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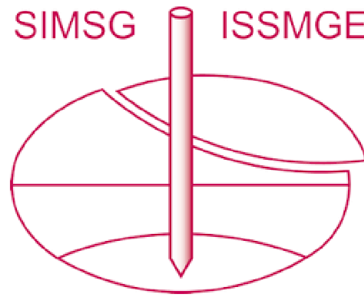

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Stability of crude urease extract for enzyme induced carbonate precipitation (EICP)

Stabilité de l'extrait brut d'uréase pour précipitations de carbonate induites par enzymes (EICP)

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ABSTRACT: The stability (urease activity) of a crude extract obtained from jack beans using a simple extraction process that employed water, rather than a buffer, as the extraction solution and without the ultracentrifugation step commonly used for enzyme extraction was evaluated when stored at room temperature and 4ºC. The stability of two commercial urease powders in aqueous solution was also evaluated. The three urease solutions were periodically tested to evaluate their activity. Results of the study showed that when stored at room temperature the number of units of urease in all three urease solutions decreased significantly over a one-year period. However, when stored at 4°C, the percentage loss of total units for the crude extract was significantly less than for the commercial urease solutions at room temperature, with a loss of less than 10% over 30 days and less than 40% over one year. The commercial urease solutions when stored at 4ºC showed greater activity losses compared to the crude extract on a percentage basis.

RÉSUMÉ : La stabilité (activité de l'urée) d'un extrait brut d'uréase obtenue à partir du pois sabre (pois de Madagascar) à l'aide d'un processus d'extraction simple à l'eau, plutôt qu'avec une solution tampon, comme solution d'extraction et sans l'étape d'ultracentrifugation couramment utilisée pour l'extraction des enzymes, a été évaluée lorsqu'il est stocké à température ambiante et à 4 °C. La stabilité de deux poudres commerciales d'uréase en solution aqueuse a également été évaluée. Les solutions d'uréase ont été périodiquement testées pour évaluer leur activité. Les résultats de l'étude montrent que lorsque les trois sources sont stockées à température ambiante, le nombre d'unités d'uréase dans chaque source a diminué de façon significative sur une période d'un an. Par contre, lorsque stocké à 4°C, la perte d'unités totales pour l'extrait brut était significativement inférieure à la perte d'unités totales quant stocké a température ambiante, avec une perte inférieure à 10 % sur 30 jours et inférieure à 40 % sur un an. Les solutions d'uréase commerciale lorsqu'elles sont stockées à 4°C ont montré des pertes d'activité plus importantes par rapport à l'extrait brut.

KEYWORDS: biocementation, carbonate precipitation, urease enzyme

1 INTRODUCTION

As part of an effort to lower the cost of the urease enzyme used in enzyme induced carbonate precipitation (EICP) for soil improvement, the stability of a crude urease-rich extract from jack beans, a urease-rich agricultural product, was evaluated and compared to that of commercially available urease. EICP has the potential to be a sustainable technique for various infrastructure construction and environmental protection applications that require binding together granular soil particles (Kavazanjian and Hamdan 2015). EICP does this through the precipitation of calcium carbonate. The precipitate is formed from a solution containing free calcium ions, urea, and urease enzyme, with the urease acting as a catalyst for the hydrolysis of the urea (Larsen et al. 2007 and Nemati et al. 2003). As noted by Khodadadi et al. (2017), one of the primary barriers to EICP being applied in practice is the cost of the free urease enzyme. To combat this cost, Khodadadi et al. (2020) showed that urease extracted from jack beans using simple and inexpensive techniques can effectively catalyze urea hydrolysis. However, storage and shelf life capacity of the extract and/or the plants from which the extract is derived need to be investigated to address the commercial viability of the simple extraction process.

2 BACKGROUND

EICP is one of a family of emerging ground improvement techniques for enhancing the strength of granular soil by precipitation of calcium carbonate from an aqueous solution. These techniques have been collectively referred to as bio-grouting (Khodadadi et al 2017). EICP employs the same chemical reaction as the more-investigated technique of microbially induced carbonate precipitation (MICP). Both EICP and MICP rely on the enzyme urease to catalyze the hydrolysis of urea to create carbonate ions in solution. If calcium ions are in the same solution, and the solution has the appropriate pH and alkalinity, the carbonate and calcium ions will merge and precipitate as calcium carbonate. The primary difference between EICP and MICP is the source of the urease. In MICP, the urease is provided by ureolytic bacteria, i.e. bacteria that contain urease within their cellular structure. These bacteria may be grown ex situ and introduced to the cementation solution (bio-augmentation) or stimulated in situ (biostimulation). In EICP, free urease is included in the cementation solution. The free urease may be extracted from vegetation, bacteria, or fungi.

