Measurement and Variability of Crude Urease Enzyme for Enzyme Induced Carbonate Precipitation (EICP) Applications

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved November 2022 by the
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ARIZONA STATE UNIVERSITY

December 2022

ABSTRACT

Two challenges in the implementation of enzyme induced carbonate precipitation (EICP) are the cost of enzyme and the variability of the enzyme. Urease enzyme costs can be lowered drastically with the use of crude extract from plant materials, but experience has shown variability in the source of the crude urease enzyme, the crude urease enzyme extraction methods, and the concentration of the EICP solution can cause significant variability in the efficacy of the EICP solution.

This thesis examines the variability in the efficacy of crude enzyme derived from jack beans (*Canavalia ensiformis*) and sword beans (*Canavalia gladiata*), two of the most commonly used sources of urease enzyme for EICP. The sources of variability investigated herein include the crude extraction method (including the effect of the bean husks on extraction) and different chemical constituent concentrations. These effects were assessed using enzyme activity measurements and precipitation efficiency tests. The activity tests were performed via spectrophotometry using Nessler's reagent. The precipitation tests looked at the influence of chemical constituent concentrations of 0.67 M calcium chloride and 1 M urea with non-fat dry milk in the EICP solutions and a higher concentration solution with chemical constituent concentrations of 2 M for both calcium chloride and urea with non-fat dry milk. The high concentration solution was selected based on preliminary testing results to maximize carbonate precipitation in one cycle of treatment. Significant sources of a decline in activity (and increase in variation) of the crude urease enzyme were found in extraction from sword beans with husks, high chemical constituent concentrations, and juicing instead of cheesecloth filtration.

This thesis also examines the accuracy of commonly used correlation factors for converting electrical conductivity to urease enzyme activity. Crude jack bean and sword bean urease enzyme activity measurement via electrical conductivity was found to have a correlation coefficient that differed from the previously reported correlation when compared to activity measured via the more accurate spectrophotometry using Nessler's reagent measurements.

ACKNOWLEDGMENTS

I would like to offer my sincerest gratitude to everyone who has helped me throughout my time at Arizona State University. Thank you to my advisor, Professor Ed Kavazanjian for his support, guidance, and patience. It was because of him, and the Center for Bio-mediated and Bio-inspired Geotechnics (CBBG) that I got involved with and excited about geotechnical engineering.

Thank you to everyone at the CBBG. My experience would not be nearly as beneficial without the community and support of the current and former professors, admin staff, and students that have helped me along the way. I would especially like to thank Dr. Kimberly Martin and (soon to be Dr.) Miriam Woolley for their mentorship, Professor Hamed Khodadadi Tirkolaei and Dr. Emmanuel Salifu for their support, and (soon to be Dr.) Vinay Krishnan and Saleh Alothman for their collaboration on preliminary testing.

Lastly, I would like to thank my family: my partner, Jason Dutro; my parents, Rachel and Craig Pearson; my brother, Jonas Pearson; and all the pets (Ditto, Daisy, Finn, and Mango) for their unwavering support.

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CHAPTER 1

INTRODUCTION

BACKGROUND

Enzyme induced carbonate precipitation (EICP) uses calcium ions and urea hydrolyzed using urease enzyme to precipitate calcium carbonate. The precipitated calcium carbonate can improve the behavior of cohesionless soils by cementing the soil particles together. This can provide beneficial improvements to address various geotechnical challenges (i.e., fugitive dust, liquefiable soils). One of the barriers to the implementation of EICP in engineering practice is the cost of urease enzyme (Almajed et al. 2021). However, a simplified method of extracting urease enzyme that was used by Nam et al. (2015) and further simplified by Martin et al. (2021) offers significant reduction in the cost of EICP for soil improvement.

This thesis addresses the issues of the consistency of crude urease enzyme extracted from common sources and crude urease enzyme activity measurement methods. There are several inconsistent factors that should be addressed with respect to crude urease enzymes and the EICP treatment solution. Jack beans (*Canavalia ensiformis*) are rich in urease enzyme and often considered the optimal agricultural source for extraction of urease enzyme (e.g., Almajed et al. (2021) and Khodadadi Tirkolaei et al. (2020)). However, many of the studies reported in the literature actually use sword beans (*Canavalia gladiata*) and do not report any difference in activity or behavior (e.g., Javadi (2021) and Javadi et al. (2021)). Furthermore, in the studies involving urease extraction from these beans the crude urease enzyme has not been extracted in a consistent matter.

For instance, differences in crude urease enzyme extraction methodology include whether or not the husk of the bean is removed as part of the extraction process and the method of filtration also varies. Additionally, the effect the varying levels of the chemical constituents used in EICP has on the crude urease enzyme in terms of precipitant yield is generally left unexplored in literature.

In addition to differences in extraction method, differences in the method used to quantify the activity of the extracted enzyme is an issue. One method of determining urease enzyme activity is the measurement of the concentration of ammonia hydrolyzed from urea by the enzyme using spectrophotometry and Nessler's reagent (e.g., Javadi et al. (2021) and Khodadadi Tirkolaei et al. (2020)). However, a simpler method that can be used for urease enzyme activity measurement is electrical conductivity measurement. The correlation used to convert electrical conductivity to urease activity is based upon testing conducted with pharmaceutical enzyme (Whiffin 2004) and may not be accurate when used with crude enzyme (because of the effects of impurities in the crude extract on electrical conductivity).

OBJECTIVES

One objective of this thesis is to discover which (if any) variations in the crude enzyme extraction process and EICP chemical constituent concentrations influence urease enzyme activity or precipitation efficiency. Knowledge of the effects of variants will serve in creating more uniform and multi-applicable results in future research and applications.

The other objective is to determine accuracy and applicability of electrical conductivity measurements for crude urease enzyme activity measurement. Electrical conductivity measurements as an alternative to spectrophotometry using Nessler's reagent simplify activity assessments, making them safer and less time consuming. Activity assessment via electrical conductivity measurement uses less specialized equipment when compared to spectrophotometry, making research and application of EICP with accurate enzyme activity concentrations more accessible.

ORGANIZATION

This thesis is organized by the following chapters:

Chapter 1. Introduction: This chapter provides an introduction to this thesis and describes the content of each chapter.

Chapter 2. Literature Review: This chapter reviews the relevant literature informing this study, providing context for the experiments conducted for this thesis.

Chapter 3. Methodologies: This chapter describes the various methods used in the experimental work conducted for this thesis.

Chapter 4. Activity and Precipitation Efficiency: This chapter presents and discusses the results of crude urease enzyme extractions and associated activity measurements and precipitation tests. These tests employed two different chemical constituent concentrations. The sources of crude urease enzymes used in this study were crude urease enzyme from sword beans and from jack beans, with and without husks. The crude urease enzyme was extracted by blending with a blender appliance and filtering

using cheesecloth, blending and filtering with a juicer appliance, and juicing alone. The beans were first prepared and soaked for 24 hours and the extraction solution was filtered through glass wool in each extraction method.

Chapter 5. Activity Measurement: This chapter presents the results and discussion of measurement of activity using both electrical conductivity measurements and spectrophotometry using Nessler's reagent. The same crude urease enzymes tested in Chapter 4 were tested in this chapter.

Chapter 6. Conclusions: This chapter summarizes the results of the experimental tests in this thesis and provides recommendations for further study.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

Enzyme Induced Carbonate Precipitation (EICP) uses urea, a source of calcium, and urease enzyme to improve some properties of soils through the precipitation of calcium carbonate in the pore fluid. Researchers (e.g., Ahenkorah et al. 2021b; Almajed et al. 2021) have drawn attention to the potential use of EICP in a variety of fields, including construction, environmental engineering, oil and gas recovery, and geotechnical engineering. In geotechnical engineering, EICP has been proposed to address problems such as poor bearing capacity, fugitive dust, heavy metal contamination, excessive seepage, surface water erosion, slope instability, levee seepage, tunnel stability, and liquefiable soils (particularly under existing buildings). Some researchers point to EICP as a more sustainable alternative to ground improvement than portland cement, however to date there are no analyses that conclusively prove this to be the case.

THE EICP PROCESS

The enzyme induced carbonate precipitation (EICP) process typically starts with calcium chloride and urea in an aqueous solution. The calcium chloride quickly dissociates into chloride and calcium ions (when below saturation), as shown in Eq. 2-1. The urea is soluble in water and is relatively stable in solution but degrades (hydrolyses) at a slow rate into ammonium and carbonate ions in the absence of a catalyst. However, as shown in Eq. 2-2, the addition of urease enzyme catalyzes the dissociation of urea in

solution, speeding up the hydrolysis process by a factor of up to 10^{14} (Ahenkorah et al. 2021b). When urease enzyme is added, the urea hydrolysis goes through several steps of dissociation, as shown in Eq. 2-3 and 2-4 (dependent on the constituent concentrations and the pH of the solution), to form ammonium ions and carbonate ions. The carbonate ions from the hydrolysis of urea combine with the calcium ions from the dissolved calcium chloride to precipitate out of solution as calcium carbonate (due to the low saturation value of calcium carbonate), as shown in Eq. 5. As shown in Eq. 2-6, the ammonium and chloride ions may also combine, forming an ammonium chloride by-product that may need to be considered in some applications.

The equations describing the EICP reaction can be written as follows (Almajed et al. 2021; Zehner et al. 2021):

- The dissolution of calcium chloride:

$$CaCl_2(s) \rightarrow Ca^{2+}(aq) + 2Cl^-(aq) \quad [2\text{-}1]$$

- The breakdown of urea:

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease Enzyme}} NH_3 + CO(NH_2)OH \quad [2\text{-}2]$$

$$CO(NH_2)OH + OH^- \rightarrow NH_3 + HCO_3^- \quad [2\text{-}3]$$

$$NH_3 + HCO_3^- \rightarrow NH_4^+ + CO_3^{2-} \quad [2\text{-}4]$$

- The precipitation of calcium carbonate:

$$Ca^{2+}(aq) + CO_3^{2-}(aq) \leftrightarrow CaCO_3(s) \quad [2\text{-}5]$$

- The formation of the ammonium chloride by-product:

$$NH_4^+(\mathrm{aq}) + \mathrm{Cl}_2^-(\mathrm{aq}) \leftrightarrow \mathrm{NH_4Cl(l)} \quad [2\text{-}6]$$

The amount of precipitated calcium carbonate depends on the concentration of the constituents and may be affected by the enzyme amount and/or activity. It is important to note that a pH between 8.0-9.0 drives the reaction in Eq. 2-3 through 2-4 to the resultant Eq. 2-5 (Ahenkorah et al. 2021b). The pH also drives the formation of the ammonium chloride by-product shown in Eq. 2-6 (as opposed to off gassing of ammonia, leaving the chloride in solution). The pH reflects a balance among processes: ammonia production raises the pH of the solution while calcium carbonate formation lowers it (Zehner et al. 2021). The rate of hydrolysis of urea is affected by the concentration of urea, temperature, pH, and inhibitors (Ahenkorah et al. 2021b).

The urease enzyme that breaks down urea is found in microbes (the source of urease enzyme for the cousin process to EICP, microbially induced carbonate precipitation, or MICP), fungi, and agricultural sources (the most common enzyme source for EICP) (Almajed et al. 2021). While there are a variety of significant agricultural sources of urease enzyme, including leaves, melon seeds, and squash (Ahenkorah et al. 2020), jack beans (*Canavalia ensiformis*), a particularly rich source of urease enzyme, have been the most thoroughly studied ((Ahenkorah et al. 2021b; Almajed et al. 2021; Nam et al. 2015). The sword bean (*Canavalia gladiata*), another rich source of urease, is a close relative of the jack bean and is sometimes incorrectly referred to as jack bean in the literature (e.g., Javadi (2021) and Javadi et al. (2021)).

Urease enzyme is available through pharmaceutical and lab supply companies. This commercial enzyme is typically extracted and purified from jack beans in a multi-step process. It can be cost prohibitive to use pharmaceutical grade urease in the EICP process. However, it has been found that, using jack beans or similar agricultural sources, a simplified crude enzyme extraction process can be just as effective, if not more so, with respect to EICP (Khodadadi Tirkolaei et al. 2020).

The amount of precipitate generated by an EICP solution can depend on a number of factors. The chemical constituents, typically calcium chloride and urea, stoichiometrically control the maximum amount of calcium chloride precipitate. However, the actual precipitation yield is often less than the maximum, i.e., the precipitation efficiency may be less than 100 percent. Ahenkorah et al. (2021b) discuss the optimization of chemical constituents to maximize precipitation efficiency and the importance of regulating the chemical constituents when comparing results from different enzyme sources and extraction procedures.

CARBONATE PRECIPITATION STRUCTURE AND MECHANICS

Enzyme induced carbonate precipitation (EICP) binds soil particles together and fills pores. It has been shown to roughen the soil surface as well, increasing interparticle friction in addition to the binding and pore-filling properties more widely attributed to enhancement of soil properties (Almajed et al. 2021). Fig. 2-1 shows scanning electron microscope (SEM) images of EICP-treated soil, illustrating the increase in soil roughness.

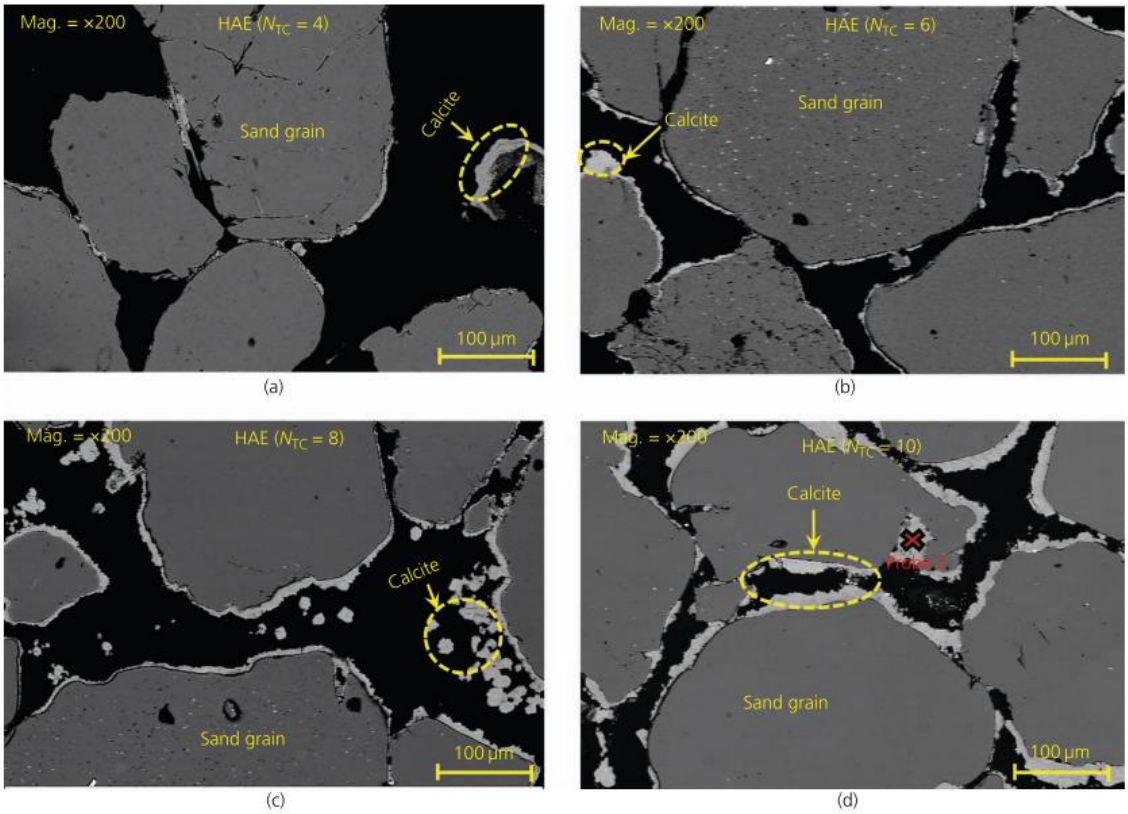

Figure 2-1: SEM imaging of EICP treated sand. (Ahenkorah et al. 2021a)

Crystallization of calcium carbonate begins with stable nucleation at the point the carbonate content of the solution reaches the supersaturated stage. The energy barrier for crystallization is typically higher for spontaneous nucleation when compared to nucleation with a surface with pre-existing (seeded) calcium carbonate crystals. The decrease in the energy required for crystallization on a seeded surface is dependent on the surface tension between the crystal and liquid phase and the solid seed. One advantage of microbially induced carbonate precipitation (MICP) compared to enzyme induced carbonate precipitation (EICP) is that the bacteria can act as seeds (Ahenkorah et al. 2022; Zehner et al. 2021). After the first treatment cycle, further cycles serve primarily to grow existing calcium carbonate crystals (Zehner et al. 2021). Zehner et al. (2021) found

that seeding the soil with foreign substrates could reduce the energy barrier to precipitation. However, these investigators also found that precipitation would happen on the surface of sand particles after pH stabilization regardless of seeding and suggested further work on modeling efforts.

One important (and somewhat surprising) improvement in the EICP process has been the addition of non-fat dry milk to the solution. Initially included in a MICP treatment solution as a stabilizer for the urease enzyme (Meyer et al. 2011), the addition of non-fat dry milk to the EICP treatment solution was found by Almajed et al. (2019) and further shown by Martin et al. (2021) to enhance the strength of EICP-treated soil by improving the distribution of the calcium carbonate precipitate (without any effect on the efficiency or yield of the solution). The increase in soil strength due to this modified EICP solution was attributed to the increased concentration of the precipitate at inter-particle contacts rather than on the particle surface. An implication of the increased strength from this modified EICP solution is that lower constituent concentrations are required to achieve a target strength and lower concentrations of the ammonium by-product are generated, lowering the cost and environmental impact of the ECIP process, as discussed by Almajed et al. (2019).

