralization fluids throughout the experiment, and as a result the increased ureolysis rate at 60°C may have caused precipitation to favor the influent. The faster rate of ureolysis and precipitation may have also resulted in the larger deposition of calcium carbonate relative to the room temperature column that was visualized in the $\mu$-CT scans and calcium digests. An increased flowrate may mitigate some of the heterogeneity seen in the 60°C column as it could lessen some advantages found close to the influent.

Conclusions
Urease immobilized on the ceramic proppant proved to be a very efficient catalyst for urea-hydrolysis induced calcium carbonate precipitation at room temperature and 60°C. The effectiveness of the immobilized urease was surprising as inactivation of the enzyme due to encapsulation during active calcium carbonate precipitation was expected. It was anticipated that the ureolytic ability would significantly decrease with each successive pulse, and the usefulness of the immobilized urease would have been drastically reduced. As rapid inactivation of the immobilized urease was not observed there appears to be a mechanism that protects the immobilized urease from inactivation during ureolysis-induced calcium carbonate precipitation. The sustained ureolytic activity had not been seen previously in other mineralization experiments utilizing urease as the catalyst, which did not use the porous ceramic proppant as the carrier. The immobilization of urease on ceramic proppant has also been shown to thermally stabilize the enzyme (Chapter 2), and the column performed at 60°C demonstrates that the immobilized urease can be an effective catalyst of mineralization even at elevated temperatures. The effective temperature range in which EICP is currently utilized may be increased using immobilized urease as it can counteract thermal inactivation and active calcium carbonate precipitation to cement proppant particles together. The ceramic proppant loaded with urease could possibly be used to solidify proppant packs in some oil fields to prevent damaging flowback. Many techniques for reducing proppant flowback used today need extreme pressures or temperatures to induce polymerization of proppant coatings and have some limitations to their usefulness (Almond, Penny and Conway
1995, Nguyen et al. 1996). Using immobilized urease at a catalyst to prevent proppant flowback could expand how this technology may be applied.

The immobilization of urease was also shown to enable the retention of high ureolytic ability after prolonged storage when compared to the suspended enzyme. Increased urease storage stability may enhance the efficiency of future large applications of urease where the production of urease through the growth of microbes or mixing of enzyme slurries may be time prohibitive. Data presented here show that the immobilized urease on the ceramic proppant may be useful in future mineralization applications and research.
CHAPTER FOUR

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

Conclusions

The research presented in this thesis highlights that calcium carbonate precipitation technologies have the possibility to be advanced into environments that are unsuitable using current UICP techniques. The first study showed that the immobilization of *S. pasteurii* urease in Ludox® gel through polymer encapsulation and the adsorption of JBM urease on a porous ceramic proppant significantly decreased the rate of thermal inactivation of the enzyme. Thermal protection of urease through immobilization was especially apparent at higher temperatures (60°C-90°C) as the rate of thermal inactivation was considerably reduced by immobilization when compared to suspended enzyme. The immobilized forms of urease could therefore be used in higher temperature environments where use of the suspended enzyme may not be feasible. The thermal fragility of urease could be challenging to some applications, and research presented here suggests a means of increasing the thermal stability of the enzyme.

The second study primarily aimed to explore how useful urease immobilized on ceramic proppant could be in advancing mineralization applications. The data showed that the immobilized urease sustained high ureolytic activity even during active calcium carbonate precipitation, a trait not observed when using traditional MICP and EICP practices. Therefore, an interesting facet of immobilized urease was revealed that could prove useful in future applications. The study also aimed to combine the protective
features and sustained ureolytic ability of the ceramic proppant in a column study at an elevated temperature (60°C). A goal of the column experiments at 60°C was to understand whether immobilized urease could show promise in applications at temperature conditions not currently accessible. The main driving force behind the entire thesis was advancing the temperature range of UICP applications and culminated with the column experiments at the elevated temperature. The immobilized urease was also able to sustain high ureolytic activity throughout the entire experiment succumbing to minimal thermal inactivation. Therefore, urease immobilized on the ceramic proppant was able to breach a thermal barrier that has heretofore hindered some UICP applications thus providing a promising way in advancing the technology.