Initial investigations into geotechnical applications of EICP relied upon commercially available urease (Hamdan and Kavazanjian 2016). However, as these commercially available enzymes were produced with very high purity for pharmaceutical purposes, the cost of the pharmaceutical-grade enzyme dominated the cost of EICP ground treatment and the cost of EICP treatment was significantly greater than the cost of MICP treatment. As part of an effort to reduce the cost of EICP treatment, Khodadadi Tirkolaei et al. (2020) demonstrated that a crude extract on urease from jack beans (a urease-rich relative of the soy bean) could effectively biocement Ottawa 20-30 sand at $1/40^{th}$ of the cost of commercially available urease. Martin et al. (2021) subsequently demonstrated that the crude urease extract was as at least effective, and sometimes more effective, at biocementation for multiple granular material types. However, for practical reasons, large quantities of the crude extract would have to be mass produced and stored if it is to be used on large infrastructure projects. As many enzymes are not stable when stored in solution (their activity, or effectiveness is reduced or lost over the time), questions about the stability of the mass produced crude extract have to be addressed.

Commercial urease is generally provided in powdered, lyophilized (freeze dried) form and has an unlimited shelf life if stored at 4ºC. However, Danial et al. (2015) evaluated the stability of commercial pharmaceutical grade urease after hydration in an aqueous solution at room temperature. These investigators observed an 85% loss of activity in the aqueous

urease solution after just 7 days and 100% after 60 days. El-Hefnawy et al. (2014) purified urease from germinating Pisum Sativum L. seeds and investigated the effects of storage in solution at 4°C for up to 60 days on urease activity. They observed the activity of the aqueous solution decreased with time even when stored at 4°C. They reported that activity decreased to 80% of the initial value on the tenth day and retained only about 14% of the initial activity after 60 days when stored at this temperature.

To address concerns over the stability of the crude urease extract, this study evaluated the effect of storage of the jack bean crude extract developed by Khodadadi et al. (2020). The stability of two commercial ureases after re-hydration and storage as an aqueous solution on urease activity was also evaluated. The aqueous solutions were stored at both room temperature (RT) and at 4°C for up to one year.

3 MATERIALS AND METHODS

3.1 *Materials*

Jack beans (Canavalia Gladiata, Sheffield's Seed) were obtained from a commercial vendor. Commercial lab grade urease enzymes from Sigma Aldrich and Fisher Scientific were also used in this study.

3.2 *Urease Extraction and Solution Preparation*

Fifty seven (57) grams of jack beans were dehusked manually to obtain fifty (50) grams of dehusked jack bean seeds which were then soaked in 200 ml tap water, rather than in a buffer as used in traditional extraction, and stored at 4°C for 24 hours. The soaked seeds were then pulverized in a blender for 2 minutes and filtered through muslin cloth to remove any coarse solids from the solution. After that, the supernatant was filtered through a thick layer of glass wool to eliminate extra excess fat. The solution obtained through this process is referred to herein as "crude extract". The ultracentrifugation step typically used for extraction (Prakash et al. 2003 and Javadi et al. 2018) was not used in the crude extraction procedure. Crude extract was stored at room temperature (RT, approximately 20°C) and at 4°C prior to activity measurement at different storage times.

3.3 *Urease Activity and Units Measurement*

Urease activity is defined as the micromoles of ammonia released per minute by 1 ml (if liquid extract) or 1 g (if powder in solution) of urease enzyme (i.e., U/ml or U/g), whereas total units, U, is a measure of the urease content of the solution (Prakash et al. 2003 and Khodadadi et al. 2020). Enzyme activity of the crude extract was assayed in 125mM urea solution (pH=7). An aliquot (4.7 ml) of DI water and 5 ml of 250 mM urea were mixed at room temperature. The urea hydrolysis reaction was then started by adding 0.3 ml of crude urease extract and the container was immediately sealed.