Phua and Røyne (2018) found that lactate had a significant effect on calcium carbonate morphology in addition to the distribution of calcium carbonate precipitate. There are three common crystalline polymorphs of calcium carbonate: calcite, vaterite, and aragonite. If there is no nucleation, it is also possible for calcium carbonate to

amorphously precipitate, though with time it may crystalize into the more thermodynamically stable phases of vaterite and calcite (Zehner et al. 2021).

Test tube studies in the absence of soil by Almajed et al. (2018) using pharmaceutical (purified) enzyme showed that the EICP precipitate was composed of both vaterite and calcite. However, studies by these investigators indicated that in the presence of soil the precipitate was primarily calcite, the most stable polymorph of calcium carbonate (Almajed et al. 2018). Fig. 2-2 shows scanning electron microscope (SEM) imaging of EICP precipitate on a clean sand after 72 hours as performed by Nam et al. (2015). They tested a control solution, a solution using lab grade purified urease, and a solution using a relatively crude extract. They found, as shown in the lower images, the crude extract resulted in aggregate precipitation including vaterite. Ahenkorah et al. (2022) draws attention to the fact that most of the studies done on calcium carbonate polymorphs haven't used a standardized EICP solution or included non-fat dry milk in the solution, complicating the comparison of results from these different studies.

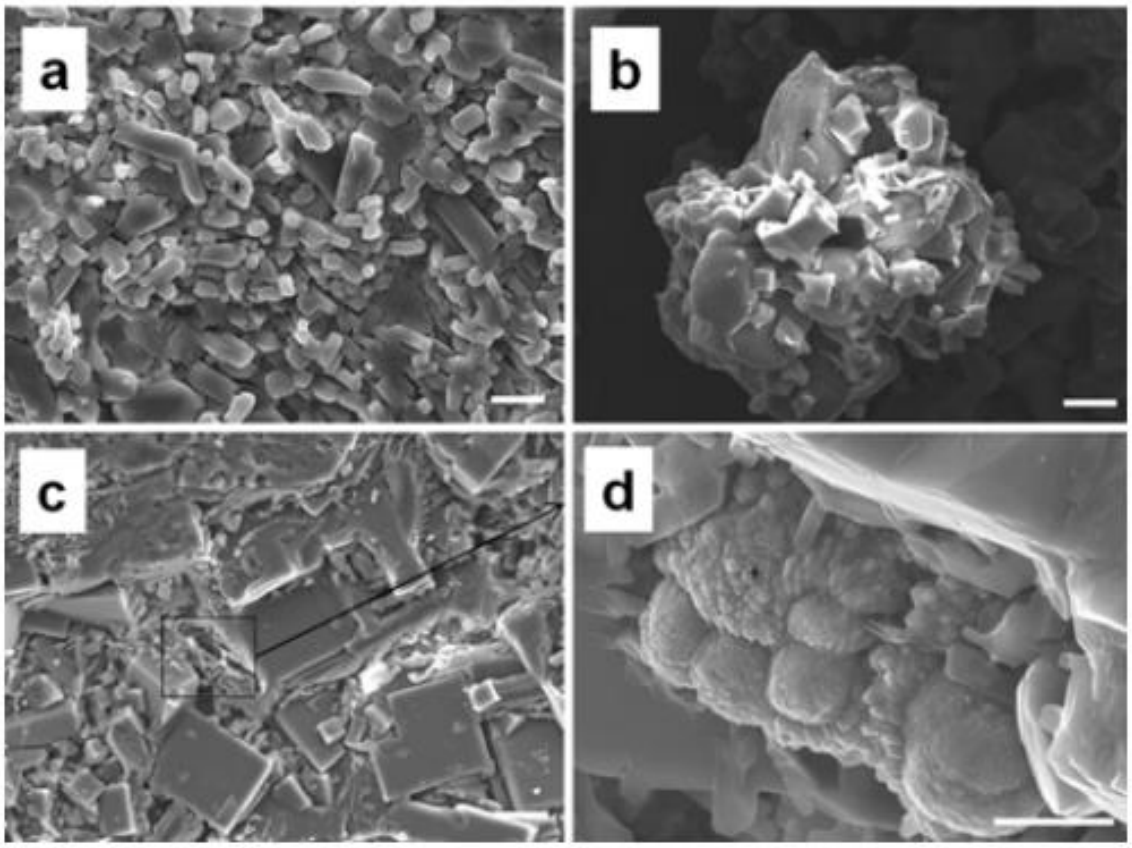

Figure 2-2: SEM imaging of a) a control solution, b) EICP using purified urease, c) EICP using crude urease extract, and d) case c magnified. (Nam et al. 2015)

The potential for variations in precipitate morphology between EICP and MICP was addressed by Ahenkorah et al. (2022) using test tube precipitation tests. The EICP solution included 4.0g/L of non-fat dry milk. Fig. 2-3 shows scanning electron microscope images of both EICP and MICP precipitate from Ahenkorah et al. (2022). These investigators found that both solutions appeared visually to contain both calcite and vaterite polymorphs, but the EICP solution contained a higher concentration of vaterite crystals than the MICP solution. However, a study by Martin et al. (2021) showed the precipitate in EICP-treated soil several months after treatment using a crude

extract and non-fat milk was almost entirely calcite. Carbonate that initially precipitated as vaterite may have transitioned to calcite by the time Martin et al. (2021) had conducted their analysis.

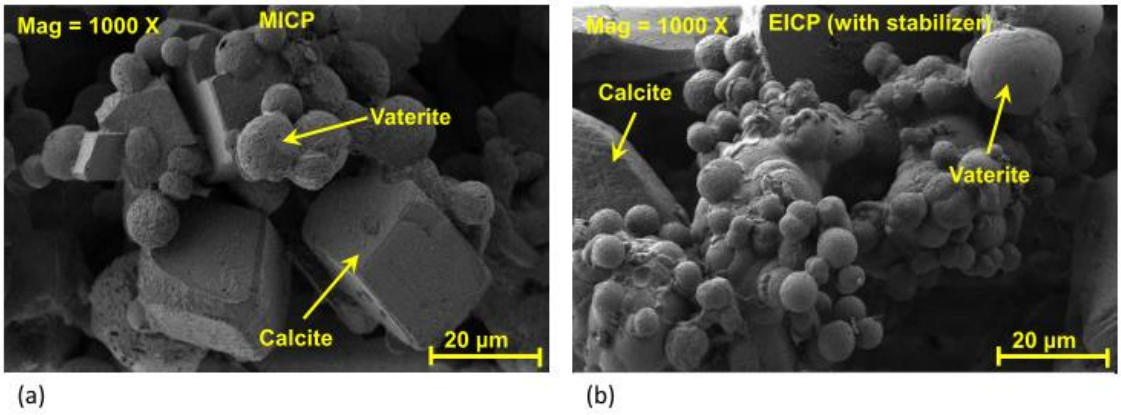

Figure 2-3: SEM imaging of a) MICP precipitate and b) EICP precipitate. (Ahenkorah et al. 2022)

MICP VS EICP

Microbially induced carbonate precipitation (MICP) and enzyme induced carbonate precipitation (EICP) employ the same chemical process and have many of the same benefits and drawbacks but do have distinctions. Almajed et al. (2021) outlines many of the drawbacks and benefits of each method. MICP is more limited by soil grain size (or more properly pore throat size), environmental concerns, and uniformity in treatment. EICP has been limited by the cost of enzyme (57-98% of the solution cost) when using pharmaceutical urease enzyme. EICP is less affected by soil grain size, as the enzyme proteins used in EICP are smaller (the basic enzyme unit is ~12nm) than the microbes used in MICP (0.5 to 3 µm), and therefore less prone to clogging. There are no concerns around the introduction of microbes to the environment when using EICP. The

release of ammonia and chloride and rise in pH can be a problem and is present in each process. Almajed et al. (2021) posits that the ammonia produced by these processes when used as a surficial treatment for dust control degrades or volatilizes over time, making it a viable option. However, in the subsurface, the ammonia tends to speciate to ammonium chloride, a regulated contaminant in some jurisdictions. The cost of development and storage of microbes used in MICP is also a factor to be considered. The longevity of MICP and EICP may vary as well. MICP may be more susceptible to degradation due to degradable biomasses creating blockages in soil pores.

Ahenkorah et al. (2022) pointed out that many of the MICP and EICP comparisons in the literature may be flawed due to variations in the chemical constituents and concentrations, the amount of solution used, the number of cycles, and the soil type treated. There seem to be nonproportional improvements in different properties in similar treatment situations. MICP has also been more extensively studied in comparison to EICP.

PRECIPITATION EFFICIENCY

The precipitation efficiency (or precipitation ratio) is a measurement of the yield of calcium carbonate precipitation divided by the theoretical (stoichiometric) maximum, usually expressed as a percent. This is presented through Eq. 3-1. Typically, precipitation efficiency is measured via test tube precipitation experiments. Test tube precipitation experiments consist of putting the EICP solution into test tubes, allowing the reaction to run its course for a set period of time, removing the supernatant, washing the precipitate (to remove soluble salts), and drying and measuring the weight of the precipitate.

Ahenkorah et al. (2020) highlights the common use of this procedure in precipitation yield and feasibility studies of EICP solutions.

$$\text{precipitation efficiency} = \frac{\text{experimental precipitate}}{\text{maximum theoretical precipitate}} \times 100\% \qquad [3\text{-}1]$$

One use of test tube precipitation efficiency studies is to determine optimum concentrations of chemical constituents and/or factors influencing enzyme activity. Ahenkorah et al. (2021b) discuss the wide variability in reported optimum precipitation ratios from test tube studies. Suffice to say, there is a large variability in reported optimum chemical constituent concentrations and ratios of constituents to enzyme activity (or amount of enzyme, in cases where activity was not measured) in these test tube studies. Ahenkorah et al. (2021b) claim the inconsistent optimum solutions are due to lack of consideration of enzyme kinetics. Furthermore, non-fat dry milk is often omitted from the treatment solutions. Ahenkorah et al. (2021b) also found that there is potential for differing amounts of ammonia and chloride ion production and pH control among the different treatment solutions reported in the literature.

Some investigators have examined alternatives to calcium chloride in test tube studies. One example is the study done by Phua and Røyne (2018). These investigators looked at the dissolution of limestone using lactic acid to generate the calcium required for EICP (with a statement that in future they would target bacteria for both limestone dissolution and urease enzyme production). They found that calcium dissolved from limestone may be a feasible source of calcium for EICP.

ENZYME SOURCE AND KINETICS

Urease enzymes are nickel-based metalloenzymes with two Ni^{2+} ions. The protein scaffold does not change between urease sources, but there is variance between the subunits of agricultural and fungal based enzymes and the subunits of bacterial based enzymes (Ahenkorah et al., 2020; Ahenkorah, Rahman, Karim, Beecham, et al., 2021). There have been reports that suggest enzyme structure may influence calcium carbonate precipitation (Nam et al. 2015).

Historically, enzyme induced carbonate precipitation (EICP) was not seriously considered as a soil improvement option, as the cost of the enzyme was prohibitive. When commercial (pharmaceutical) enzyme was used, enzyme costs made up 57-98% of the process costs (Almajed et al. 2021). However, the use of crude enzymes (Nam et al. 2015; Martin et al. 2021) has made EICP much more cost effective.

In the studies by Nam et al. (2015), the crude enzyme was processed using a phosphate buffer, filter paper, and centrifugation, yielding 2mL of crude extract per 50g of jack beans. They found activity similar to the purified enzyme commercially available at the time (Nam et al. 2015). Martin et al. (2021) report on a major simplification of the extraction process, significantly changing or removing each step used by Nam et al. (2015). The process developed by Martin et al. (2021) consists of soaking the jack beans in tap water, running the beans through a blender until the mixture reaches the desired consistency, and filtration of the resulting mixture using cheesecloth and glass wool. Martin et al. (2021) report that the crude enzyme was as or more effective than the purified pharmaceutical enzyme for biocementation via EICP. Other investigators have

reported on crude extraction of urease from soybeans (e.g., Shu et al. (2022)). However, it is often unclear what extraction procedure is used in these studies.

Enzyme kinetics, including enzyme activity, are critical to understand to accurately predict, control, and monitor the EICP process. Enzymes are complex and have a limited lifetime, this can make them a source for variability that is not always considered in EICP studies. There is potential for a further decrease in cost with an accurate understanding of enzyme kinetics (Ahenkorah et al. 2021b).

Urease enzyme activity (reaction rate) is typically presented in terms of Units (U). A unit is the amount of the enzyme needed to hydrolyze 1 μmol of urea in a minute at a pH of 7.0 and a temperature of 25 °C (Ahenkorah et al. 2021b). Parameters influencing reaction rate (or activity) include the pH and temperature of the solution. Various inhibitors can also play an important role in reaction rates (Ahenkorah et al. 2021b).

There are a number of urease inhibitors that have a range of strengths, including urea and ammonia which acts as weak inhibitors. The inhibitors are generally separated into competitive, mixed, and uncompetitive inhibitors. Competitive inhibitors prevent the urease from binding with the substrate by binding to the enzyme at an active site. Mixed inhibitors bind with the urease enzyme, but at a non-active site. Uncompetitive inhibitors bind to the urease-substrate complex. Mixed and uncompetitive inhibitors are common in systems similar to EICP but aren't thoroughly understood in the context of EICP. Competitive inhibitors have been studied more thoroughly. They can strongly reduce the rate of the reaction and significantly change the precipitation distribution and structure

(Ahenkorah et al. 2021b). Most studies that have considered inhibitors have considered them in the context of the chemical constituents or the soil, not the crude enzyme.

Activity clearly effects precipitation efficiency, and this effect should not be ignored. Ahenkorah et al. (2020) performed test tube precipitation tests over varying activity concentrations for equimolar 0.5M chemical constituent concentration and 1.0M chemical constituent concentration, both without non-fat dry milk, the results of which are summarized in Fig. 2-4. Ahenkorah et al. (2021a) performed a wider range of similar tests and found the optimum enzyme activity to solution concentration ratio was 20kU/mol at 25-30°C and an initial pH of 8. The results of this later study are shown in Fig. 2-5.

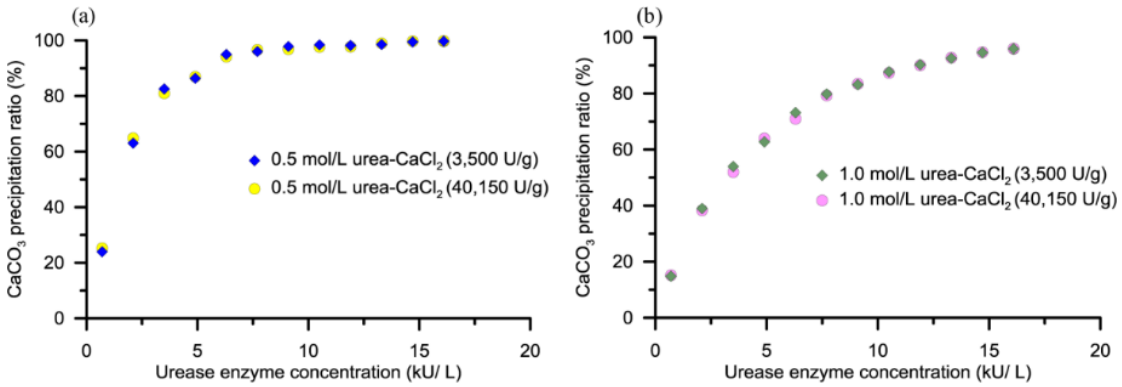

Figure 2-4: Precipitation efficiency vs enzyme concentration for a) 0.5M equimolar chemical constituents omitting non-fat dry milk, b) 1M equimolar chemical constituents omitting non-fat dry milk. (Ahenkorah et al. 2020)

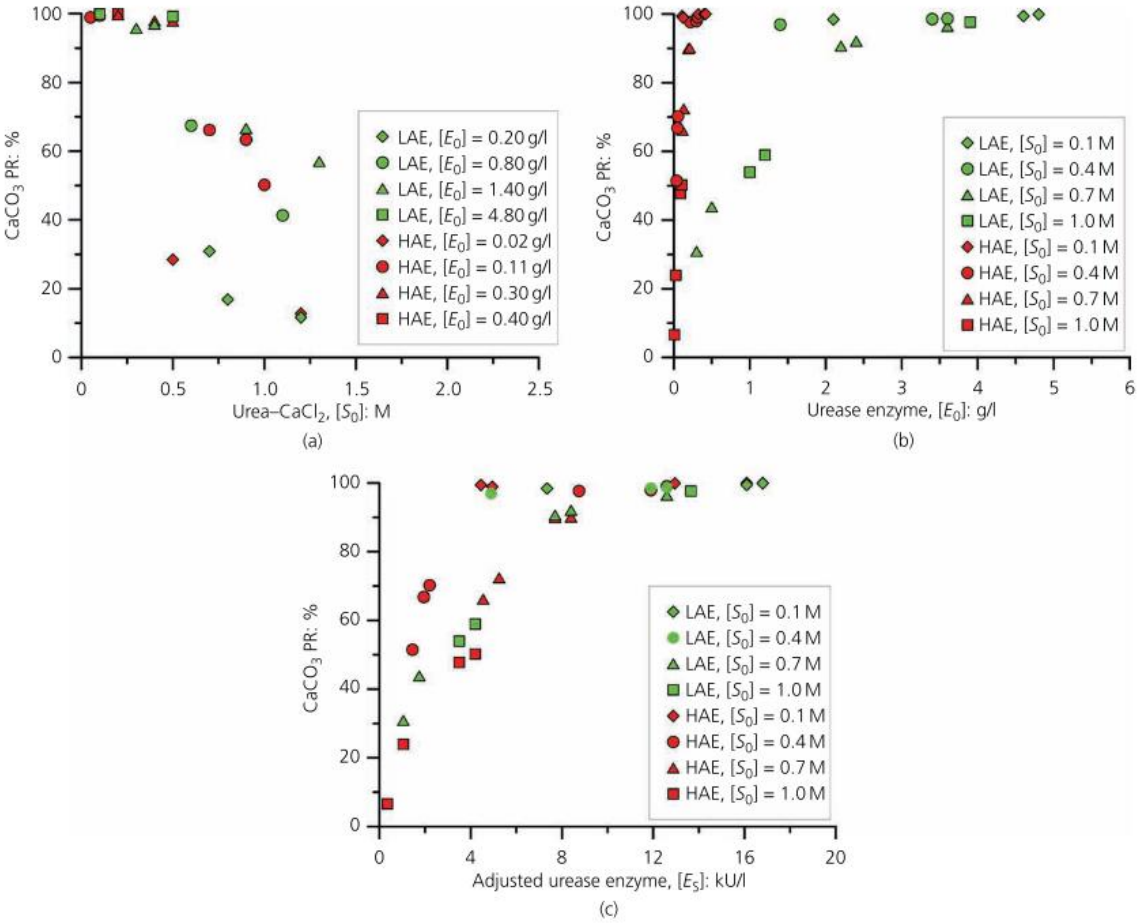

Figure 2-5: Precipitation efficiency vs a) chemical constituent concentration, b) g/L of enzyme, and c) enzyme activity using low activity enzyme (LAE) and high activity enzyme (HAE). (Ahenkorah et al. 2021a)

The relationship between the enzyme activity and chemical constituent concentrations were found to be reasonably approximated using an exponential function by Ahenkorah et al. (2021a). Ahenkorah et al. (2021a) also found that a ratio of enzyme activity to constituent concentration beyond 20kU/mol had little to no effect on the precipitation efficiency in all test cases. However, these investigators cautioned that the reported efficiencies were likely specific to the conditions of their experiments (an

ambient temperature of 25-30°C with an initial pH of 8) and would likely be different at different pH and temperature. The effects of temperature and pH on activity are illustrated in Fig. 2-6. Peak enzyme activity for crude enzymes in these tests occur around 48°C and a pH of 7.5. These values vary based on crude enzyme source (Javadi 2021).