Suggestions for Future Work

Further investigation of the effectiveness of urease immobilized on the ceramic proppant should occur. Higher temperature conditions such as those greater than 60°C should be examined to understand at which temperature the immobilized urease becomes unusable. Insight would be provided into the entire range of temperatures that the immobilized urease may be used. The other protective attributes of this immobilized form should be addressed. Immobilization has been shown to protect the enzyme from high concentrations of heavy metals, high and low pH, and other inhibitors (Marzadori et al. 1998, Krajewska 1991, Pandya et al. 2005) Additional protective features should be explored as they could reveal other areas that immobilized urease may be suited for. The protection from heavy metals could be particularly useful in some environmental
remediation applications as harsh conditions are often encountered. Also, pH can be a problem when using urease in application for carbon dioxide sequestration. Future experiments could further provide knowledge into how immobilized urease may be used in conjunction with traditional techniques.

Further research should be conducted in exploring the mechanism that appears to protect the immobilized enzyme from inactivation during active calcium carbonate precipitation. Knowledge of how the immobilization process protects urease from this inactivation could allow for exploitation of this unique ability in future applications. The engineering of a form of urease that may be able to escape inactivation by suspected calcium carbonate entombment could be a breakthrough in establishing innovative applications.

Enzyme immobilization has been researched intensively and as a result there are numerous ways in which urease has been or could be immobilized (Krajewska 2009b). For future work, additional immobilization techniques could be investigated in their effectiveness in promoting calcium carbonate precipitation and how valuable they could be to UICP applications. Each carrier material can have unique attributes that could help advance UICP technologies.

Future work could involve the immobilization of different enzymes in the porous ceramic proppant and other carrier materials as well for developing different technologies rather than only focusing on UICP. Many new and untried applications of immobilized enzymes could be developed, and the scientific knowledge of immobilization could be enhanced.
APPENDICIES
APPENDIX A

SPECTROPHOTOMETRIC ASSAY FOR QUANTIFYING CALCIUM CONCENTRATION
Calcium concentration can be an important measurable quantity that gives vital insight in many experiments but can be relatively work intensive to measure. Measuring calcium concentration was made easier through the development of a colorimetric assay based around an assay already developed for microbiology (World Health Organization 2000). It was noted that nitric acid was preferable to sulfuric acid in making dilutions as calcium sulfate precipitated when using sulfuric acid. Precipitation of calcium sulfate decreased the actual calcium ion concentration that was measured so that the reported value of calcium concentration was less than the actual value. To verify that this assay worked for experiments performed in the lab three replicates of four different calcium solutions were prepared. The calcium concentration was measured using this assay and compared to results obtained through Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The reported values of each method were then statistically compared using equal variance t-tests with a confidence interval of 95% and a null hypothesis of no difference in the means. It was necessary to verify that the calcium assay and ICP-MS measured calcium concentration values that were not significantly statistically different. (Table A.1).

Table A.1: Calcium concentrations measured with the new calcium assay and ICP-MS, the p-values determined by equal variance t-tests at the 95% confidence interval, and the percent error of the calcium assay from the ICP-MS values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calcium Assay Value (g/l)</th>
<th>ICP-MS Value (g/l)</th>
<th>P-value</th>
<th>Percent Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.8 ± 0.2</td>
<td>29.8 ± 0.5</td>
<td>0.056</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>14.2 ± 0.4</td>
<td>15.1 ± 0.3</td>
<td>0.042</td>
<td>5.9</td>
</tr>
</tbody>
</table>
Table A.1. shows that the calcium concentration measured by the calcium assay and ICP-MS were not significantly statistically different as the estimated p-values were greater than the 0.05 of the confidence intervals. Therefore, the calcium assay was as accurate as ICP-MS in measuring calcium ion concentration. It was also important to understand how the calcium assay may be inhibited by common lab chemicals. Several different compounds and media types were tested to discover whether they inhibited the calcium assay. The compounds tested were iron trichloride and urea while the media types tested were calcium mineralization media (CMM+ and yeast extract). Iron trichloride was chosen as soluble iron may be present in some reactors that include iron piping, and urea was tested as it is used often included in lab experiments. Both media types were chosen because they for many experiments and are a good representative source of organics that may inhibit the calcium assay. Each solution was mixed with calcium chloride dihydrate to obtain a final calcium ion concentration of 13.3 g*L⁻¹ of Ca²⁺. The calcium concentration of each solution was measured with the calcium assay, and the result was compared using an equal variance t-test to a solution of 13.3 g*L⁻¹ Ca²⁺ mixed into DI water (Table A.2). The t-tests were performed using a 95% confidence interval and a null hypothesis that there was no difference between the means.
Table A.2: Calcium concentrations measured with the calcium assay for each test solution and the p-value for an equal variance t-test as compared to a standard solution of 13.3 g/l Ca^{2+}.

