

Article

Longevity of Raw and Lyophilized Crude Urease Extracts

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Abstract: The stability (longevity of activity) of three crude urease extracts was evaluated in a laboratory study as part of an effort to reduce the cost of urease for applications that do not require high purity enzyme. A low-cost, stable source of urease will greatly facilitate engineering applications of urease such as biocementation of soil. Inexpensive crude extracts of urease have been shown to be effective at hydrolyzing urea for carbonate precipitation. However, some studies have suggested that the activity of a crude extract may decrease with time, limiting the potential for its mass production for commercial applications. The stability of crude urease extracts shown to be effective for biocementation was studied. The crude extracts were obtained from jack beans via a simple extraction process, stored at room temperature and at 4 °C, and periodically tested to evaluate their stability. To facilitate storage and transportation of the extracted enzyme, the longevity of the enzyme following freeze drying (lyophilization) to reduce the crude extract to a powder and subsequent re-hydration into an aqueous solution was evaluated. In an attempt to improve the shelf life of the lyophilized extract, dextran and sucrose were added during lyophilization. The stability of purified commercial urease following rehydration was also investigated. Results of the laboratory tests showed that the lyophilized crude extract maintained its activity during storage more effectively than either the crude extract solution or the rehydrated commercial urease. While incorporating 2% dextran (*w/v*) prior to lyophilization of the crude extract increased the overall enzymatic activity, it did not enhance the stability of the urease during storage.

Keywords: enzyme induced carbonate precipitation; EICP; storage; stability; activity; freeze drying; lyophilization; urease



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1. Introduction

The enzyme urease has a variety of industrial applications, including biocementation of soil and pet stain removal. However, commercial sources of the enzyme are either highly purified and expensive laboratory and food grade powder or unprocessed meal from urease-rich agricultural products. This hinders the potential for industrial applications that require kilogram-scale supplies of the free enzyme. In particular, the adoption of enzyme induced carbonate precipitation (EICP), an emerging technique for biocementation of soil and repair of cracks in concrete, is hindered by the high cost of commercially available urease [1]. A low-cost source of free urease enzyme would greatly facilitate the adoption of this potentially sustainable technique for various infrastructure construction and environmental protection applications that require binding together granular soil particles.

EICP binds granular soil particles together 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 (i.e., for ureolysis) [2,3]. As noted by Khodadadi et al. [4], 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. [4] showed that urease extracted from jack beans using a simple,

inexpensive technique can effectively catalyze ureolysis. However, the storage and shelf life of the crude extract needs to be investigated to address the commercial viability of the simple extraction process for mass production of urease.

Generally, enzymes are not stable in an aqueous solution: their activity is reduced or lost over time. 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). Total units, U, is therefore a measure of the urease content of a urease solution. Danial et al. [5] stored commercial lab grade purified urease after hydration in an aqueous solution at room temperature for 60 days. These investigators observed an 85% loss of activity in the solution after just 7 days and 100% loss of activity after 60 days. El-Hefnawy et al. [6] purified urease from germinating *Pisum Sativum* L. seeds and investigated the effects of storage in aqueous 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 [5,7]. Sehgal et al. [8] first soaked jack beans for 20 to 24 h in water and then the beans which were swollen were husked and frozen at −20 °C. Upon thawing the seeds after 4 to 6 months of storage and extracting the enzyme, they observed a loss of approximately 3% to 5% in urease activity for the soaked and frozen seeds. Das et al. [9] found that when purified urease was stored in 0.1 M Tris–acetate buffer with pH of 6.8 at −20 °C, the activity half-life was about 50 days and the half-life decreased to 30 days when the urease was kept at 4 °C. They also observed that by adding of 5 mM Dithiothreitol (DTT) the half-life at −20 °C increased to 3 months [10].