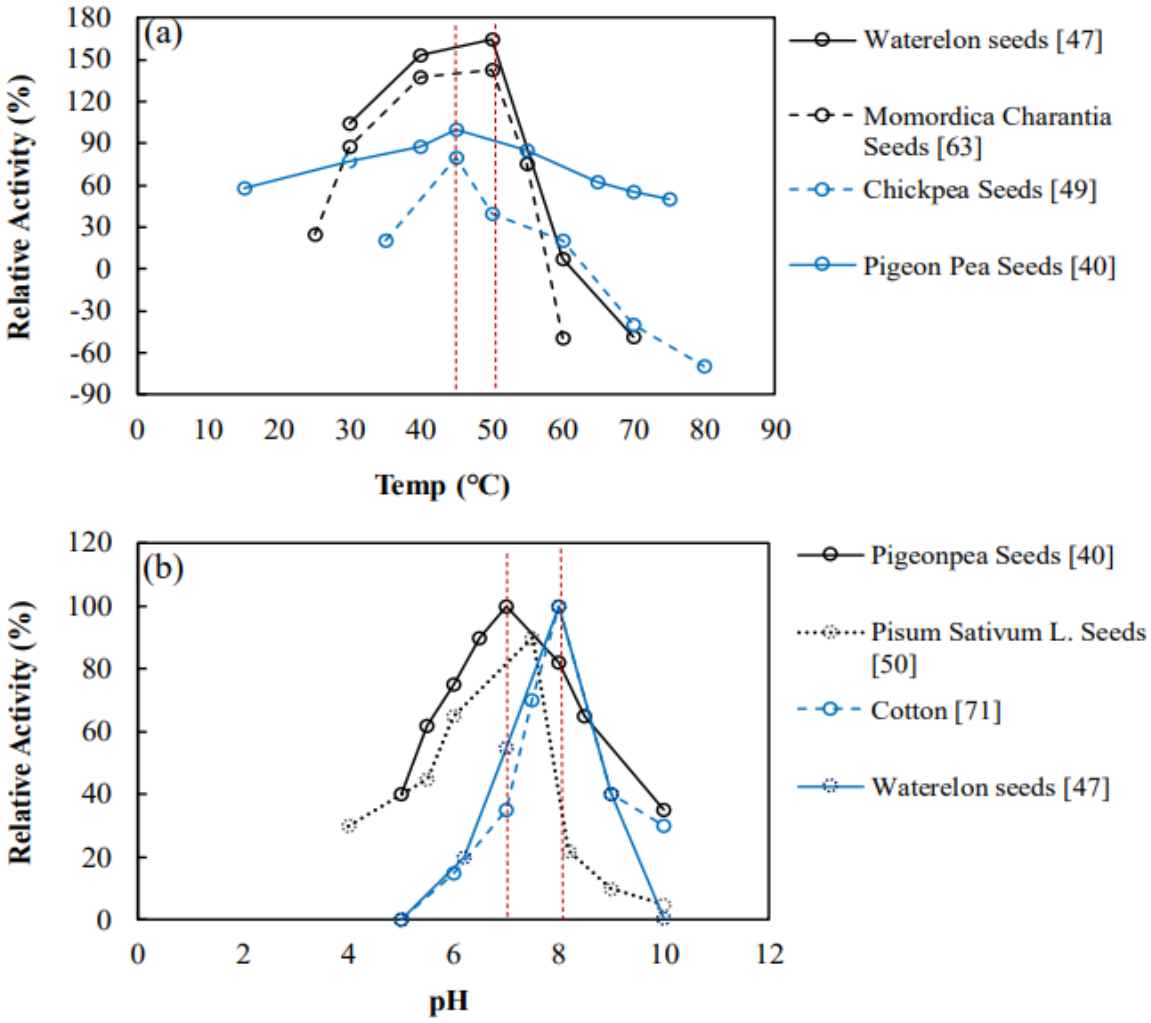

Figure 2-6: Relative urease activity for various crude extracts for different a) temperatures and b) pH values. (Javadi 2021)

There are multiple methods of measuring activity, but one of the most common and accurate is spectrophotometry using Nessler's reagent (Khodadadi Tirkolaei et al. 2020). The initial velocity of the reaction is used to determine urease activity. Fig. 2-7 shows the ammonia calibration curve for spectrophotometry using Nessler's reagent and the activity curve for a commercially available enzyme. The red line displays the initial velocity, the slope of which is the activity. An alternative method gaining traction due to its simplicity is electrical conductivity and pH, but urease enzyme activity is often ignored in studies that employ this method (Ahenkorah et al. 2021a).

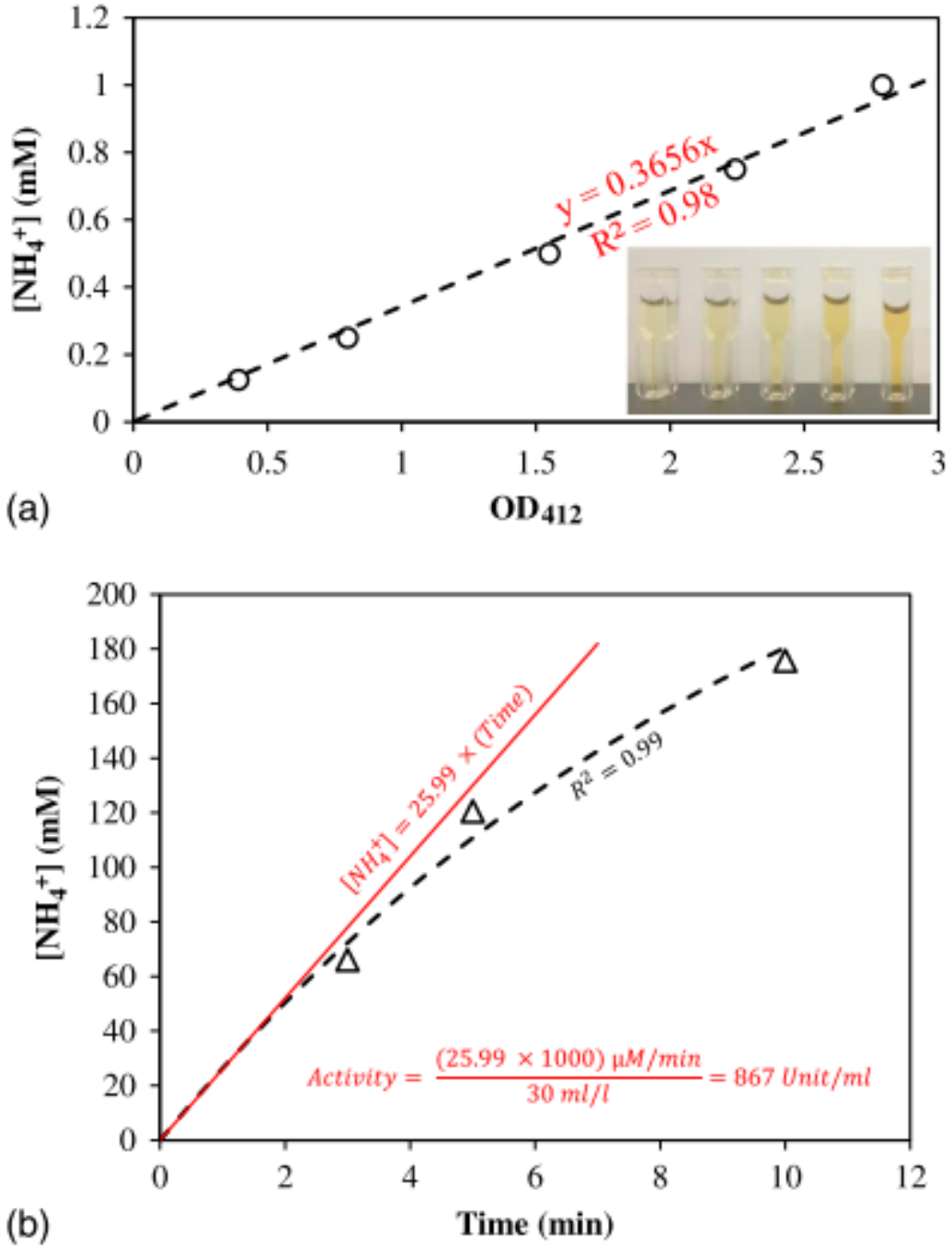

Figure 2-7: Urease enzyme activity graphs using spectrophotometry of a) the calibration curve and b) activity of commercially available enzyme. (Khodadadi Tirkolaei et al. 2020)

The measurement of activity using spectrophotometry is based on a linear relationship between the optical density (OD) measurement and amount of ammonia colored through Nessler's reagent. This relationship is dependent on the amount of Nessler's reagent and the optical distance (OD) used. Using an OD of 412nm and 100µL of Nessler's reagent, Khodadadi Tirkolaei et al. (2020) found a conversion factor of 0.3656 with an R^2 value of 0.98, as illustrated in Figure 2.7a.

Whiffin (2004) developed a correlation factor of 11.1 for the conversion of electrical conductivity measurements to the amount of urea hydrolyzed with a reported R^2 value of 0.998 for a commercially available urease enzyme. This is a different definition of activity than typically used. When using activity measurements as previously defined, the conversion factor becomes 22.2. The correlation factor was not tested for crude urease enzyme. Ahenkorah et al. (2020) provide additional information on using electrical conductivity and pH to measure urease activity. Notably, Ahenkorah et al (2020) made their activity measurement using both calcium chloride and urea as the chemical constituents, rather than just urea (as done in most previous studies). When using both constituents, the electrical conductivity and pH are affected by both the ammonium and calcium carbonate generated by using both constituents. These constituents act against each other with respect to their influence on electrical conductivity and pH. These investigators used both low activity and high activity commercially available purified urease in their study. The results of their experiments are presented in Fig. 2-8.

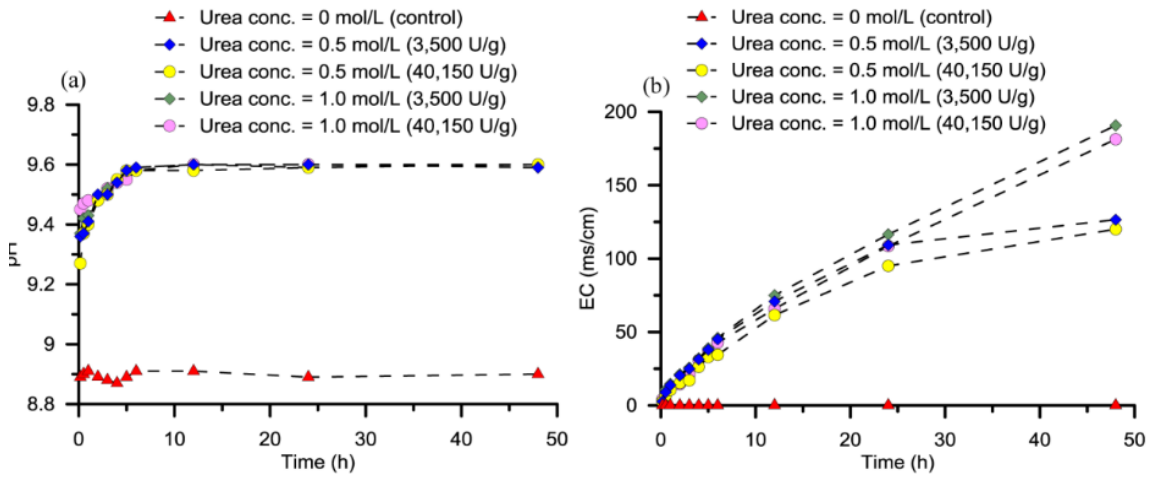

Figure 2-8: Plots of a) pH and b) electrical conductivity versus time. (Ahenkorah et al. 2020)

The work by Ahenkorah et al. (2021a), shown in Fig. 2-8, provides evidence of continuous activity with a gradual decay in electrical conductivity measurements, limited by amount of urea after 24 hours. This work was not compared to other measurements of activity but suggested (based upon the varying amounts of high and low activity enzymes) that electrical conductivity measurements may be effective in showing the presence of the activity qualitatively but not quantitatively.

ENZYME STORAGE

Storage of the enzyme in solution at room temperature has been shown to result in a reduction in enzyme activity when using sword beans (Javadi et al. 2021). However, Javadi et al. (2021) showed that enzyme activity can be maintained for at least one year by using lyophilization (freeze drying), with and without preservatives. Using dextran and sucrose at a concentration of 2.37% by weight, tests by these investigators showed no notable loss in activity over a year of storage. Dehusking sword beans prior to extraction

was also found to increase the longevity of the enzyme without any effect on initial activity. They also found storage at 4°C, whether freeze dried or in solution, greatly improved the activity retention. However, these investigators did not test precipitation efficiency or potential soil interactions. The results from Javadi et al. are summarized in Fig 2-9.

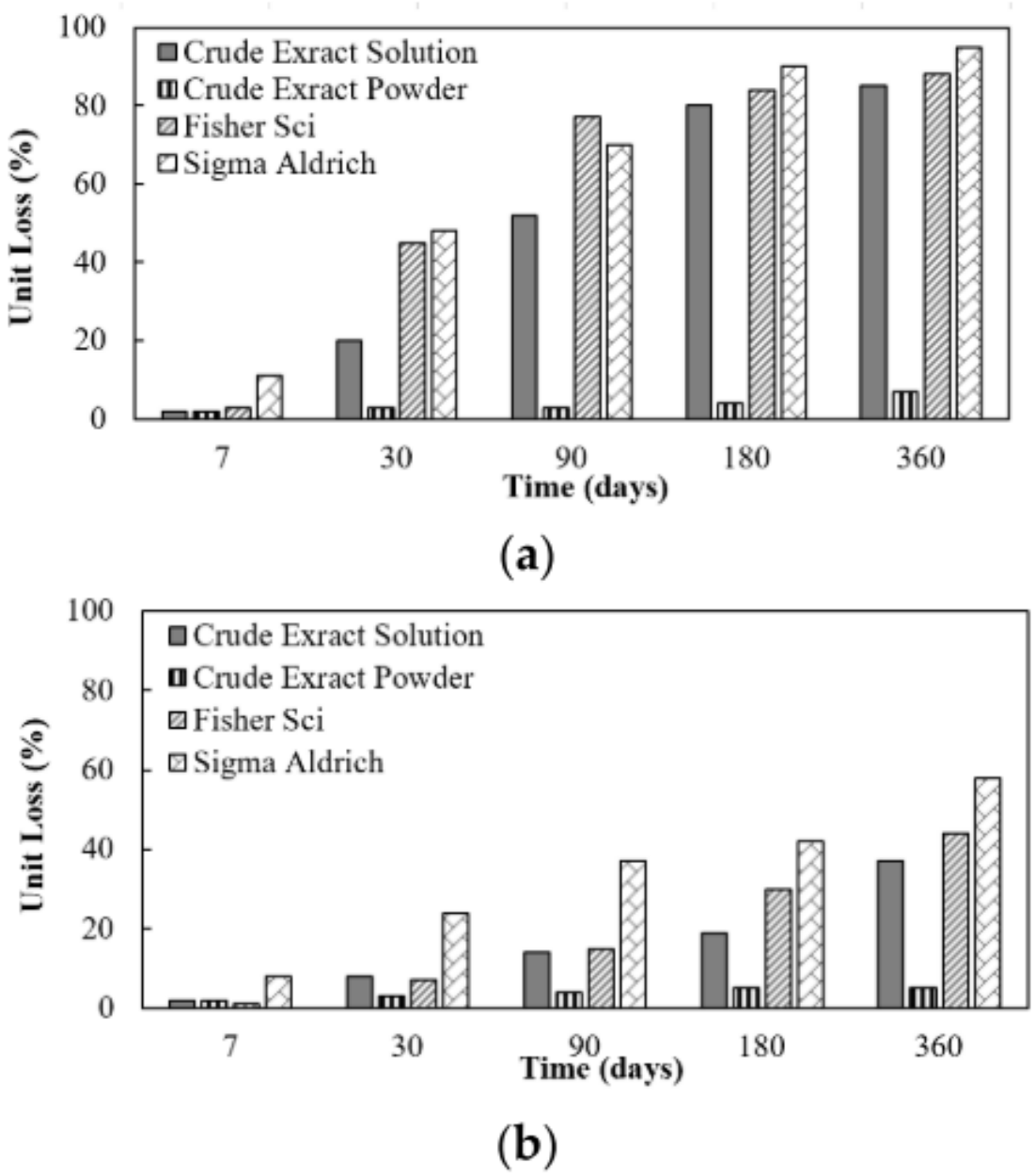

Figure 2-9: Percentage of unit activity loss for various cases at a) room temperature and b) 4 °C Storage. (Javadi et al. 2021)

CHAPTER 3

METHODOLOGIES

The methods used in the experimental work this thesis covers are crude urease extraction methods, test tube precipitation testing, spectrophotometry using Nessler's reagent for activity measurement, and electrical conductivity and pH measurements for evaluation of urease activity.