<table>
<thead>
<tr>
<th>Solution plus 13.3 g/l Ca^{2+}</th>
<th>Measured Calcium Concentration (g/l)</th>
<th>P-value (95% Confidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 g/l Iron Trichloride</td>
<td>14.1 ± 0.3</td>
<td>0.23</td>
</tr>
<tr>
<td>60 g/l Urea</td>
<td>13.8 ± 0.02</td>
<td>0.42</td>
</tr>
<tr>
<td>CMM +</td>
<td>13.1 ± 0.2</td>
<td>0.18</td>
</tr>
<tr>
<td>15 g/l Yeast Extract</td>
<td>13.3 ± 0.3</td>
<td>0.46</td>
</tr>
</tbody>
</table>

The results of the inhibition study show that no compounds or media types tested had a statistically significant influence on the calcium assay. The compounds and media types tested did not seem to inhibit the calcium assay, and it was concluded that the calcium assay can be used in lab experiments to reliably measure calcium concentration. The new calcium assay will save time and money for the lab group and will be a great addition to the techniques that are already available. The following is the developed method that was produced to measure calcium concentration.

**Calcium Assay Method**

**Reagent 1 – Color Reagent**

1. Add 25 mL of Milli-Q® water to a 125 mL flask.
2. Add 7.5 ml of stock solution (12.1 M) HCl to the water.
3. Add 13 mg of o-cresolphthalein complexone and mix until completely dissolved.
4. Add 125 mg 8-hydroxyquinilone and mix until completely dissolved.

5. Bring final volume up to 125 mL using Milli-Q® water.

Reagent 2 – AMP Buffer

1. Add 75 mL of Milli-Q® water to a 125 mL flask.
2. Add 19 mL of 2-amino-2-methyl-1-propanol to the water and mix until the solution is clear.
3. Add stock solution (12.1 M) HCl slowly until the pH is adjusted to 10.7.
4. Bring final volume was up to 125 mL using Milli-Q® water.

Standards:

0, 5, 10, 20, 40, 60, 80 mg/L were made with CaCl$_2$$\cdot$2H$_2$O.

(Samples and standards are diluted in 5% trace metal grade nitric acid.)

Procedure:

1. Distribute 125 µL of Reagent 1 into required number of wells in 96-well plate.
2. Add 10 µL of all samples and standards to the wells in triplicate.
3. Distribute 125 µL of Reagent 2 into the wells.
4. Place the 96-well plate on the rotating shaker plate at 450 rpm for 1 minute.
5. Incubate at room temperature (22°C) for 10 minutes.

Measurement:

Measure the absorbance with plate reader at 575 nm.
The calcium assay has been found to be extremely sensitive, and as a result many tips and tricks have been developed to ensure dependable results. The following is a more in-depth procedure with tips and tricks for each stage of the procedure.