At time intervals of 3, 5, and 10 min 5 ml of 15% trichloroacetic acid was added into the sealed system (water, urea, and crude urease extract) to stop the reaction. After stopping the reaction, each bottle was opened, and the solution was diluted 100-fold using DI water in a volumetric flask. Then 2 ml of this solution was mixed in a cuvette containing 100µl of Nessler's reagent. After 2 minutes, the cuvette was placed in a spectrophotometer to measure the optical density of the solution at a wavelength of 412 nm (i.e., OD412) using a Spectronic 21UVD spectrophotometer.

All activity measurements were made at room temperature. Solutions stored at 4°C were allowed to equilibrate at room temperature before measuring their activity. Urease activity is known to be temperature dependent, with a peak value typically between 40°C and 50°C (Das et a. 2002, Mohamed et al. 1999, Pervin et al., 2013, Krishna et al. 2011). While the decreased activity at lower temperatures may be an important consideration in determining how much urease is required for a particular application in a particular environment, it was not considered a significant factor in evaluation of the relative activity of a given solution as a function of storage time and storage temperature.

Aqueous solutions were prepared from the commercial ureases of Fisher Scientific and Sigma Aldrich (their high activity urease), and then stored and tested in the same manner as the crude extract. Fisher enzyme solution was prepared with 0.15 g of the enzyme powder (with an activity of 2,500 U/g) dissolved in 10 ml DI water, resulting in an initial value of 23,000 total units of urease. Sigma enzyme solutions was prepared with 0.05 g and of the enzyme powder (with an activity of 15,000 U/g) similarly dissolved in 10 ml DI water, resulting in an initial value of 150,000 total units.

4 RESULTS

Figure 1 shows the measurements of total urease units versus time for jack bean crude extract and the two commercial enzymes at both room temperature and 4°C. Figure 2 presents this data as the percentage of activity lost. The results indicate that the activity of all three enzyme sources reduced with the storage time. The loss of activity was more much pronounced at room temperature than at 4°C, as expected. For example, after storage of crude extract for 1 year, urease activity was reduced by approximal 35% when stored at 4°C (Figure 2b). However, the activity loss was over 90% when the crude extract was stored at room temperature (Figure 2a). Furthermore, when stored at 4°C the crude extract retained its activity for a significantly longer period of time than the commercial enzymes in solution. For instance, the loss of activity after 180 days at 4°C for the crude extract was less than 20% whereas for the sigma enzyme the percentage of unit loss was above 40% over the same time period (Figure 2b).

These results are in agreement with the study of Schneider et al. (1980) which found the urease in a crude extract is sufficiently stable, but the stability decreases considerably in a purified state.