CRUDE UREASE ENZYME EXTRACTION

A variety of procedures were used to extract urease enzyme from jack beans and sword beans. This section will be organized as outlined in Fig. 3-1.

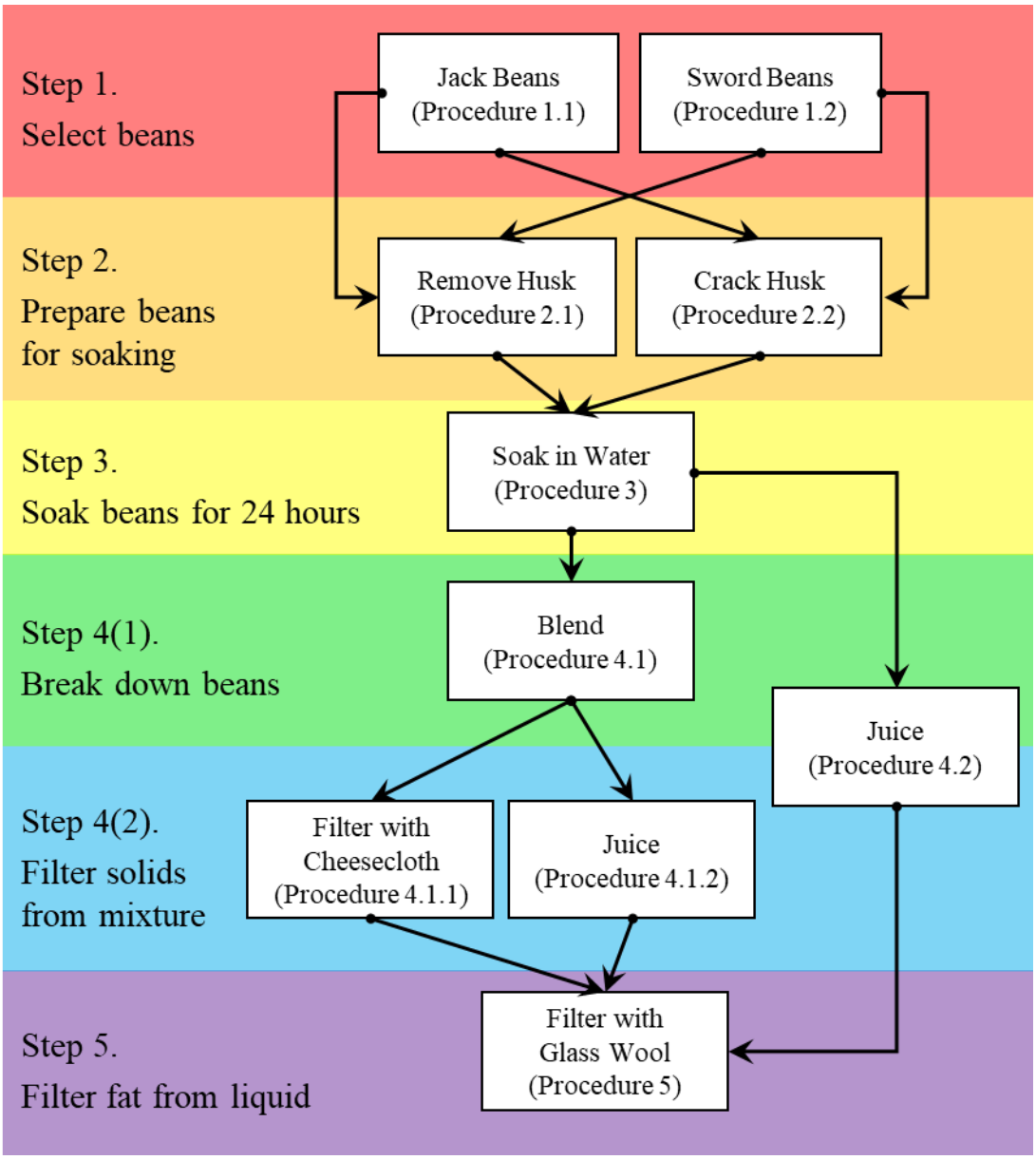

Figure 3-1: Procedural flow chart for crude urease enzyme extraction.

STEP 1: SELECT BEANS

PROCEDURE 1.1 & 1.2: SELECT BEANS

Two types of beans were compared in the experiments reported herein: jack beans (*Canavalia ensiformis*) and sword beans (*Canavalia gladiata*). The same extraction procedures were used on both bean types. A minimum of 25.0 g of the bean flesh (not

including the weight of the husk) was used for each extraction to provide an adequate amount for processing. The maximum amount of dehusked bean used was 26.0 g, with an average of 25.4 g used for extraction.

STEP 2: PREPARE BEANS FOR SOAKING

In order to allow the soaking solution to penetrate the husk of the bean, the husk must be removed, or broken in some way and the weight recorded. Two different techniques were used to accomplish this: husks were either removed or cracked.

PROCEDURE 2.1: REMOVE HUSKS

To remove the husk, the bean was first cracked with pliers. The husk was then peeled from the flesh of the bean. The weight of both the flesh of the beans and the husk of the beans is recorded.

PROCEDURE 2.2: CRACK HUSKS

The husk was cracked using pliers to allow the penetration of water. The total bean weight is recorded. The same beans are used as in Procedure 2.1 (for each bean type, respectively) and the weight of the bean flesh was estimated using the average ratio of bean flesh to total weight found using the tests from Procedure 2.1. The average ratio of bean flesh to total weight was 84% for jack beans with a standard deviation of 3.5% and 77% with a standard deviation of 3.3%. Note, an alternative method that has been used for penetrating the husks prior to soaking (not used in this experimentation) is to place beans in a blender and blend for 2 minutes.

STEP 3: SOAK BEANS

PROCEDURE 3: SOAK IN WATER

The beans (with or without husk) are then placed in deionized water with a volume (in milliliters) equal to 4 times the weight of the flesh of the beans (in grams). Fig. 3-2 shows sword beans and jack beans soaking.

a) b

Figure 3-2: a) Sword beans with cracked husks soaking and b) jack beans with cracked husks soaking, shortly after addition of water.

The mixture is covered with Parafilm to prevent evaporation and contamination and left to soak at 4°C for approximately 24 hours.

STEP 4: BREAK DOWN BEANS & FILTER SOLIDS FROM MIXTURE

This step includes two distinct processes, breaking down the beans to release the enzyme and filtering the solids from the mixture. In Procedure 4.1, this is accomplished through two separate processes, blending with a blender appliance and filtering either manually with cheesecloth or with a juicer appliance. In Procedure 4.2, they are combined into a single step of juicing with a juicer appliance. The liquid produced after each step is measured by volume.

PROCEDURE 4.1: BLEND USING BLENDER APPLIANCE

The beans and water were emptied into the pitcher of the household blender pictured in Fig. 3-3. The lid is put on the blender and the beans are blended for a minimum of 2 minutes, or until the mixture has no large chunks (>2-4mm). The mixture is not blended for more than 5 minutes. This prevents the breakdown of urease enzyme proteins. The breakdown of the urease enzyme proteins can be indicated by a change in the texture of the mixture, to a smoother texture. Each of the extractions were checked for the presence of any indication of urease enzyme protein breakdown.

Figure 3-3: Blender used for crude urease enzyme extraction.

PROCEDURE 4.1.1: FILTER USING CHEESECLOTH

A funnel is lined with 2 pieces of cheesecloth cut into squares and placed on top of each other at an approximate angle of 45°, forming an 8-pointed star and placed atop a glass beaker. The blended mixture is poured into the funnel, being careful not to overfill. The points of the cheesecloth lining are gathered, and the mixture is squeezed using a

twisting motion until liquid production ceases. The cheesecloth and solids are disposed of.

PROCEDURE 4.1.2 & 4.2: JUICE USING JUICER APPLIANCE

The blended mixture (for Procedure 4.1.2) or beans and water (for Procedure 4.2) was emptied from the blender into a household juicer appliance (pictured in Fig. 3-4) and juiced according to manufacturer's direction into a glass beaker. The solids are separated by the juicer into the designated compartment. They are then disposed of.

a) b)

Figure 3-4: Juicer appliance pictured a) with cover and b) with cover removed.

While the method of blending using a blender and filtering solids with cheesecloth is a method used in literature (Javadi et al. 2021; Khodadadi Tirkolaei et al. 2020; Martin et al. 2021), extraction using a juicing appliance have not. Juicing could potentially lower the material usage and time needed for crude urease enzyme extraction but could present differences in the crude urease enzyme due to the differences in how juicers break down plant tissues and filter solids.

STEP 5: FILTER FAT FROM LIQUID

PROCEDURE 5: FILTER USING GLASS WOOL

A funnel is lined thoroughly with glass wool fiber. The filtered liquid is poured through the glass wool, separating the fats from the liquid, and crude urease enzyme is collected in a closed container and stored at 4°C.

UREASE ENZYME ACTIVITY MEASUREMENT

ELECTRICAL CONDUCTIVITY & pH

Twenty-seven milliliters (27 mL) of 1.1 M urea was placed in a small (40mL) glass beaker. A cover of Parafilm, reinforced with duct tape, with holes for benchtop sensor probes was placed atop the beaker to minimize ammonia off-gassing. The probes were then placed in the beaker such that they were submerged in the solution. The set-up is pictured in Fig. 3-5. Probe readings were allowed to stabilize for 60-120 seconds (based on continuous readings). After lifting the cover slightly, 3 mL of crude urease enzyme (at 4 °C) was added to beaker and cover closed. The beaker was then swirled to mix the solution. The mixture was then left undisturbed for 10 minutes with the electrical conductivity and pH probes recording measurements taken every 10 seconds.

a) b)

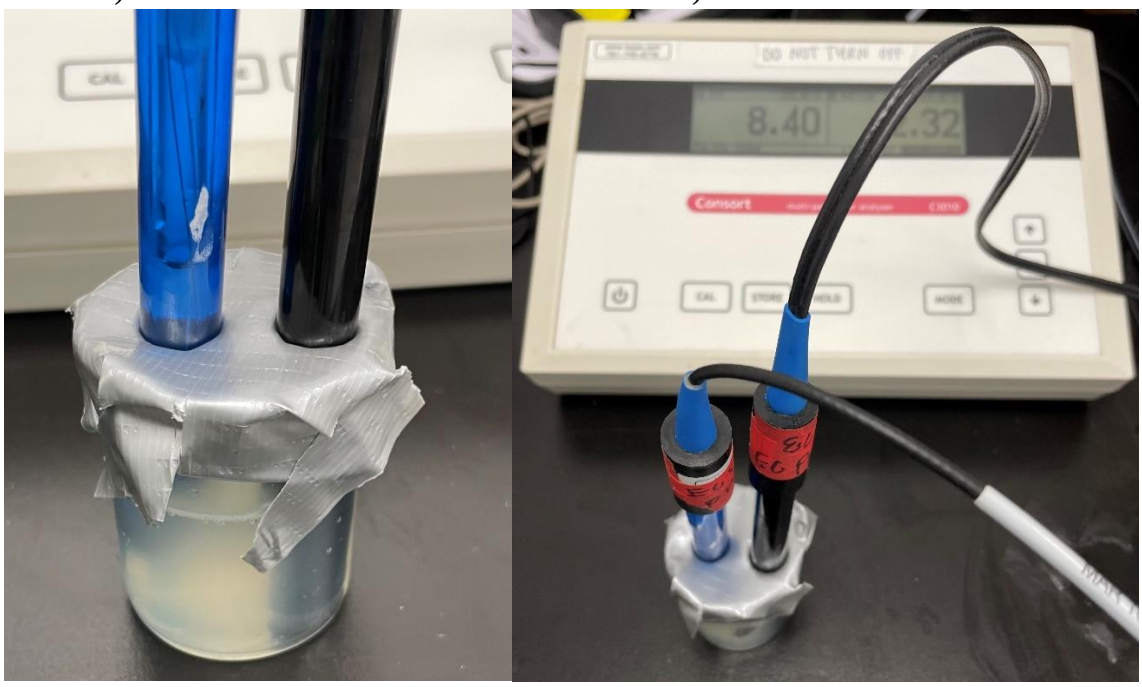

Figure 3-5: Images of electrical conductivity set-up, a) presents a close-up view while b) presents a wider view.

SPECTROPHOTOMETRY USING NESSLER'S REAGENT

Fifteen milliliters (15 mL) of 300 mM urea and 15 mL of 15% trichloroacetic acid solutions were prepared. Three 15 mL glass serum bottles were prepared with 4.7mL of deionized water and 5mL of prepared urea in each. Three 100 mL volumetric flasks were prepared with 99 mL of deionized water in each. Three syringes were prepared with ½ inch 14 G needles and filled with 5 mL of the 15% trichloroacetic acid.

Thirty microliters (30 µL) of the crude urease enzyme were added to one of the 15 mL serum bottles and a timer set to either 3, 5, or 10 minutes was started. The serum bottle was quickly capped using a rubber cap and sealed using an aluminum seal with the aluminum seal crimper. The sample is set aside until 5-10 seconds before timers'

completion. At the point the timer had 5-10 seconds left, one of the syringes containing trichloroacetic acid was inserted into the rubber cap of the 15 mL serum bottle with the sample, but not yet dispensed.

At the time the timer reaches completion, the syringe of trichloroacetic acid was dispensed into the sample, while gently shaking to mix and thoroughly halt hydrolysis. As the bottle was sealed, the air inside increased in pressure and the plunger of the syringe was allowed to extend, allowing fluid into the syringe. The syringe was removed from the serum bottle and set aside. The aluminum seal was removed from the serum bottle using the seal crimper, and the rubber cap removed. The excess mixture in the syringe was reinserted to the serum bottle mixture and the syringe disposed of. One milliliter (1 mL) of the mixture in the serum bottle was added to one of the prepared 100 mL volumetric flasks. The flask was covered using a piece of Parafilm and shaken to mix.

The process from adding the crude urease enzyme to the 15 mL serum bottle was repeated two more times with different timers, for a total of three times for each crude urease enzyme sample. The timers were set to 3, 5, and 10 minutes.

One microliter (1µL) of Nessler's reagent was added to each of 4 disposable cuvettes. Two milliliters (2mL) of deionized water were added to one of the cuvettes. This was the calibration cuvette. Two milliliters (2 mL) of each of the diluted samples in the volumetric flasks were added to the other 3 cuvettes. Using a spectrophotometer, the optical density at a wavelength of 412 nm (OD_{412}) was measured for each of the sample cuvettes after calibration with the calibration cuvette.

TEST TUBE PRECIPITATION TEST

Six centrifuge tubes were labeled and weighed (for tests done in triplicate). The amount of solution for each precipitation test for each crude urease enzyme was limited by the amount of crude urease enzyme produced. The majority of the samples were prepared using 50 mL total EICP solution for each test tube. However, the experiments for crude urease enzyme extracted from sword beans (with and without husk) via blending followed by juicing were prepared with 25 mL of total solution in each test tube, respectively. The test tube precipitation experiments for crude urease enzyme extracted from jack beans without husk extracted via blending followed by juicing were prepared using 30 mL in each tube, the experiments for crude urease enzyme from jack beans with husk extracted via blending followed by juicing with 10 mL in each tube, and the experiments for crude urease enzyme from jack beans with husk extracted via juicing without blending were prepared with 20 mL total EICP solution in each tube. Each of the solutions were prepared using deionized water.

For the experiments using 50 mL of solution in each tube, 100 mL with a concentration of 3 M calcium chloride dihydrate and 3 M urea was prepared. This is part of the solution for the high concentration precipitation tests, concentrations determined through preliminary testing to maximize amount of precipitate. Another 100 mL solution was prepared with concentrations of 1.5 M urea and 1 M calcium chloride. This is part of the solution for the standard concentration precipitation tests, concentrations as determined by Almajed et al. (2019) to maximize the ratio of precipitate to crude urease enzyme used. Fifty (50 mL) of a solution with a concentration of 4 g/L non-fat dry milk

and an estimated 33,750 U/L (based upon measurements taken via spectrophotometry with Nessler's reagent) of crude urease enzyme was prepared. This is the other part of the solution for the high yield precipitation tests. Another 50 mL solution with a concentration of 4 g/L non-fat dry milk and an estimated 10,500 U/L (based upon measurements taken via spectrophotometry with Nessler's reagent) was prepared. This is the other part of the solution for the standard yield precipitation tests.

For the experiments using 50 mL of EICP solution in each tube, 33 mL of the high precipitation yield, or standard precipitation yield urea-calcium chloride solution was combined with 17 mL of the non-fat dry milk-urease high precipitation yield or standard precipitation yield solution, respectively. This was repeated for each of the six test tubes (three with high yield precipitation solutions and three with standard yield). The tests using less than 50 mL of total solution in each tubes scaled back volumetric amounts according to the amount of solution used in each tube.