**Method:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Materials, tips, and tricks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Dilute all samples that are to be analyzed in 5% trace metal grade nitric acid, to an appropriate concentration within the quantification limits of the assay.</td>
<td>Dilution tubes: microcentrifuge tubes, falcon tubes, etc. 5% trace metal grade nitric acid made by diluting the stock solution (assumed to be 67.5% nitric acid) to the appropriate concentration with Milli-Q® water.</td>
</tr>
<tr>
<td>2.</td>
<td>Pipette 125 µl of Calcium Reagent 1 into a 96 well microtiter plate.</td>
<td>Calcium Reagent 1 is stored in an opaque bottle at room temperature in an acid cabinet. 0.1 mL pipette tips, multichannel pipettor.</td>
</tr>
<tr>
<td>3.</td>
<td>Pipette 10 µl of the seven different standards into the appropriate wells on the microtiter plate. Standards can be found in the refrigerator. Use standards: 0, 5, 10, 20, 40, 60, 80 mg/l for the assay.</td>
<td>Standards are made from CaCl₂•2H₂O (Calcium chloride dihydrate) diluted in 5% trace metal grade nitric acid. 0.1 pipette tips, 0-0.1ml pipettor.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Pipette 10 µl of each sample into their corresponding wells of the microtiter plate.</td>
<td>Calcium Reagent 2 is stored in an opaque bottle in the refrigerator. 0.1 ml pipette tips, multichannel pipettor. Discard pipette tips after each column to ensure no contamination is transferred to reagent reservoir.</td>
<td></td>
</tr>
<tr>
<td>4. Pipette 125 µl of Calcium Reagent 2 into the 96 well microtiter plate. Change tips after each column to mitigate calcium contamination between wells.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Place 96 well microtiter plate on the rotating shaker plate for one minute at 450 RPM. Then incubate at room temperature, 22°C, for 10 minutes.</td>
<td>Shaker plate.</td>
<td></td>
</tr>
</tbody>
</table>
| 6. Place 96 well microtiter plate on the plate reader and measure the absorbance at 575 nm using the protocol: 575 Calcium Assay. The resulting data can be converted from absorbance readings to concentrations when related to the standard curve.  

*Note always run a standard curve for every plate.* | Plate reader. |
| 7. Discard all excess Calcium Reagent 1 and 2 into the appropriate hazardous waste container. Discard all solution from microtiter plate and rinse the microtiter plate with DI water then discard waste into the hazardous waste container. | Calcium Assay waste container. |
Standard Calculations:

Stock solution: (Made by mixing 0.0588 g of CaCl$_2$•2H$_2$O in 50 ml of 5% trace metal grade nitric acid) contains 320 mg/l of Ca$^{2+}$.

*Remember when making standards to vortex each solution well before transferring it in order to obtain a more homogenous and therefore accurate solution.

<table>
<thead>
<tr>
<th>Standard Identification</th>
<th>Standard Concentration (mg/l)</th>
<th>Standard or Stock to add (µl)</th>
<th>5% trace metal grade nitric acid to add (µl)</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prime</td>
<td>160</td>
<td>600 Stock</td>
<td>600</td>
<td>Into microcentrifuge tube.</td>
</tr>
<tr>
<td>A</td>
<td>80</td>
<td>600 Prime</td>
<td>600</td>
<td>Into microcentrifuge tube.</td>
</tr>
<tr>
<td>B</td>
<td>60</td>
<td>450 Prime</td>
<td>750</td>
<td>Into microcentrifuge tube.</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>600 A</td>
<td>600</td>
<td>Into microcentrifuge tube.</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>600 C</td>
<td>600</td>
<td>Into microcentrifuge tube.</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>600 D</td>
<td>600</td>
<td>Into microcentrifuge tube.</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>600 E</td>
<td>600</td>
<td>Into microcentrifuge tube.</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>0</td>
<td>600</td>
<td>Into microcentrifuge tube.</td>
</tr>
</tbody>
</table>

Making Reagents: Remember the assay can be quite sensitive so to ensure the best results it is recommended that one always stays “calcium sterile” while making the reagents. This means to let all glassware air-dry using no paper towels and being careful not to touch anything that may have contained calcium to the tools being used to make the reagents. Before starting, soak all necessary glassware in a 5% nitric acid bath to remove
residual calcium. Then wash with soap and DI water, and finally rinse all glassware with Milli-Q® water and let air dry. Perform all mixing in the fume hood to protect yourself from gaseous compounds and wear appropriate personal protective equipment (PPE).