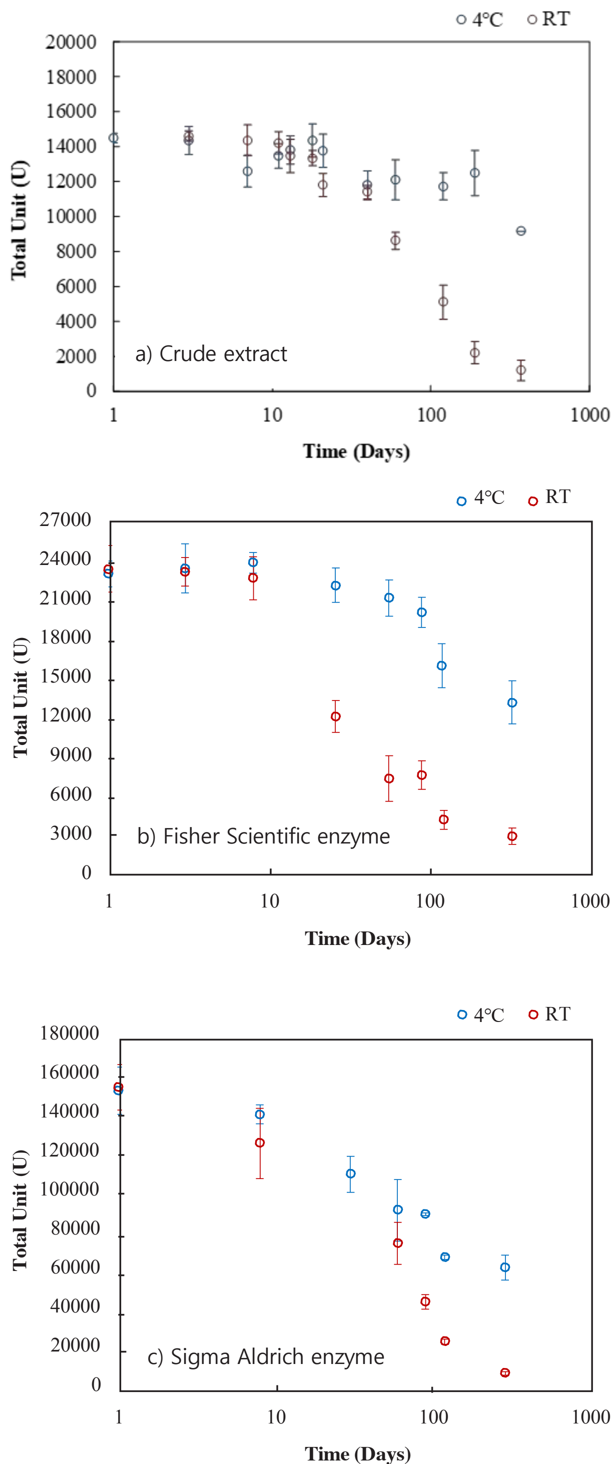

Figure 1. Urease activity measurements for aqueous solutions stored at room temperature and 4°C for crude extract, Fisher Scientific, and Sigma Aldrich aqueous solutions.

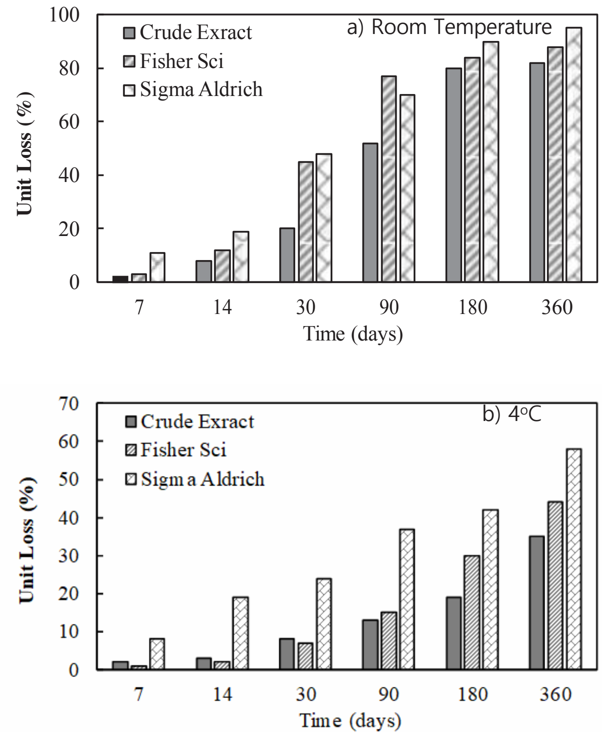

Figure 2. Percentage of unit loss of the jack bean crude extract and Fisher Scientific and Sigma Aldrich aqueous solutions at different times when stored at room temperature and 4°C.

5 CONCLUSIONS

Urease enzyme is an essential ingredient in the emerging ground improvement technique of EICP (enzyme induced carbonate precipitation). It has been demonstrated that the cost of obtaining urease, and thus the cost of ground improvement, can be significantly reduced by use of a crude urease extract from jack beans. However, enzymes in solution are known to lose their activity (their effectiveness) over time, raising concerns about the ability to mass produce and store the crude extract over the duration of a typical ground improvement project.

The stability of three urease solutions, including a crude extract made from jack beans through a simple extraction process and two commercially available lab grade enzymes in aqueous solution, was evaluated following storage at room temperature and at 4°C for up to one year. All three urease sources lost most of their activity when stored at room temperature for one year. The crude extract showed remarkable stability when stored at 4°C, losing less than 10% of total urease units in 30 days, less than 20% of the total units after 180 days, and less than 40% after one year. However, the stability of the crude extract was superior compared to both commercial enzymes. It should be noted, however, that the two commercial enzymes retain a much higher percentage of the total units if left in their "as purchased" lyophilized (i.e., powder) form rather than in an aqueous solution.

The results presented herein suggest that crude urease extract prepared at the beginning of a biocementation ground improvement product will remain stable for the duration of the project if stored at 4°C. This finding enhances the practical viability of using crude extract for EICP ground improvement projects. Ongoing work is looking at the potential for reducing the crude extract to a powder by lyophilization (freeze drying) to further facilitate mass production, storage, and transportation of urease for ground improvement.

6 ACKNOWLEDGEMENTS

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