Each tube was shaken vigorously for several seconds after combining solutions. Fig. 3-6 shows examples of solutions after shaking and after precipitation occurs.

a) b)

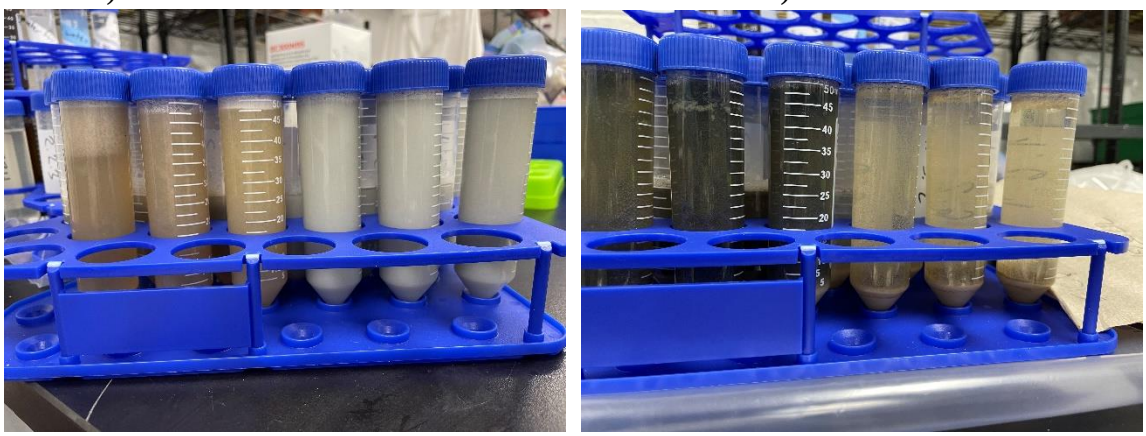

Figure 3-6: Precipitation experiments at high (2M calcium chloride-urea) and standard (0.67M calcium chloride, 1M urea) chemical constituent concentrations a) immediately after mixing and b) 72 hours after mixing.

The filled centrifuge tubes were left at room temperature (around 24 °C) for 72 hours to allow for complete precipitation. After the 72 hours elapsed, each tube was shaken vigorously for several seconds to release any precipitate stuck to the sides of the tube before centrifuging for five minutes at 5000 RPM. The tubes were carefully removed and uncapped. The supernatant fluid was carefully poured out of each tube individually, taking care not to disturb the precipitate.

Each tube was then filled with deionized water and mixed at high-speed using a benchtop vortex mixer interspersed with vigorous shaking, until no precipitate remained stuck to the bottom of the tube. The tubes were capped, and centrifugation was repeated for five minutes at 5000 RPM. The tubes were carefully removed from the centrifuge and uncapped. The water was carefully drained from each tube, taking care not to disturb the precipitate.

The caps were set aside, and the tubes placed in the oven at 33-38°C for 24 hours. After 24 hours, the tubes were removed from the oven and allowed to cool. The caps were placed on the matching sample and the samples were weighed. Fig. 3-7 displays a tube after the process is complete.

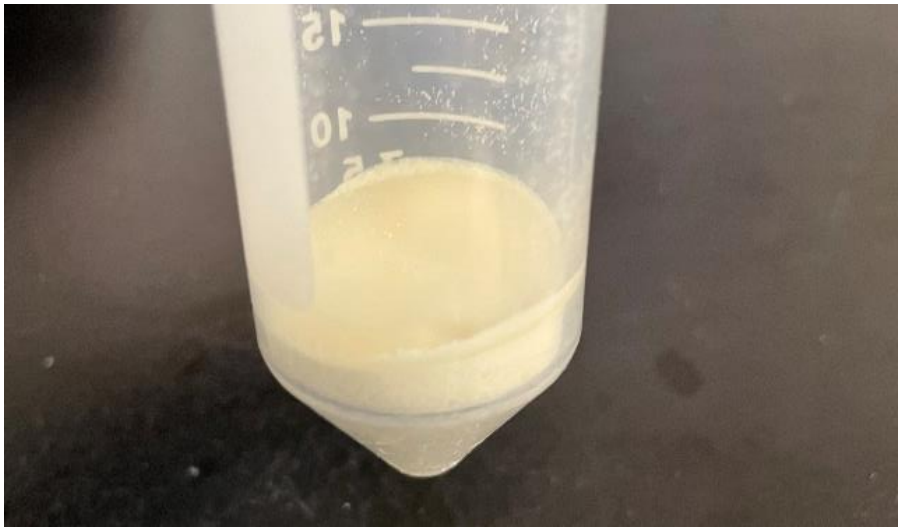

Figure 3-7: Precipitate in a centrifuge tube.

CHAPTER 4

ACTIVITY & PRECIPITATION EFFICIENCY

INTRODUCTION

This chapter examines the effects of the different extraction methodologies used in this work on urease enzyme activity and precipitation efficiency. Activity was measured using spectrophotometry with Nessler's reagent. Precipitation efficiency included non-fat dry milk at a rate of 4.0 g/L and was evaluated at two different concentrations of chemical constituents and enzyme activity. The extractions performed for this experiment were done for the variables outlined in Chapter 3. Methodologies. These variables included: source (jack beans or sword beans); presence or omittance of husks; blending before filtration using a blender or skipping the blending step; and filtration using cheesecloth or a juicer appliance. Each grouping of variables was soaked in deionized water at a ratio of 4 mL water per 1g bean flesh for 24 hours and filtered through glass wool to separate fats from the crude urease solution, regardless of source, husk presence or omittance, or extraction method.

The concentrations for the first solution tested in this research were based upon the solution used by Almajed et al. (2019) and Martin et al. (2021). This solution had a urea concentration of 1M and a calcium chloride concentration of 0.67 M. The crude urease enzyme concentration was roughly 10,500 U per liter of solution. This solution will be referred to as the standard yield solution.

The concentrations for the second solution tested in this research, referred to herein as the high concentration solution, were based upon the preliminary testing

presented in Fig. 4-1. In the tests presented in Fig. 4-1, equimolar concentrations of urea and calcium chloride were used. Fig. 4-1a shows the influence of urease enzyme activity on the precipitation efficiency for a concentration of 1.5 M and Fig. 4-1b shows the effect of chemical concentration on precipitation ratio for a urease activity of 33,750 U/L. One test tube test was performed for each point on Fig. 4-1 (which may have not adequately addressed increased variance at higher levels). The crude urease enzyme used in the tests was from dehusked sword beans using the method hereon referred to as the common extraction method. The common method uses a blender to break down the beans and cheesecloth to filter out the solids. As with all the methods of extraction, the beans were soaked before processing and the solution was filtered with glass wool. The activity of the crude urease enzyme used was not measured but assumed to be 270 U per mL of the crude urease enzyme, based on past results.

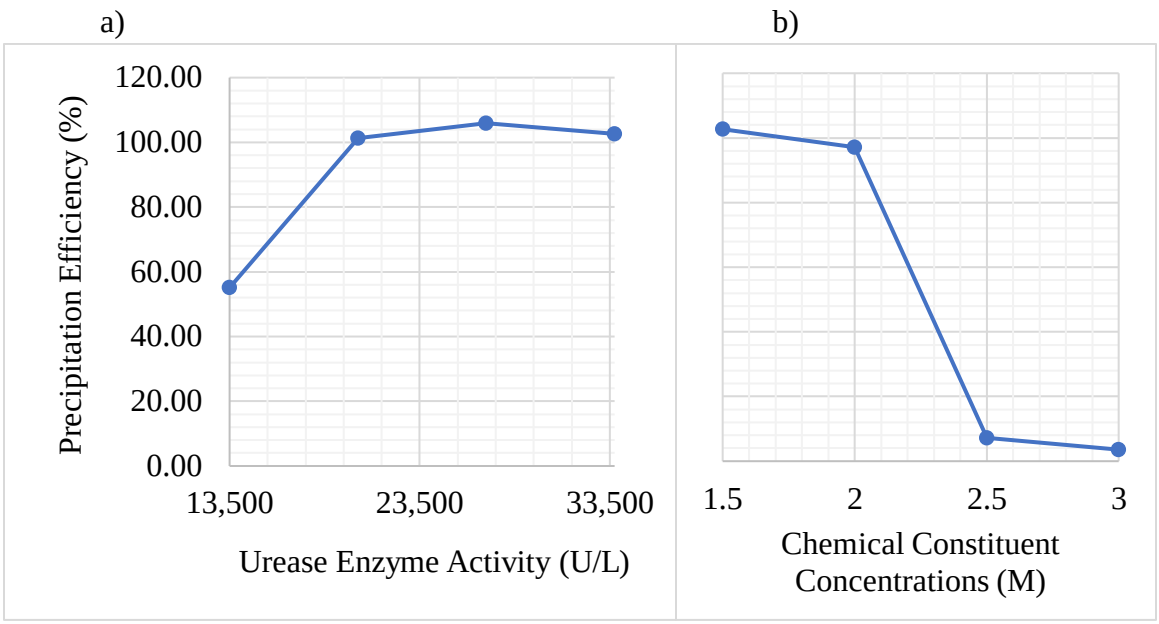

Figure 4-1: Precipitation efficiency for a range of a) urease activity concentrations using an equimolar urea-calcium chloride concentration of 1.5 M and b) equimolar chemical constituent concentrations using an estimated 33,750 U/L concentration of crude urease enzyme.

Based upon the precipitous drop in precipitation efficiency from the 2 M solution to the 2.5 M shown in Fig. 4-1b, an equimolar 2 M calcium chloride-urea solution was selected, with a urease enzyme activity of roughly 33,750 U per liter of solution. The difference in precipitation efficiency between the 1.5 M solution and the 2 M solution was within a reasonable margin of error.

As the activity of the enzymes was initially approximated, the activity concentrations used for the precipitation efficiency tests varied. The actual activity concentrations used for each of the precipitation efficiency tests and each crude urease source, husk option, and extraction method are shown in Table 4-1.

Table 4-1: Activity concentrations for precipitation efficiency tests.

		Activity Concentration (U/L)			
		Sword Beans		Jack Beans	
		Dehusked	With Husk	Dehusked	With Husk
Standard	Blender, Cheesecloth	16,895	11,919	11,701	11,486
	Blender, Juicer	11,553	13,075	12,230	12,258
	Juicer	12,593	12,243	12,698	11,712
High	Blender, Cheesecloth	54,305	38,312	37,611	36,920
	Blender, Juicer	37,136	42,026	39,311	39,402
	Juicer	40,477	39,351	40,816	37,645

RESULTS

BEAN COMPOSITION AND WATER ABSORPTION

Sword beans (*Canavalia gladiata*) and jack beans (*Canavalia ensiformis*) are pictured in Fig. 4-2 and are from the same family of beans. They have the same carrier protein form of urease enzyme. They both are sometimes called jack bean: the term has been used interchangeably in literature in relation to EICP.

Figure 4-2: Sword beans pictured right, jack beans pictured left, beans with husks pictured above dehusked beans.

Despite this familial relationship, jack beans and sword beans are distinct species of beans with clear differences in appearance, size, and texture. Jack beans are smaller (approx. 1.5 cm in length), have thin (under 0.5 mm), low plasticity (breaks with bending), white husks. Sword beans are larger (approx. 2.5cm in length), have thicker (approx. 1mm), higher plasticity (bends approx. 45° before breaking), maroon husks. The

average husk weight is shown in Table 4-2 and was found to be 16% of the total weight for jack beans, and 23% of the total weight for sword beans.

Table 4-2: Averages and standard deviation for each bean component for sword beans and jack beans as a percentage of total weight of the beans.

		Percentage of Total Weight	
		Sword Beans	Jack Beans
Flesh	Average	77.1%	83.7%
	Standard Deviation	0.33%	
Husk	Average	22.9%	16.3%
	Standard Deviation	0.35%	

The volume of water absorbed by each bean upon soaking differed. The dehusked jack beans absorbed 32% of the total volume of water using the 4 mL deionized water to 1 g bean flesh ratio, while the dehusked sword beans absorbed 24% of the total volume of water. The jack bean husks (assuming same average absorption for the bean flesh as in dehusked scenario) absorbed 3.0 mL of water per 1 g husk, while sword beans (under the same assumption) absorbed 2 mL of water per 1 g husk. This is shown in terms of the normalized volume of water absorbed from 100 mL of soaking water in Table 4-3 and by percentage of soaking water absorbed by volume in Fig. 4-3.

Table 4-3: Averages and standard deviation for water absorbed by bean components.

		Water Absorbed (mL / 100mL)	
		Sword Beans	Jack Beans
Flesh	Average	24	32
	Standard Deviation	1.4	1.3
Husk	Average	15	15
	Standard Deviation	1.2	3.8

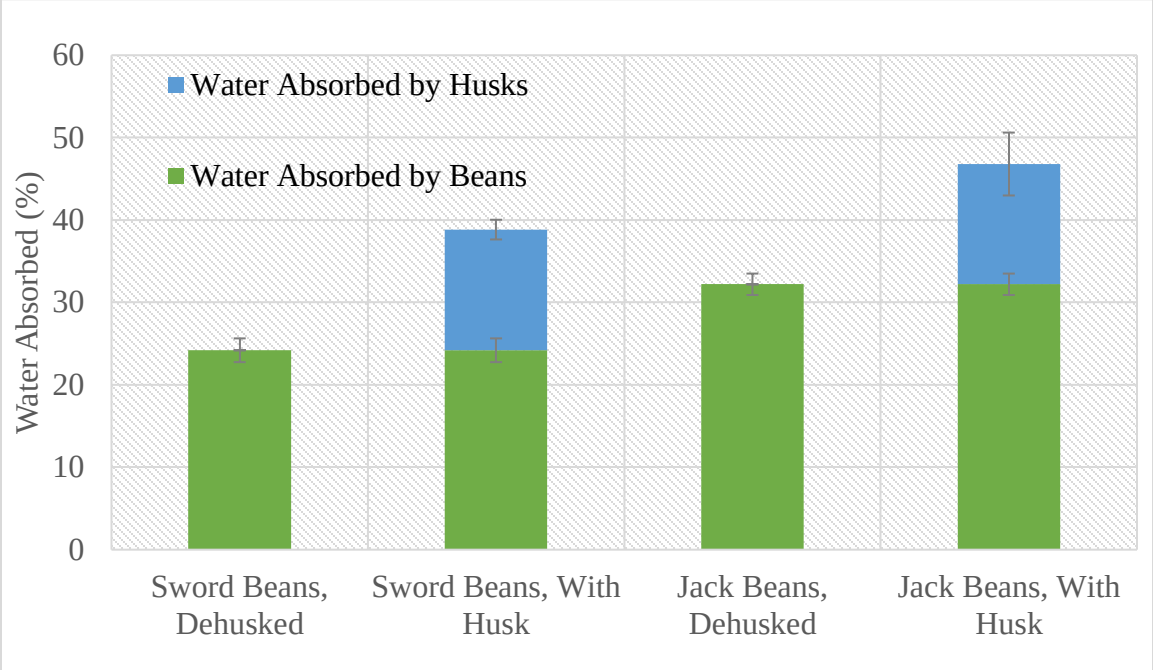

Figure 4-3: Water absorbed in percentage of soaking water volume by beans and husks, respectively after 24 hours of soaking.

CRUDE UREASE ENZYME YIELD AND ACTIVITY

The crude urease enzyme yield for each case is presented in Table 4-4 and Fig. 4-4 (using the procedure described in Chapter 3. Methodologies). Results are based upon one

sample in each category. The commonly used extraction method (blending, followed by cheesecloth) resulted in the highest crude enzyme yield (43-63 mL per 25 g bean flesh) in all cases except for the crude urease enzyme extraction from beans with husks. Blending combined with juicing provided the lowest yield (12-38 mL per 25 g bean flesh) in each case. These low yield values correspond to a decrease in yield of 25-37mL per 25 g of bean flesh and a percentage decrease of 40-73% in each case when compared with the commonly used extraction method.

The yields from juicing alone ranged from a 36% decrease to a 6% increase when compared to the commonly used extraction method. Using the common method of extraction, crude urease enzyme from jack beans with husks had a yield decrease by 32% when compared to crude urease enzyme from jack beans without husk. Crude urease enzyme from sword beans with husks experienced a 19% decrease when compared to the yield from crude urease enzyme without husks.

Table 4-4: Equivalent crude urease enzyme yield.

	Crude Enzyme Yield (mL) / 25g Bean Flesh			
	Sword Beans		Jack Beans	
	Dehusked	With Husk	Dehusked	With Husk
Blender, Cheesecloth	55	45	63	43
Blender, Juicer	18	18	38	12
Juicer	35	47	51	35

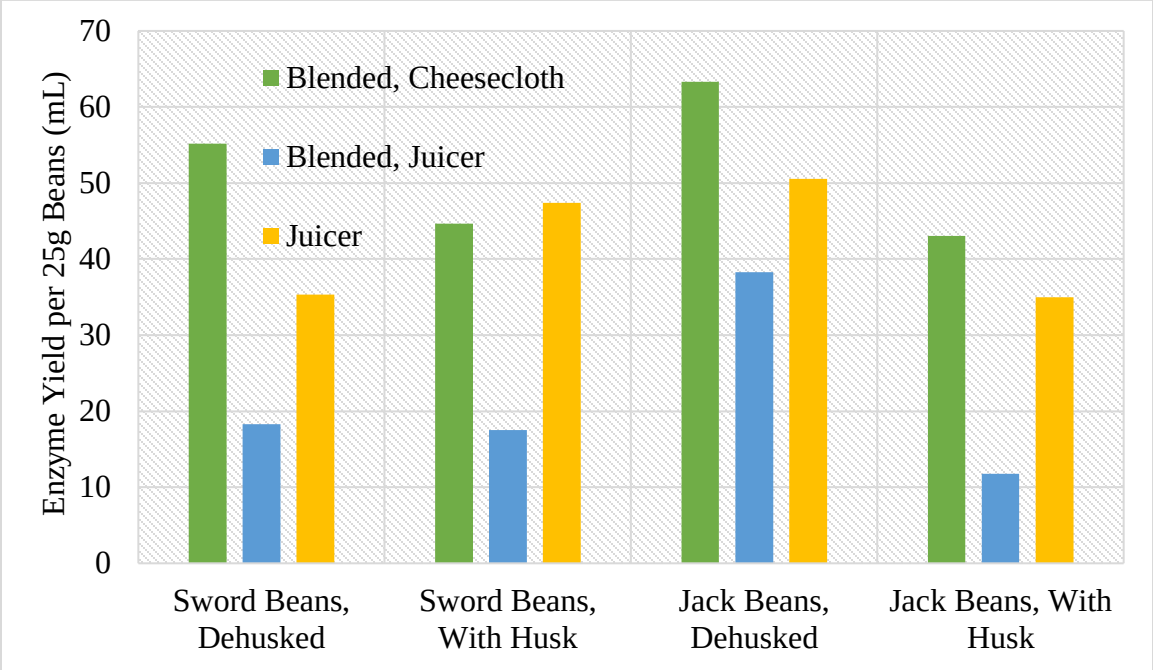

Figure 4-4: Equivalent crude urease enzyme yield in mL for 25 g of bean flesh.