Reagent 1: Color Reagent

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Measure 125 mg of 8-hydroxyquinilone and 13 mg of o-cresolphthalein complexone using an accurate balance.</td>
<td>Accurate balance.</td>
</tr>
<tr>
<td>2.</td>
<td>Measure 25 ml of Milli-Q® water using a 250 ml graduated cylinder and add it to a 125 ml bottle.</td>
<td>125 ml bottle, 250 ml graduated cylinder</td>
</tr>
<tr>
<td>3.</td>
<td>Pour about 10 ml of stock solution (12.1 M) HCl into a 20 ml beaker using a glass funnel for safety. Then add 7.5 ml of this solution to the 125 ml bottle containing the water using a 10 ml serological pipette. Save residual HCl for later use.</td>
<td>20 ml beaker, glass funnel, 10 ml serological pipette.</td>
</tr>
<tr>
<td>4.</td>
<td>Add the o-cresolphthalein complexone to the 125 ml bottle until completely dissolved. Then add the 8-hydroxyquinilone to the mixture until dissolved.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Pour the mixture into a 250 ml graduated cylinder and bring the final volume up to 125 ml using Milli-Q® water. Then pour this solution back into the 125 ml bottle.</td>
<td>250 ml graduated cylinder</td>
</tr>
<tr>
<td>6.</td>
<td>Cover the bottle in tin foil and store in the blue acid cabinet at room temperature. Stable for around 1 month.</td>
<td>Tin foil</td>
</tr>
</tbody>
</table>

*For Reagent 2 precise pH adjudgments are needed, so before making the reagent move the pH meter into the fume hood and calibrate it for the 7-10 range. Make sure to clean
the probe off well with Milli-Q® water and remember do not wipe the probe with a paper towel as this may contaminate the reagent.

Reagent 2: AMP Buffer

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Measure 75 ml of Milli-Q® water with a 250 ml graduated cylinder and add it to a 125 ml bottle.</td>
<td>125 ml bottle, 250 ml graduated cylinder.</td>
</tr>
<tr>
<td>2.</td>
<td>Pour about 20 ml of 2-amino-2-methyl-1-propanol into a 40 ml beaker, and then add 19 ml of this to the 125 ml bottle using a 25 ml serological pipette. Mix until the solution is clear.</td>
<td>50 ml beaker, 25 ml serological pipette.</td>
</tr>
<tr>
<td>3.</td>
<td>Add the leftover stock solution of 12.1 M HCl very slowly dropwise into the 125 ml bottle using a 1 ml syringe until the pH reads 10.7 being careful not to go lower than 10.7. If necessary, pour more stock HCl into the 20 ml beaker to use. Remember to never pour leftover acid back into the bottle but dispose of it accordingly.</td>
<td>1 ml syringe</td>
</tr>
<tr>
<td>4.</td>
<td>Pour solution into a 250 ml graduated cylinder, and then bring the total volume up to 125 ml using milli-Q water. Pour this back into the 125 ml bottle.</td>
<td>250 ml graduated cylinder</td>
</tr>
<tr>
<td>5.</td>
<td>Cover the 125 ml bottle in tin foil and place in the fridge. Stable for around 1 month.</td>
<td>Tin foil</td>
</tr>
</tbody>
</table>


APPENDIX B

INHIBITION STUDIES ON JBM UREASE WITH DIFFERING CONCENTRATIONS
OF DIVALENT HEAVY METAL CATIONS AND SODIUM CHLORIDE
Ureolytic inhibition of copper chloride, zinc chloride, cobalt chloride, and sodium chloride (Fisher) on urease sourced from jack bean meal (JBM) was investigated. Urea hydrolysis studies were performed using JBM exposed to different concentrations of these potential inhibitors to determine whether activity was altered.

Materials and Methods

A solution of 40 g*L⁻¹ urea plus a specific concentration of an inhibitor was prepared in DI water, and another solution of 10 g*L⁻¹ JBM and the specific concentration of the inhibitor in DI water was also prepared. Both solutions were made for each concentration of each potential inhibitor. The metal inhibitors were mixed in 10, 100, and 1000 µM solutions of the metal ion itself, and the sodium chloride solutions were mixed to be 9, 50, and 100 g*L⁻¹ of the salt itself. Batch studies like those performed in Chapter 2 were conducted, and the same sampling and analytical procedures were followed. However, the urease was in its suspended form when the two solutions were mixed. Treatments of JBM without any inhibitors were also tested to obtain the initial ureolytic activity of urease. The results of the batch studies of each inhibitor can be seen in Table B.1. The pH of each suspension was measured to determine whether hydrogen ion concentration may be causing inhibition (Table B.2).