Powderization of an aqueous medium by freeze drying (lyophilization) is often used to facilitate transportation and storage of biological materials. In particular, lyophilization is a common method for preparing protein samples for long-term storage. However, the freezing and drying processes can cause protein denaturation [11,12]. Preservation of protein structure and function during lyophilization may require stabilization against both freezing and drying with appropriate additives. Dextran and sucrose have both been used for this purpose, though they protect proteins during these stresses by distinctly different mechanisms [12,13]. Schneider et al. [14] reported that purified commercial urease in the form of powder retained only 20% of its activity upon re-hydration immediately after lyophilization and less than 1% following rehydration after 6 weeks of storage at 33 °C. However, urease stabilized with the addition of dextran prior to lyophilization retained 100% of its activity following rehydration after 3 months storage.

Scope of This Study

As part of an effort to lower the cost of the urease enzyme used in EICP for soil improvement, the stability of three crude urease extracts from jack bean, a urease-rich agricultural product, was evaluated and compared to the stability of commercially available urease powder. The effect of dehusking the jack beans and of filtration on the urease activity of the crude extract after storage as an aqueous solution for a period of up to one year was also evaluated. Three crude extract solutions were prepared: (a) a solution prepared using dehusked beans followed by filtration; (b) an unfiltered solution prepared using dehusked beans; and (c) an unfiltered solution prepared using whole crushed jack beans (without removal of the husks prior to crushing the beans). The effect of storage of the crude extract as an aqueous liquid, of the crude extract upon rehydration following storage in the form of powder (i.e., after lyophilization), and of two commercial urease powders when dissolved in an aqueous solution on urease activity was also evaluated. The effect of storage of the extract in aqueous solution and after rehydration of commercial powdered urease was evaluated both at room temperature (RT) and at 4 °C. In addition, the effect of treatment of the crude extract with dextran and with dextran and sucrose before lyophilization on its stability was investigated.

2. Experimental Program

2.1. Materials

Jack beans (*Canavalia Gladiata*, Sheffield's Seed) were obtained from a commercial vendor. Commercial lab grade urease enzymes from Sigma Aldrich (U1500, Type III, powder, 42,700 U/g activity, Sigma Aldrich, St. Louis, MO, USA) and Fisher Scientific (U2125, powder, no activity reported on the label, Fisher Scientific, Hampton, NH, USA) were also used in this study.

2.2. Urease Extraction and Solution Preparation

Jack beans with and without husk were soaked in tap water, rather than in a buffer as used in traditional extraction, and stored at 4 °C for 24 h. Dehusking was performed manually, a laborious and time consuming procedure. The ratio of jack beans to tap water was 1:4 (*w/v*). For example, to obtain 20 g of dehusked jack beans, approximately 23 g of jack bean were manually dehusked (husk comprises approximately 14% of the mass of jack beans) and the 20 g of dehusked jack beans were then soaked in 80 mL tap water. The soaked jack beans were then pulverized in a kitchen blender for 2 min and filtered through a muslin cloth to remove any coarse solids from the solution. The beans tested without dehusking, referred to herein as whole crushed beans, were simply soaked, without dehusking, and then pulverized and filtered as described above. After that, the remaining solution (i.e., the supernatant) was filtered through a thick layer of glass wool to reduce the amount of fats present. The solution obtained through this process is referred to herein as "crude extract." In one case the crude extract from dehusked beans was filtered through a 0.25 µm filter. This extract is referred to as the filtered crude extract. Note that the ultracentrifugation step typically used for extraction was not used in the crude extraction procedure.

Thirty (30) mL of crude extract made from dehusked jack beans and 30 mL made from whole crushed beans with measured activities of 286 and 141 U/mL, respectively, were stored at RT and at 4 °C prior to additional activity measurement after different storage times. Both unfiltered and filtered solution made from the dehusked beans were tested (they had approximately the same activity) and stored. The solution made from the whole crushed beans was not filtered.