Table 4-5 and Fig. 4-5 display the activity as measured using spectrophotometry with Nessler's reagent for each crude urease enzyme extraction case in units per milliliter of crude urease enzyme. Results are reliant on one sample in each category. Crude urease enzyme from dehusked jack beans using the common method had an activity 43% lower than the activity of crude urease enzyme from dehusked sword beans using the common method of extraction.

Table 4-5: Activity of crude urease enzymes.

	Activity (U) / Crude Urease Enzyme (mL)			
	Sword Beans		Jack Beans	
	Dehusked	With Husk	Dehusked	With Husk
Blender, Cheesecloth	400	367	226	322
Blender, Juicer	266	413	315	317
Juicer	386	221	144	120

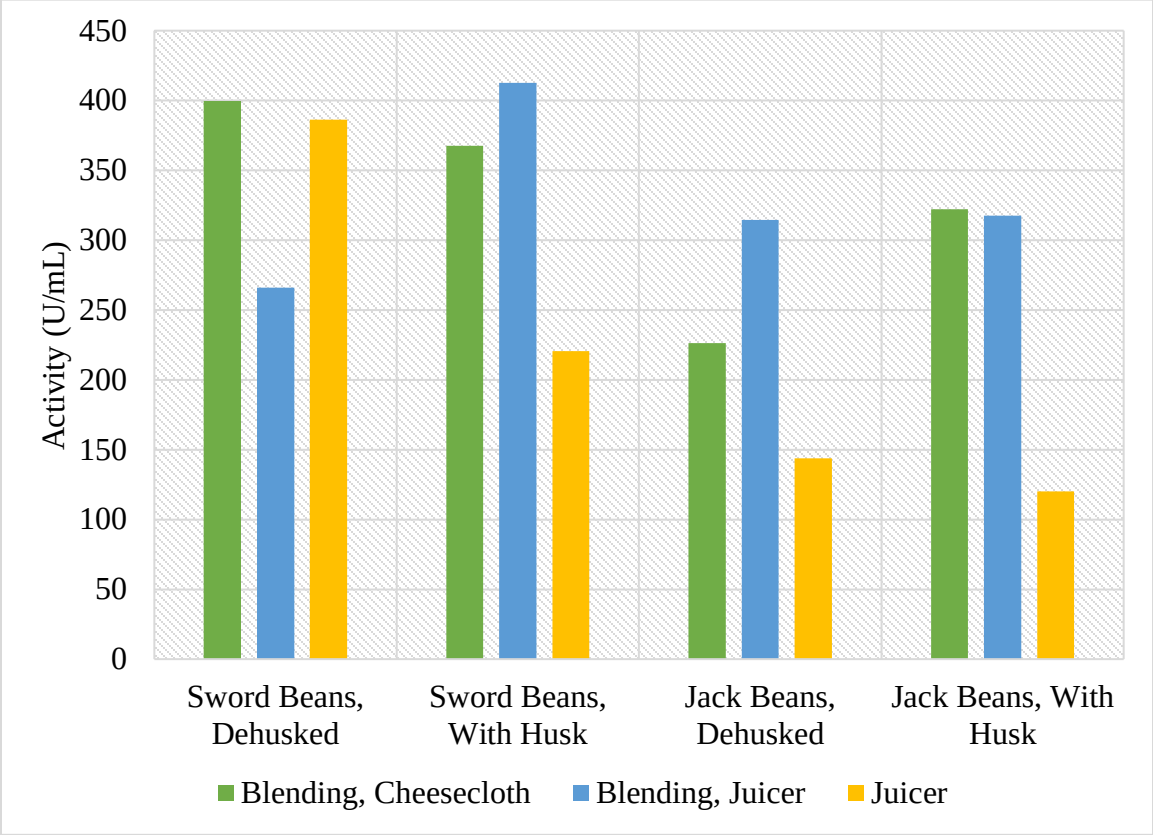

Figure 4-5: Activity of crude urease enzyme via spectrophotometry using Nessler's reagent.

When compared to the crude urease enzyme from dehusked jack beans and sword beans extracted using the common method, the crude urease enzyme from the jack beans

with husk extracted using the common method experienced a 42% increase in activity, the crude urease enzyme extracted from sword beans with husks saw no significant change in activity. Extraction of crude urease enzyme via juicing alone results in an average 34% decrease in crude urease enzyme activity when compared to the other extraction methods involving blending.

Table 4-6 and Fig. 4-6 display the products of the data presented in Fig. 4-4 and Fig. 4-5 (the yield in mL of crude enzyme per 25 g of bean flesh and activity per mL of crude enzyme, respectively) in terms of the total urease enzyme activity yield per 25g of bean flesh. Crude urease enzyme extracted using methods involving the juicer (alone and as an alternative to cheesecloth) provided varying total activity yields. Crude urease enzyme extracted with the juicer alone (without blending, each extraction method included soaking and filtration with glass wool) averaged a 28% higher total activity yield when compared to the crude urease enzyme extracted with blending and juicing. When compared to the common extraction method, there is 47% reduction in the total activity yield for extraction with the juicer alone, and a 58% reduction for extraction with the juicer and blending.

The crude urease enzyme from beans with husk extracted using the common method yielded 26% lower total activity for sword beans and had a 3% increase for jack beans in respects to the respective crude urease enzymes from beans without husks.

Table 4-6: Total activity of crude urease enzymes.

	Total Activity (U) / 25g Bean Flesh			
	Sword Beans		Jack Beans	
	Dehusked	With Husk	Dehusked	With Husk
Blender, Cheesecloth	22,042	16,414	14,315	13,854
Blender, Juicer	4,862	7,220	12,039	3,740
Juicer	13,649	10,466	7,264	4,210

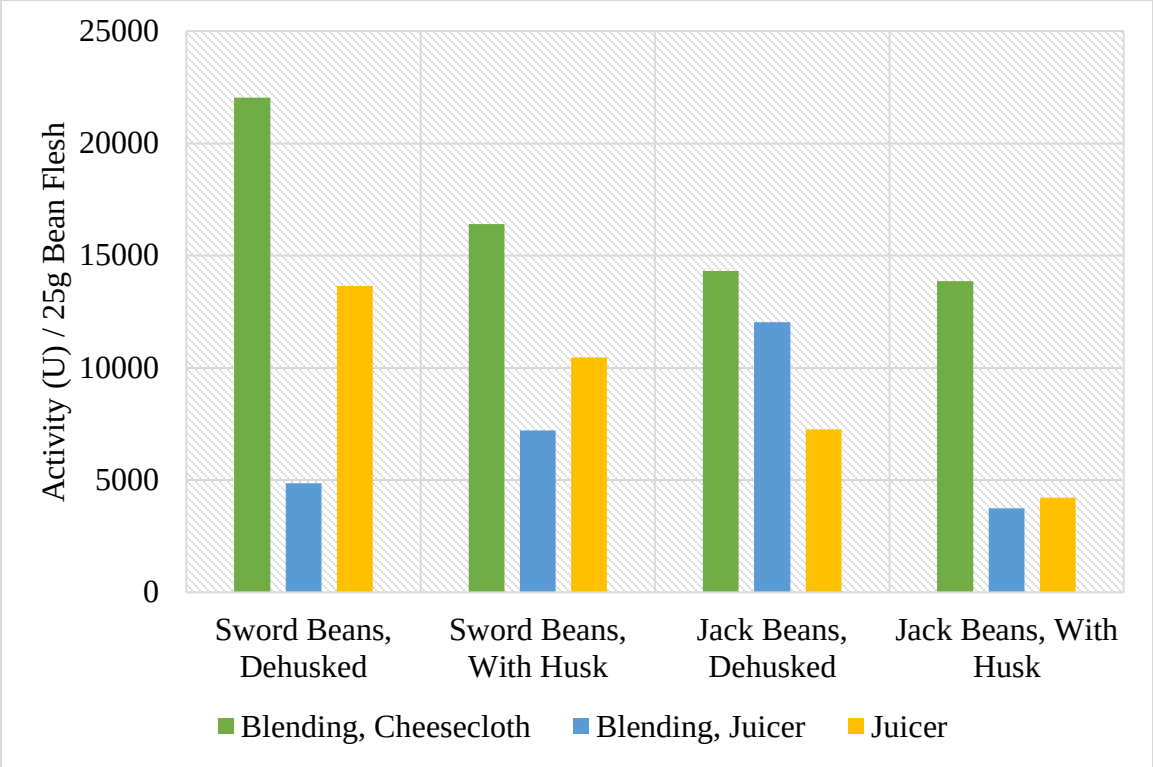

Figure 4-6: Equalized total activity of crude urease enzyme via spectrophotometry using Nessler's reagent per 25 g bean flesh.

PRECIPITATION EFFICIENCY

Table 4-7 and Fig. 4-7 shows the results of the test tube precipitation efficiency tests (the precipitation efficiency is the experimental precipitation yields over the maximum theoretical yields) for the standard concentration, 0.67 M calcium chloride, 1

M urea solution. Tests were done in triplicate. The results had no correlation with the variation in activity concentration for the solution as presented in Table 4-1, and were internally consistent within each sample triplicate group, with an average standard deviation of 5%. Therefore, a correction for total activity was deemed unnecessary. There were two samples with significantly higher precipitate yields than the maximum possible yield in this set of testing. If these data are omitted, the results are consistent within an average 2% internal standard deviation.

Table 4-7: Precipitation efficiency averages and standard deviations for standard yield solutions of 0.67 M calcium chloride, 1 M urea solution.

		Standard Yield Solution Precipitation Efficiency (%)			
		Sword Beans		Jack Beans	
		Dehusked	With Husk	Dehusked	With Husk
Blender, Cheesecloth	Average	103%	92%	105%	101%
	Standard Deviation	2.1%	2.5%	0.4%	0.6%
Blender, Juicer	Average	91%	101%	104%	102%
	Standard Deviation	5.7%	0.5%	1.0%	4.0%
Juicer	Average	98%	99%	106%	95%
	Standard Deviation	3.6%	7.1%	1.1%	1.4%

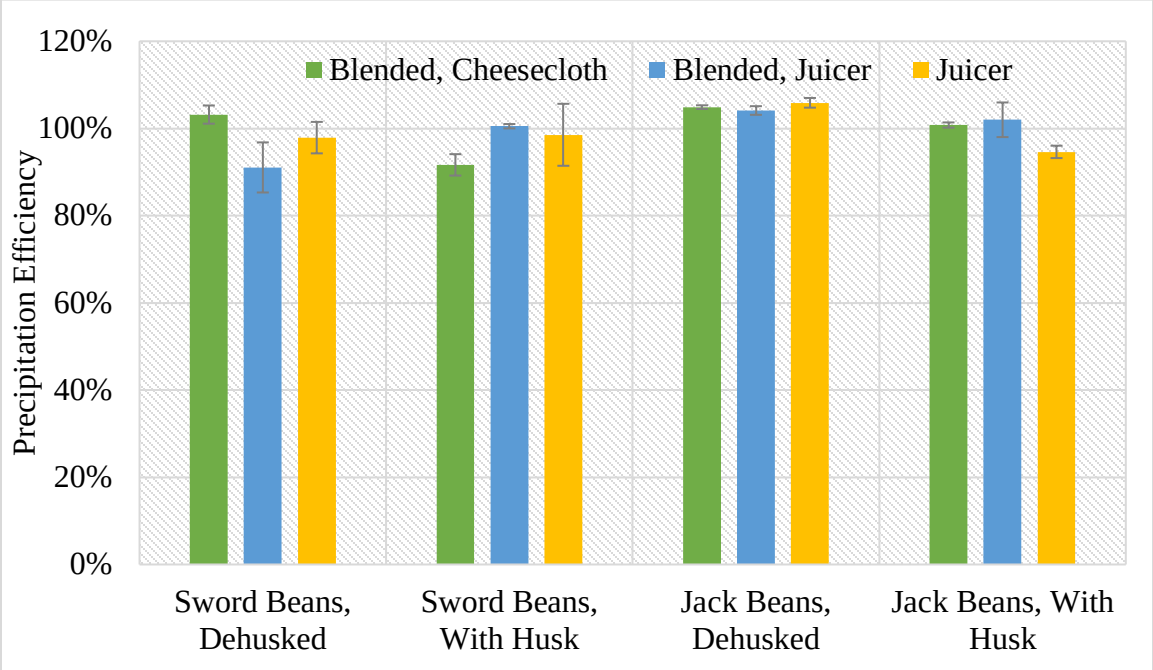

Figure 4-7: Precipitation efficiency tests using standard yield solution of 0.67 M calcium chloride and 1 M urea.

Table 4-8 and Fig. 4-8 show the precipitation efficiency for the equimolar 2 M solution. The variation in crude urease enzyme concentration did not correlate with the precipitation efficiency. Tests were done in triplicate. The high yield solutions showed much higher variation when compared to the standard yield solution. The average standard deviation for each sample category was 12%. Regardless of extraction method, the precipitation efficiency of the solution experiences a sharp decline in the sword beans with husk case, with juicing alone providing a higher precipitation efficiency than the other two methods (the common method and blending and juicing).

Table 4-8: Precipitation efficiency averages and standard deviations for high yield solutions of equimolar 2 M calcium chloride-urea solution and 33,750 U/L crude urease enzyme.

		High Yield Solution Precipitation Efficiency (%)			
		Sword Beans		Jack Beans	
		Dehusked	With Husk	Dehusked	With Husk
Blender, Cheesecloth	Average	85%	22%	87%	76%
	Standard Deviation	7.3%	5.2%	18.7%	11.3%
Blender, Juicer	Average	66%	28%	70%	62%
	Standard Deviation	25.8%	7.6%	2.6%	22.5%
Juicer	Average	81%	46%	83%	70%
	Standard Deviation	6.1%	9.1%	19.2%	7.8%

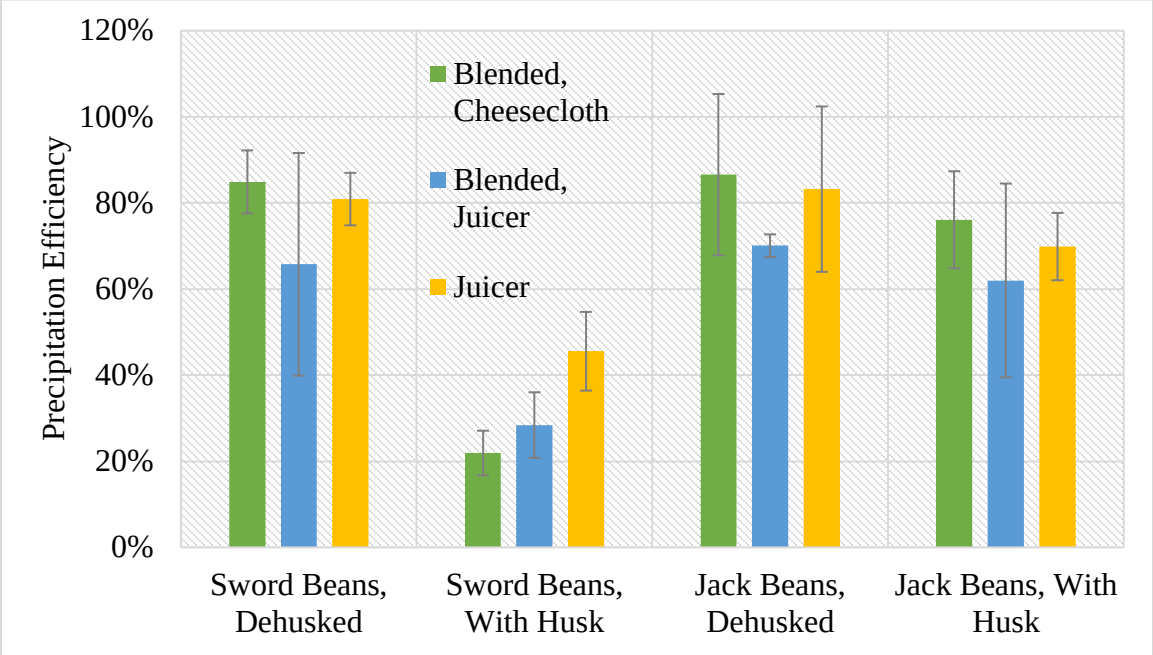

Figure 4-8: Precipitation efficiency tests using high yield solutions of equimolar 2 M calcium chloride-urea solution and 33,750 U/L crude urease enzyme.

DISCUSSION

SWORD BEANS VS JACK BEANS

The jack beans absorbed more water over the 24-hour soaking period than the sword beans. While potentially due to innate properties of the beans, another potential cause for this result may be differing levels of initial hydration. All tests done with jack beans were taken from the same batch of jack beans and all sword beans were, likewise, taken from the same batch.

The jack beans had a higher crude urease enzyme yield than the sword beans. This is counterintuitive, as the jack beans had absorbed more water. This result suggests that the jack bean may break down more easily when compared to sword beans. Alternatively, the smaller size of the jack beans may lend them to more thorough

blending before the breakdown of the urease enzymes. Another potential explanation is variability inherent to the data, as all data is based on one test and organic materials have an inherent natural variability.