Table B.1: The fraction of initial activity of each concentration of each inhibitor as compared to the initial, noninhibited urease activity.

<table>
<thead>
<tr>
<th>Inhibitor and Concentration</th>
<th>Fraction of Initial Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co²⁺ 10 µM</td>
<td>0.74 ± 0.13</td>
</tr>
</tbody>
</table>
Table B.1: Continued

<table>
<thead>
<tr>
<th>Inhibitor and Concentration</th>
<th>pH of Initial Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co$^{2+}$ 100 µM</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td>Co$^{2+}$ 1000 µM</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Cu$^{2+}$ 10 µM</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>Cu$^{2+}$ 100 µM</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>Cu$^{2+}$ 1000 µM</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>Zn$^{2+}$ 10 µM</td>
<td>1.05 ± 0.03</td>
</tr>
<tr>
<td>Zn$^{2+}$ 100 µM</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>Zn$^{2+}$ 1000 µM</td>
<td>0.23 ± 0.19</td>
</tr>
<tr>
<td>NaCl 9 g*L$^{-1}$</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>NaCl 50 g*L$^{-1}$</td>
<td>0.73 ± 0.14</td>
</tr>
<tr>
<td>NaCl 100 g*L$^{-1}$</td>
<td>0.57 ± 0.22</td>
</tr>
</tbody>
</table>

Table B.2: pH of the solutions containing the JBM and the specific metal inhibitor at the specific concentration.

<table>
<thead>
<tr>
<th>Inhibitor and Concentration</th>
<th>pH of Initial Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co$^{2+}$ 10 µM</td>
<td>6.44</td>
</tr>
<tr>
<td>Co$^{2+}$ 100 µM</td>
<td>6.38</td>
</tr>
<tr>
<td>Co$^{2+}$ 1000 µM</td>
<td>5.81</td>
</tr>
<tr>
<td>Cu$^{2+}$ 10 µM</td>
<td>6.36</td>
</tr>
<tr>
<td>Cu$^{2+}$ 100 µM</td>
<td>6.10</td>
</tr>
<tr>
<td>Cu$^{2+}$ 1000 µM</td>
<td>4.62</td>
</tr>
<tr>
<td>Zn$^{2+}$ 10 µM</td>
<td>6.42</td>
</tr>
</tbody>
</table>
Table B.2: Continued

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn$^{2+}$ 100 μM</td>
<td>6.16</td>
<td></td>
</tr>
<tr>
<td>Zn$^{2+}$ 1000 μM</td>
<td>4.62</td>
<td></td>
</tr>
</tbody>
</table>

Table B.1 shows that some of the metals ions indeed appear to inhibit ureolysis by JBM as the fraction of initial activity decreased when the metal ions were present. As metal ion concentration increased JBM ureolytic activity seemed to decrease. Sodium chloride, at concentrations of 50 and 100 g*L$^{-1}$, also seems to exhibit inhibition of ureolysis by JBM. Sodium chloride is a common solute in the subsurface where EICP technology is often employed, so urease inhibition by sodium chloride is important to understand. Table B.2 shows that the pH of the metal ion solutions was within the range of JBM activity, meaning pH was likely not the reason why a significant loss in ureolytic activity was observed (Pettit et al. 1976). These data may help in implementing EICP as they highlight solutes that may inhibit ureolysis activity.
APPENDIX C

SUPPLEMENTAL UREA AND PH DATA FOR THE PROPPANT COLUMNS
Figure C.1: Influent (●) and effluent (■) urea concentration data for the first room temperature column.

Figure C.2: Influent (●) and effluent (■) pH data for the first room temperature column.
Figure C.3: Influent (●) and effluent (■) urea concentration data for the second room temperature column.

Figure C.4: Influent (●) and effluent (■) pH data for the first room temperature column.
Figure C.5: Influent (●) and effluent (■) urea concentration data for the first 60°C column.