Aqueous solutions prepared from commercially available powdered urease purchased from Fisher Scientific and from Sigma Aldrich and from lyophilized crude extract were also stored and tested in the same manner as the liquid crude extract. A solution containing 355 enzyme units was prepared by dissolving 0.15 g of Fisher Scientific enzyme with a measured activity of 2500 U/g in 10 mL of deionized (DI) water. A solution containing 770 enzyme units was prepared by dissolving 0.05 g of high activity Sigma Aldrich enzyme with a measured activity of 15,000 U/g in 10 mL of DI water. A solution containing 560 enzyme units (U) was prepared by dissolving 0.15 g of the lyophilized crude extract in 10 mL DI water.

2.3. Urease Activity Measurement

Enzyme activity of the crude extract was assayed in a 300 mM urea solution (pH = 7). An aliquot (4.7 mL) of DI water and 5 mL of 300 mM urea were mixed at room temperature in a vial. The ureolysis reaction was then started by adding 0.3 mL of crude urease solution to the DI water-urea solution and the vial was immediately capped and shaken gently at time intervals of 3, 5, and 10 min. 5 mL of 15% trichloroacetic acid was immediately added to the reaction solution (i.e., to the water, urea, and urease solution) and then the vials were opened to stop the reaction. After stopping the reaction, 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 100 µL of Nessler's reagent. After 2 min, the cuvette was placed in a spectrophotometer to measure the optical density of the solution at a wavelength of 412 nm (i.e., OD₄₁₂) to determine the concentration of ammonium.

2.4. Lyophilization

Each 20 g of dehusked jack beans when mixed with 80 mL of tap water yielded 32 mL of jack bean crude extract after the extraction process described above. Fifty (50) mL test tubes containing the crude extract were then placed in a $-80\text{ }^{\circ}\text{C}$ freezer. After freezing, the samples in the test tubes were capped with parafilm (leaving small holes on the top of each tube) and then placed onto the racks of a Labconco Freezone 6 lyophilizer and processed at a vacuum pressure of 50 mT (millitorr) at $-50\text{ }^{\circ}\text{C}$. The lyophilizer uses a condenser that is kept colder than the samples and a vacuum to drive the sublimation process, extracting water vapor from the test tube and depositing it upon the coil. The samples were retained in the lyophilizer until the extract turned to powder, indicating that the residual moisture has been removed. The mass of the powder from lyophilizing 32 mL of crude extract was approximately 3.91 g. After the lyophilization, the powdered extract was stored at RT and $4\text{ }^{\circ}\text{C}$ until it was rehydrated.

2.5. Whole Bean Storage and Dextran and Sucrose Stabilization

In addition to the effect of storage on the activity of the hydrated commercial enzymes, the liquid extract, and the lyophilized crude extract following rehydration, the effect of storage on unprocessed jack beans stored at RT and $4\text{ }^{\circ}\text{C}$ was also examined. The jack beans were stored whole and then periodically sampled to create crude extract and make activity measurements in the same manner as described above (i.e., using the Nessler method). In addition, as part of the effort to enhance the stability of the crude extract, the effect of adding (a) dextran and (b) dextran with sucrose in the crude extract prior to lyophilization was evaluated. Various concentrations of dextran and dextran with sucrose were added to the crude extract solutions. The concentrations of dextran and sucrose used in this study were based upon previous work on enzyme stabilization using these substances [15].

3. Results

3.1. Storage Capability of Whole Jack Beans

The results of activity measurements on the stored whole beans are presented in Figure 1 in terms of the total units in 38 mL of crude extract. As illustrated in Figure 1, the decrease in total units of urease in the crude extract prepared using beans stored both at $4\text{ }^{\circ}\text{C}$ and at RT was negligible.

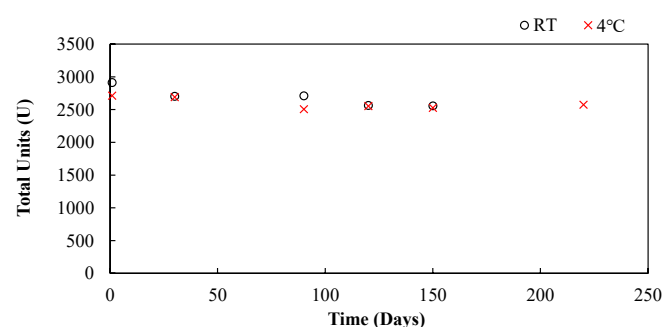


Figure 1. Urease activity measurements of jack bean crude extracts after storage of the whole beans at RT and $4\text{ }^{\circ}\text{C}$.