The activity of the crude urease enzyme from jack beans without husks, extracted using the common method, was found to be 43% lower than that of crude urease enzyme from sword beans without husks, extracted using the common method. This was based off of one sample each and may not be representative of all jack beans and sword beans.

Considering the total activity yield of the crude enzymes, sword beans without husks, extracted using the common method, have a 54% higher total activity compared to jack beans. The difference in total activity is not as pronounced when taking husks and other extraction methods into account, but still averages 35% and should be considered when selecting beans for enzyme induced carbonate precipitation (EICP).

There was not a notable difference in precipitation efficiency between crude urease enzymes from jack beans and sword beans without husks, extracted using the common method.

INCREASING CHEMICAL CONSTITUENT CONCENTRATIONS

The standard solution (0.67 M calcium chloride, 1 M Urea) averages a precipitation efficiency of 91-106% and an average standard deviation of 2% with the removal of two outlying samples for beans without husks. The high yield solution (equimolar 2 M calcium chloride-urea, 33,750 U/L) had an average precipitation efficiency of 79% with a 12% average standard deviation for each case. The decrease of

precipitation efficiency using the high yield solution may be due to the higher production of ammonia (which acts as an inhibitor) or the increased pH of the solution. However, the cause of the increase in standard deviation is unclear when compared to the standard yield solution.

The theoretical maximum precipitation yield for 1 L of the standard solution is 67 g, while the theoretical maximum precipitation yield for 1 L of the high yield solution is 200 g. Considering the average precipitation efficiency, the amount of precipitate that can be expected from the standard solution and high yield solution are 70.3 g (approx. 69-66 g) and 157 g (approx. 138-176 g), respectively. This results in a precipitate yield ratio (high yield to standard yield) of 2.2, i.e., that the 2 M solution will precipitate over twice as much carbonate per treatment cycle than the standard solution on average.

The high precipitation yield solution uses 3.2 times the amount of enzyme that the standard yield uses. Depending upon the price of enzyme, the disproportional amount of enzyme needed to precipitate enzyme in high yield solutions could make high yield solutions inefficient in comparison to multiple treatment cycles. However, multiple treatment cycles have the drawback of increasing the time needed for treatment and additional application costs, resulting in a trade-off of costs that must be evaluated to determine the preferred solution concentration.

The differing activity levels may have had an impact on the precipitation efficiencies but were not correlated with trends in variance or efficiency.

THE EFFECTS OF HUSKS

Jack beans and sword bean husks absorbed the approximately the same average percentage of the water, 15% by normalized volume of the total added water, though jack bean husks had a more significant standard deviation of 3.8% compared to sword bean's 1.2% standard deviation. The higher standard deviation for the jack beans may be due to a higher natural variation in the properties, e.g., initial moisture content, of jack beans.

When extracted using the common method (blending followed by filtration using cheesecloth), both jack beans and sword beans with husk experience a decrease in crude enzyme volume yields when compared to crude urease enzyme from beans without husk, with the decrease being more pronounced in jack beans. The crude enzyme activity from jack dehusked beans increases near proportionally to the decrease in yield, resulting in no change in total urease content (i.e., the capacity for biocementation). The sword beans, however, saw a slight decline in biocementation capacity after husking. This could be due to a constituent of the sword bean husks acting as an inhibitor to the urea enzyme.

The standard yield precipitation solution did not show significant differences in precipitation efficiency for crude urease enzyme extracted from beans with or without husks using the common method (blending followed by filtration using cheesecloth). The high yield solution, however, had significant differences between crude urease enzyme extracted from beans with or without husks. Crude urease enzyme from sword beans with husks, extracted using the common method, had an average precipitation efficiency of 45% less than crude urease enzyme extracted without husks using the common method, while crude urease enzyme from jack beans, extracted using the common method, had an

11% decrease from extraction with husks to extraction without husks. This could also potentially be due to husks acting as an inhibitor to the urease enzyme and having a greater effect at higher enzyme concentrations.

VARIABILITY AMONG CRUDE EXTRACTION METHODS

The common method of urease enzyme extraction (blending followed by filtration using cheesecloth) resulted in the highest yield of crude urease enzyme, except in the case of sword beans with husk. However, the crude urease enzyme yield for both methods involving the juicer have very high losses in volume. Our experience shows these losses to be dependent on the juicer model (i.e. grater sizes, side angles, motor capacity) and bean size and is highly variable. Therefore, while the average total urease yield is notably higher for crude enzymes extracted from juicing alone compared to crude enzymes extracted from juicing with pre-blending, it is difficult to draw a definitive conclusion.

The common method and blending followed by juicing did not result in significant differences in the average crude urease enzyme activity compared to the method of juicing alone. However, there was a significant decrease in activity for juicing without prior blending when compared to juicing following blending and the common method of blending and cheesecloth filtration. Juicing alone likely did not result in breaking down the beans adequately, as it is not as disruptive as blending. This was evident in the chunks in the solid in the discard from the juicer. This issue could possibly be alleviated with a different juicer, or a specially designed appliance.

Except for the crude urease enzyme extracted from sword beans without husk, the standard yield precipitation tests did not reveal any differences in precipitation efficiency. The crude urease enzyme from sword beans without the husks, extracted using blending followed by juicing had a precipitation efficiency of 91%, 10% lower than the average of the other two methods. This difference was similar in the high yield precipitation efficiency tests (except in the crude urease enzyme from sword beans with husks).

While all high yield solutions had low precipitation efficiencies, for the case of crude urease enzyme from sword beans with husks extraction via juicing alone provided the highest precipitation values, followed by blending and juicing. Upon visual analysis, the sword bean husks are less likely to be fully processed in the juicer. It is unclear whether the jack bean husks are any less likely to be fully processed due to the similarity of the color of the husk to the bean flesh.

CONCLUSIONS REGARDING CRUDE EXTRATION METHODS

With the results of all tests considered, the common extraction method appears to be the most efficient with respect to enzyme yield. However, the common method requires much more labor and material and requires intensive filtering through cheesecloth and can only be done with approximately 25g of bean mixture at a time. Other mechanical filtration methods may be able to filter out the solids more efficiently without any negative effects (reduction in activity, enzyme yield, or precipitation efficiency) on the resultant crude urease enzyme. It may be less expensive and more sustainable (without the added use and life cycle of single-use cheesecloth) to juice the beans, with or without blending. A specialized juicer (capable of juicing smaller masses

without loss) may bring juicing without blending on par with the common method in terms of enzyme activity yield.

CHAPTER 5

ACTIVITY MEASUREMENT

INTRODUCTION

The activity of a urease enzyme is quantified by Units (U), defined as amount of ammonia (in µM) that can be formed through hydrolysis of urea by the urease enzyme in one minute. The actual rate of hydrolysis is not constant but can be reasonably accurately modeled by a quadratic equation (Eq. 5-1) in most cases. However, the initial velocity of the reaction is used to represent the activity. The initial velocity is the maximum velocity of the activity and does not consider the decay of the activity, which can vary with testing conditions. The initial velocity (A in Eq. 5-1) is usually calculated through quadratic regression to measurements over the first ten minutes of the reaction.

$$y = Ax + Bx^2 \qquad [5\text{-}1]$$

Commonly, spectrophotometry using Nessler's reagent is used to make the measurements that are used to determine enzyme activity. The method of spectrophotometry measurement is discussed in more depth in Chapter 3: Methodologies. Electrical conductivity (EC) is a factor that depends upon the amount of ammonia in solution. Due to its relative simplicity, EC measurement has been used as a method to measure urease enzyme activity, albeit less commonly than spectrophotometry and not always quantitatively. EC is an appealing method of measurement as it requires less time, resources, and safety measures than spectrophotometry using Nessler's reagent. This chapter discusses the use of EC as an alternative to spectrophotometry using Nessler's

reagent for measurement of the activity of crude urease enzyme extracted from sword beans and jack beans using various extraction methods.

RESULTS

Fig. 5-1 shows measurements taken with spectrophotometry using Nessler's method. The data has been converted from OD_{412} (optical density at 412 nm) readings to mM of ammonia formed through the hydrolysis of urea by crude urease enzyme extracted from various sources and methods (as further defined below the figure title) through dilution factors and the conversion factor 0.3656 found by Khodadadi Tirkolaei et al. (2020). The legend in Fig. 5-1 uses acronyms to organize samples based upon source bean, husk presence (or lack thereof), and extraction method. The acronyms used are defined in the text below the figure title.

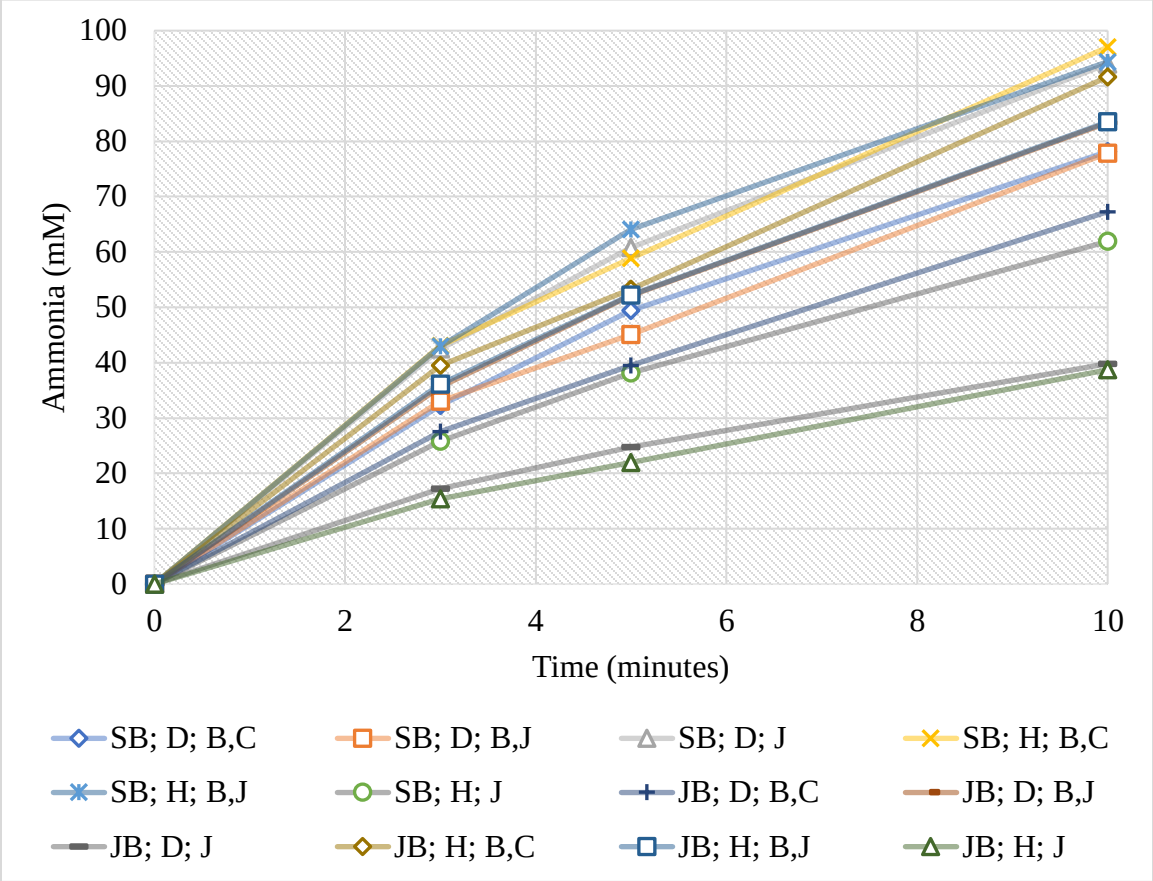

Figure 5-1: Measurements taken using spectrophotometry with Nessler's reagent and correlated with ammonia concentration using a correlation factor of 0.3656 from Khodadadi Tirkolaei et al. (2020).

Acronyms used:

SB: Extracted from sword beans

JB: Extracted from jack beans

D: Extracted from dehusked beans (beans without husk)

H: Extracted from beans with husks present

B,C: Extracted using a blender to break down beans, and cheesecloth to filter out solids (all extract is also filtered through glass wool to separate out fats)

B,J: Extracted using a blender to break down beans, then mixture was ran through a juicer to filter out solids (all extract is also filtered through glass wool to separate out fats)

J: Extracted using a juicer to break down beans and filter out solids (all extract is also filtered through glass wool to separate out fats)

The data presented in Fig. 5-2 is based upon the data presented in Fig. 5-1. Fig. 5-2 presents the linear portion of the quadratic fit with the decay component removed (the slope being the initial velocity) of the ammonia formed through the hydrolysis of urea by the crude urease enzymes over time. This value defines the activity of the urease enzyme. The initial velocity was found through quadratic regression performed on each test data. Each quadratic fit for the data from spectrophotometry using Nessler's reagent had an R^2 value higher or equal to 0.9988. Fig. 5-1 and 5-2 are displayed with the same scale for ease of comparison. There is some variation between the position of the samples relative to other samples between Fig. 5-1 and 5-2 (the measured ammonia concentration and initial velocity of the measured ammonia concentration, respectively). The variation in positioning is not correlated with any variations in crude extraction method.

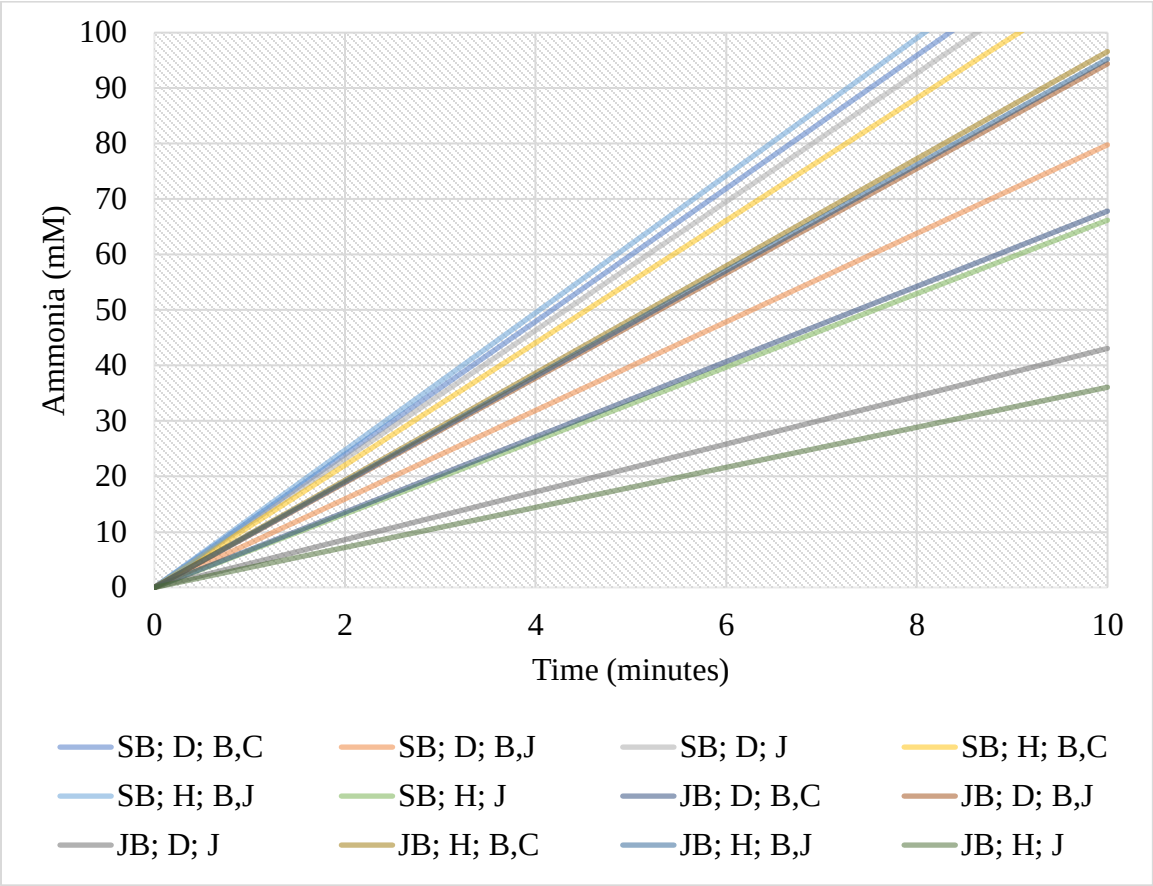

Figure 5-2: Ammonia formed through the hydrolysis of urea, without decay component, using the crude urease enzyme denoted in the legend, measurements taken with spectrophotometry using Nessler's reagent.

Figure 5-3 presents the adjusted electrical conductivity (adjusted for dilution) based upon EC measurements for the same EICP solutions reported in Fig. 5-1 and 5-2 (measurements taken using spectrophotometry using Nessler's reagent). The EC measurements generally follow the same decay as measurements taken via spectrophotometry using Nessler's reagent shown in Fig. 5-1. When performing quadratic regressions on the adjusted EC measurements, the quadratic fits to the data based on EC measurements have slightly lower R^2 values (equal to or less than 0.9901) than the R^2

values for quadratic regressions performed on measurements taken via spectrophotometry using Nessler's (equal to or less than 0.9988).