Figure C.6: Influent (●) and effluent (■) pH data for the first 60°C column.
Figure C.7: Influent (●) and effluent (■) urea concentration data for the second 60°C column.

Figure C.8: Influent (●) and effluent (■) pH data for the second 60°C column.
APPENDIX D

DETERMINATION OF UREASE SOURCE FOR CERAMIC PROPPANT EXPERIMENTS
Heat inactivated *Sporosarcina pasteurii* was immobilized on the ceramic proppant to understand whether the proppant could successfully immobilize *S. pasteurii*. A culture of *S. pasteurii* was grown to a specific optical density following the procedure outlined in Chapter 2. Samples of 25 mL of the adjusted culture were placed in conical tubes and subjected to 60°C for 5 hours. The prolonged exposure to 60°C killed the bacteria but did not fully inactive the enzyme. The heat exposed sample was then utilized as the urease source and was immobilized on the porous proppant following the procedure outlined in Chapter 2. The first order thermal inactivation rate coefficient was then calculated at 80°C following the procedure outlined in Chapter 2. The value obtained was then compared to suspended *S. pasteurii* urease and Jack Bean meal urease immobilized on the ceramic proppant (Table D.1).

Table D.1: The values of the first order thermal inactivation rate coefficient at 80°C for suspended urease and JBM urease and heat killed *S. pasteurii* immobilized on ceramic proppant.

<table>
<thead>
<tr>
<th>Urease Form</th>
<th>First Order Thermal Inactivation Rate Coefficient (hr(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspended <em>S. pasteurii</em> Urease</td>
<td>9.77</td>
</tr>
<tr>
<td>Immobilized Heat Killed <em>S. pasteurii</em> Urease</td>
<td>5.77</td>
</tr>
<tr>
<td>Immobilized JBM Urease</td>
<td>2.26</td>
</tr>
</tbody>
</table>

Examining Table D.1 is can be seen that the value for the first order thermal inactivation rate coefficient of heat killed *S. pasteurii* urease was 5.77 hr\(^{-1}\) while the value
for the immobilized JBM urease was 2.26 hr\(^{-1}\). The ceramic proppant was therefore able to thermally protect the JBM urease more effectively than the heat killed \textit{S. pasteurii} urease.

Also, the ureolytic activity of the heat killed \textit{S. pasteurii} and JBM immobilized on the proppant was estimated following the procedure outlined in Chapter 2. The activities were then compared using an equal variance t-test with the null hypothesis being that the means are the same and a confidence interval of 95%. Data can be visualized in Table D.2.

<table>
<thead>
<tr>
<th>Urease Form</th>
<th>Ureolytic Activity (g*L(^{-1})min(^{-1}))</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Killed \textit{S. pasteurii} Urease</td>
<td>0.059 ± 0.004</td>
<td>9.20*10(^{-6})</td>
</tr>
<tr>
<td>JBM Urease</td>
<td>0.159 ± 0.005</td>
<td></td>
</tr>
</tbody>
</table>

The p-value in Table D.2 is much less than the confidence interval of 0.05 meaning that the null hypothesis can be rejected, and the two means are not statistically significantly similar. The ureolytic activity of the JBM urease immobilized on the ceramic proppant is pointedly higher than the activity of the heat killed \textit{S. pasteurii}. Therefore, it was concluded that the JBM would be used in experiments containing the ceramic proppant as the activity was high and it seemed that the JBM urease was thermally protected more effectively than the heat killed \textit{S. pasteurii} urease.
APPENDIX E

SUPPLEMENTAL X-RAY COMPUTED MICRO-TOMOGRAPHY SCANS OF THE 60°C PROPPANT COLUMNS
Figure E.1: Supplemental μ-CT scans of the 60°C columns. Column trial one (grey), column trial two (blue), and column trial two scanned upside down and then reconstructed right-side up (orange).

The red and orange data sets seen in Figure E.1 represent scans of the same column trial. Two scans were done to understand whether the jumps seen in the data were artefacts of the μ-CT scan. However, the scans line up relatively well highlighting that the jumps were truly found in the column and were not articles of the scan. Also, the scan of column trial one exhibits the jumps in similar places to column trial two showing that the jumps occurred in both trials.