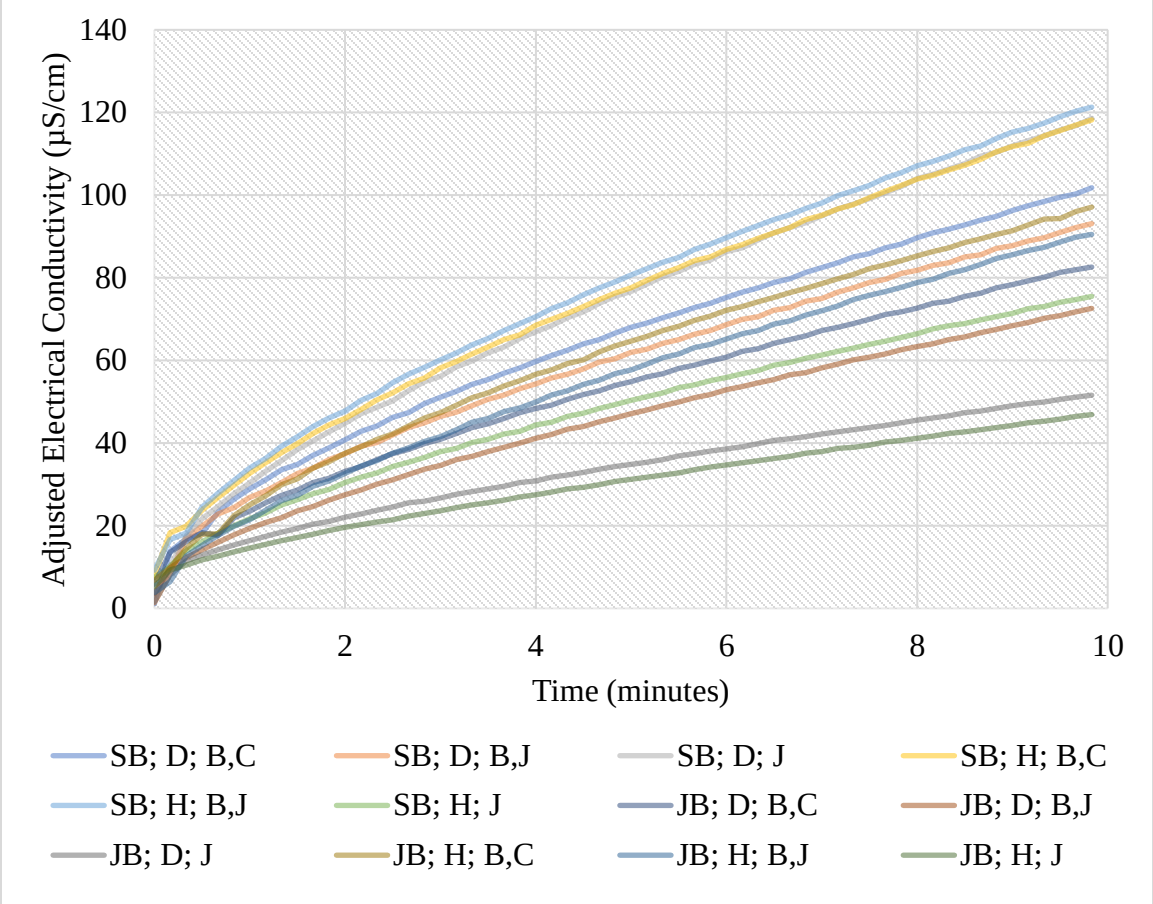

Figure 5-3: Measurements taken of EC and adjusted for units and crude urease enzyme concentration.

The data presented in Fig. 5-4 are the linear components of the quadratic regressions with the decay components removed. The slope of the lines presented in Fig. 5-3 (adjusted EC measurements) are the initial velocities which define the activity measurement. Fig. 5-3 and 5-4 are displayed using a similar scale for ease of comparison. When comparing Fig 5-3 and 5-4, beyond approximately 45 seconds, there is some

variation between the position of the samples relative to each other. The variation in position is not correlated with any variations in crude extraction method.

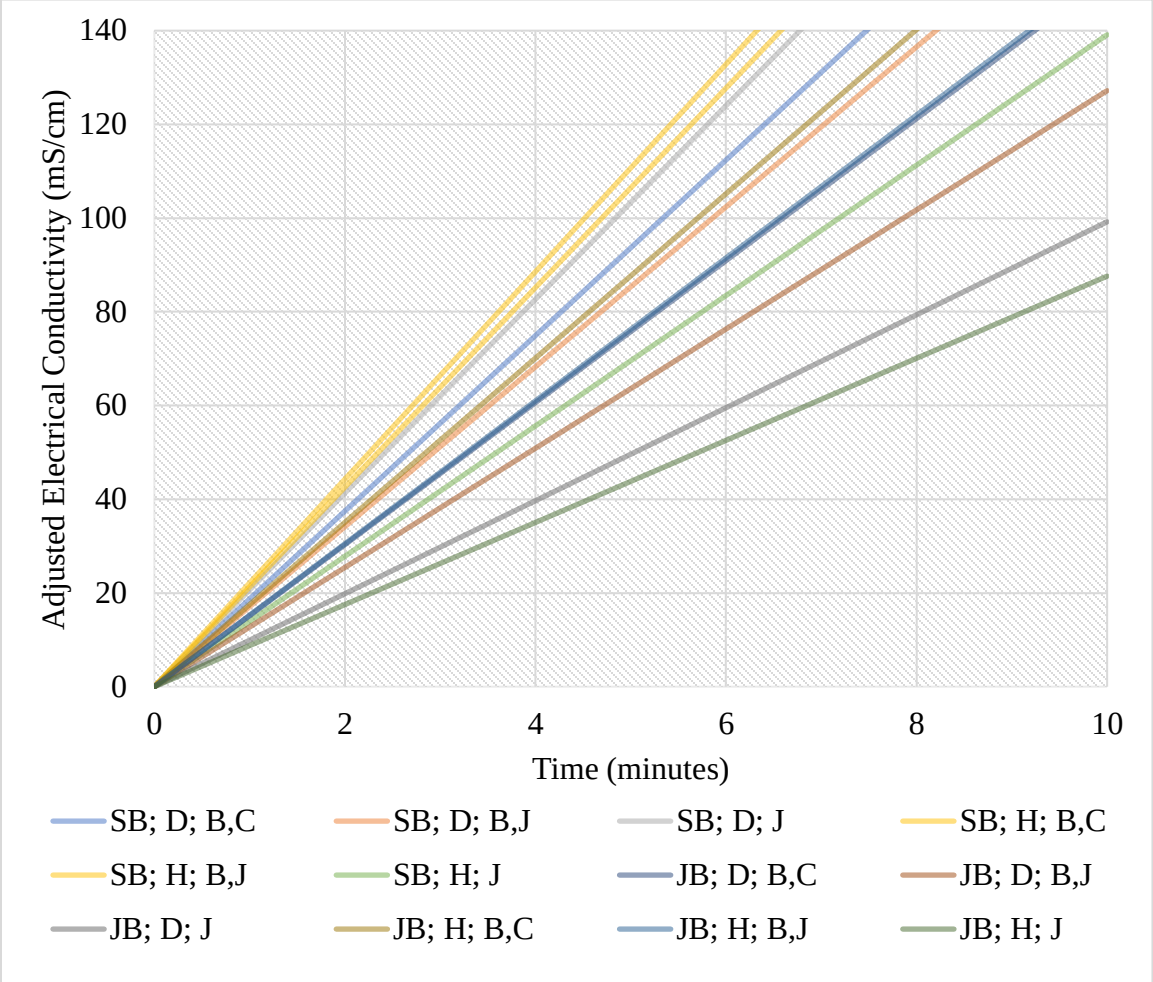

Figure 5-4: Linear component of adjusted EC regression without decay component.

As Fig. 5-2 (the initial velocity of data from the spectrophotometer measurements) and Fig. 5-4 (the initial velocity of the adjusted EC measurements) are presented with different units on the y-axis, it is not possible to compare them quantitatively. However, when comparing Fig. 5-2 and 5-4 qualitatively, some of the samples are in different positions relative to the other samples. This indicates that there is

a variation between the measurement of activity of samples with EC and measurement of activity of samples using spectrophotometry with Nessler's reagent. The variation in position is not correlated with any variations in crude extraction method.

Table 5-1 summarizes the following information for each crude urease enzyme sample (classified by bean type, husk presence (or lack thereof), and extraction method, acronyms are defined below the title of Fig. 5-1):

- Measured Activity: This is the activity measured with a spectrophotometer using Nessler's reagent and correlated using the factor (0.3656) from Khodadadi Tirkolaei et al. (2020). It is presented in terms of urease activity units (U).
- Adjusted Initial EC Velocity: This is the adjusted initial velocity of the quadratic fit for the EC measurements. These values are presented in terms of µS/cm.
- Correlation Factor: This is the correlation factor between the adjusted EC measurement's initial velocity and the activity measured using spectrophotometry with Nessler's reagent for each respective sample. The average correlation factor was found to be 17.9 with a standard deviation of 3.11, resulting in a standard deviation for urease enzyme activity of approximately 56 U for an average EC-based activity of 288 U.
- EC-based Activity: This is the activity using the EC measurements. One value in this table is based upon the correlation factor of 17.9 found in this study, and one uses the 22.2 correlation factor (stated in text as 11.1, converting to amount of urea hydrolyzed instead of the resulting ammonia formation typically used for activity measurement) established by Whiffin (2004) based upon measurements

from commercially available enzymes in a solution that did not contain calcium chloride. Both are presented in terms of urease activity units (U).

- Activity Ratio: This is the ratio of EC-based activity to measured activity and is presented for both correlation factors. These values represent the percentage of the activity inferred from electrical conductivity (using each respective correlation factor) to the activity measured using spectrophotometry. The ratio of EC-based activity to the measured activity when using a correlation factor of 22.2 (found by Whiffin (2004)), was 1.28 while the ratio using the average correlation factor for these tests (17.9) averaged 1.03.

Table 5-1: Summary of crude urease enzyme analysis for crude urease enzyme from sword beans.

		SB					
		D			H		
		B,C	B,J	J	B,C	B,J	J
	Measured Activity	400	266	386	367	413	221
	Adjusted Vo	18.7	17.1	20.7	21.3	22.1	13.9
	Correlation Factors	21.3	15.6	18.7	17.3	18.6	15.9
17.9 Correlation Factor	EC-Based Activity	335	306	370	381	396	249
	Activity Ratio	1.16	0.85	1.04	0.96	1.04	0.87
22.2 Correlation Factor (Whiffin, 2004)	EC-Based Activity	416	379	459	473	491	309
	Activity Ratio	1.04	1.42	1.19	1.29	1.19	1.4

Table 5-2: Summary of crude urease enzyme analysis for crude urease enzyme from jack beans.

		JB					
		D			H		
		B,C	B,J	J	B,C	B,J	J
	Measured Activity	226	315	144	322	317	120
	Adjusted Vo	15.2	12.7	9.9	17.5	15.2	8.8
	Correlation Factors	14.9	24.7	14.5	18.4	20.8	13.7
17.9 Correlation Factor	EC-Based Activity	271	228	178	314	273	157
	Activity Ratio	0.8	1.28	0.76	1.03	1.14	0.7
22.2 Correlation Factor (Whiffin, 2004)	EC-Based Activity	336	282	220	389	338	194
	Activity Ratio	1.49	0.9	1.53	1.21	1.07	1.62

DISCUSSION

Measurement of crude urease enzyme activity using EC results in a standard deviation of 56 U when compared with measurements taken using spectrophotometry with Nessler's reagent using the 17.9 average correlation factor found for these tests with an average activity of 288 U. The standard deviation across all activity measurements taken using spectrophotometry with Nessler's reagent was 93 U, with an average of 291 U while the standard deviation across all EC-based activity measurements (using a

conversion factor of 17.9) was 74 U with an average of 288 U. This results in a difference of approximately 6.7% in standard deviations across all taken activity measurements. This difference in the standard deviation suggests there may be a systematic bias when using EC-based activity measurements. Using EC-based measurement of crude urease enzyme activity may be a viable option in cases where the uncertainty associated with this relatively high standard deviation is acceptable. However, based on the high standard deviation and potential systematic bias, EC-based activity measurement is not ideal for measurements of crude urease enzyme activity where accuracy is desired.

The differences between EC-based activity measurement and activity measurements based upon spectrophotometry using Nessler's reagent, as evidenced in the differences of relative position in Fig. 5-2 and 5-4, is likely due to the natural variation in the composition of the beans, and therefore, in crude urease enzyme composition. This variation may influence electrical conductivity. There was no correlation of the ratio of EC-based activity to activity measured via spectrophotometry with Nessler's reagent with any of the crude urease enzyme groupings (source bean variety, the presence or absence of husk for extraction, and extraction methods), suggesting that there are no direct relationships between these factors and EC measurements. The decay rates of the activity for each sample and measurement method were not consistent, as evidenced by the differences in relative positioning to other samples in comparison of Fig. 5-1 and 5-2, and Fig. 5-3 and 5-4.

Urease enzyme activity measurement using EC measurements and the 22.2 correlation factor from Whiffin (2004) should be used with caution when using crude

urease enzyme similar to the ones explored in these experiments, as the 22.2 correlation factor resulted in a urease activity that was too high in all but one case. Using the correlation factor of 22.2 recommended by Whiffin (2004) to convert EC measurements to activity could result in underestimating the amount of crude urease enzyme needed for an EICP treatment and potentially result in lower levels of precipitation or undesired distribution of precipitate.

CHAPTER 6

CONCLUSIONS

SUMMARY

Understanding the factors influencing crude urease enzyme activity, and the effect the crude urease enzyme and the chemical constituent concentration has on precipitation efficiency is important for the successful implementation of enzyme induced carbonate precipitation (EICP) using crude urease enzyme for ground improvement methods. Little prior work has been done to assess the influence of the extraction method, the difference between crude urease extracted from jack beans (*Canavalia ensiformis*) or sword bean (*Canavalia gladiata*), or chemical constituent concentration on the efficacy of the EICP process. The accuracy of the commonly used technique of evaluating enzyme activity based upon electrical conductivity measurements on crude urease extract has also not been studied in depth. These factors are systematically investigated in this thesis.

After reviewing literature on the EICP process and urease enzyme extraction and activity measurement methods in Chapter 2, the experimental techniques used in this thesis are described in Chapter 3. Chapter 4 then addresses the influence of crude urease enzyme source and extraction method on the activity of the crude enzyme extract and the effect of chemical constituent concentration, bean type, and extraction method on precipitation efficiency. In Chapter 5, the accuracy of the electrical conductivity method and conversion factor for the measurement of crude urease enzyme activity is evaluated.

FINDINGS

In Chapter 4, Activity & Precipitation Efficiency, crude urease enzyme from sword beans presented a higher total activity yield (EICP potential) than crude urease extract from jack beans. This may make them a more appealing alternative to jack beans for some EICP applications.

Crude urease enzyme extracted through juicing the beans without blending them first resulted in a significant decline in activity in comparison to the extraction methods with blending. This may be due to inadequate breakdown of the bean flesh using the juicer appliance.

There was an approximate drop of 40% in precipitation efficiency when using a 2 M equimolar solution of urea and calcium chloride from near 100% precipitation efficiency with a 0.67 M urea, 1 M calcium chloride solution with crude urease enzyme. Decreases in precipitation efficiency can counter act any benefit from using high concentration solutions, so this decrease in precipitation efficiency needs to be carefully considered when using higher concentrations solutions to optimize the amount of carbonate that can be precipitated in one cycle of treatment.

The high yield EICP solution tested is less efficient in terms of enzyme consumption to precipitation yield. While the high yield solution precipitated 2.3 times as much calcium carbonate in one cycle of treatment when compared to the standard yield solution, the high yield solution used 3.3 times the amount of the crude urease enzyme that the standard yield solution used.

High yield EICP solutions (equimolar 2 M calcium chloride-urea, 4 g/L non-fat dry milk, and roughly 33,750 U/L of crude urease enzyme) using crude urease enzymes from beans without husk increase the internal standard deviation (within the group of samples done in triplicate) of precipitation efficiency by 9.6% and lowers the average precipitation efficiency from 101% to 79% when compared to standard yield precipitation solutions (0.67 M calcium chloride, 1 M urea, 4 g/L non-fat dry milk, and roughly 10,500 U/L crude urease enzyme) using crude urease enzymes from beans without husk. Even when considering the decrease in precipitation efficiency, the high yield solution will form 2.3 times as much calcium carbonate in one cycle of treatment when compared to the standard yield solution.

Crude urease enzyme extracted from beans with husks had a lower precipitation efficiency when compared to crude urease enzyme from beans without husks. Crude urease enzymes extracted with husks from sword beans saw the most dramatic decrease in precipitation efficiency when used in high yield EICP solutions. There was a 30% decrease in the average precipitation efficiency for crude urease enzyme from sword beans with husk compared to a 23% decrease in average precipitation efficiency average for crude urease enzyme from sword beans without husk across all extraction methods.

Crude urease enzyme extracted through blending and filtered using a juicer appliance resulted in a decline in precipitation efficiency when compared to the other extraction methods (juicing alone and blending with filtration via cheesecloth). This decline was more pronounced in the high concentration EICP solutions.

In Chapter 5, Activity Measurement, it was found that the correlation between electrical conductivity (EC) the activity of crude urease enzyme appears to be relatively inaccurate, with an error of up to 30% when compared to measurements taken using spectrophotometry with Nessler's reagent (which is considered more accurate). This variability may be due to natural variation in the composition of the beans used for crude urease enzyme extraction.

EC-based measurements for activity were done for crude urease enzyme from sword beans and jack beans, with and without husks, using each of the extraction methods (juicing, blending and filtering with juicing, and blending and filtering with cheesecloth). Each variation in source and extraction was grouped and considered against the variation between EC-based activity measurement and activity measured with spectrophotometry using Nessler's reagent. There was no significant correlation found between any of the groupings and the variation in activity.

To convert EC measurements to urease enzyme activity measurements, a conversion factor of 22.2 (Whiffin, 2004) is commonly used. This factor is based upon pharmaceutical enzyme. An EC conversion factor of 17.9 was found to be more appropriate for the crude urease enzyme tested and should be considered for use in evaluating the activity of similar crude urease enzymes from EC measurements.

RECOMMENDATIONS FOR FUTURE STUDY

- A cost analysis should be conducted to establish the optimum crude urease enzyme source, extraction method, and concentrations of urea and calcium chloride in an EICP solution.

- Further research on optimizing enzyme concentrations for high yield enzyme solutions is advisable.
- Further research and/or development of appliances for crude urease extraction should be considered to improve extraction yield.
- Further research of EC-based activity measurement focused on the correlation between measurement variance and crude enzyme composition should be conducted.

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