3.2. Storage Capability of Crude Extract Solution

Figure 2 shows the activity measurements in terms of total urease units (U) versus time for the three different crude extract solutions (whole crushed beans, dehusked beans-unfiltered, dehusked beans-filtered) stored at RT and $4\text{ }^{\circ}\text{C}$. The results show a significant decrease in the activity of all three enzyme solutions with storage time, presumably due to degradation of the enzyme. However, crude extract activity from whole crushed beans decayed more rapidly at both temperatures compared to the dehusked beans. In addition, the extract from whole crushed beans had a significantly lower number of total units than

the extracts from dehusked beans, possibly because the presence of the husks interferes with the proteins in the solution, which are essential to the efficiency of urease activity [16]. It is postulated that the faster decay of the urease extract made from whole crushed beans is a result of this mechanism. There was little difference in the decay rate of the unfiltered and filtered extracts made from dehusked beans.

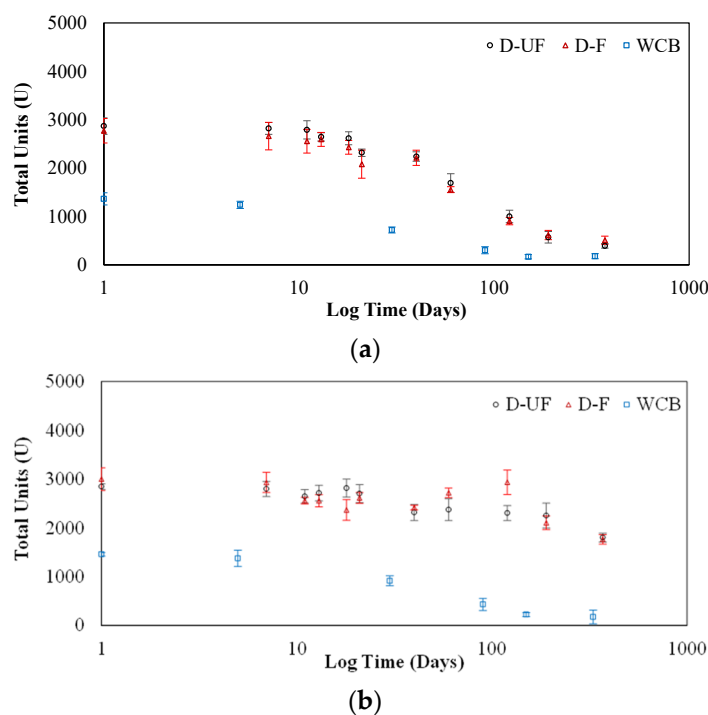


Figure 2. Urease activity measurement determined at different storage times for three different sources of prepared crude extract at (a) room temperature and (b) 4 °C. (D-UF) Dehusked Unfiltered, (D-F) Dehusked Filtered, (WCB) Whole Crushed Beans.

Figure 3 compares the percentage of units lost for the three sources of crude extract solution at different storage times at (a) RT and (b) 4 °C. The extract made from whole crushed beans lost approximately 90% of its activity in approximately 200 days at RT and 4 °C. Both enzyme solutions made from dehusked beans (i.e., the filtered and unfiltered extracts) stored at RT lost on the order of 85% of their activity in approximately 365 days. In contrast, when stored at 4 °C the solutions made from dehusked beans (filtered and unfiltered), were able to retain approximately 60% of their activity after storage for a period of up to 365 days. Filtering the crude extract from dehusked beans did not affect the measured total units of the extracts at either storage temperature.

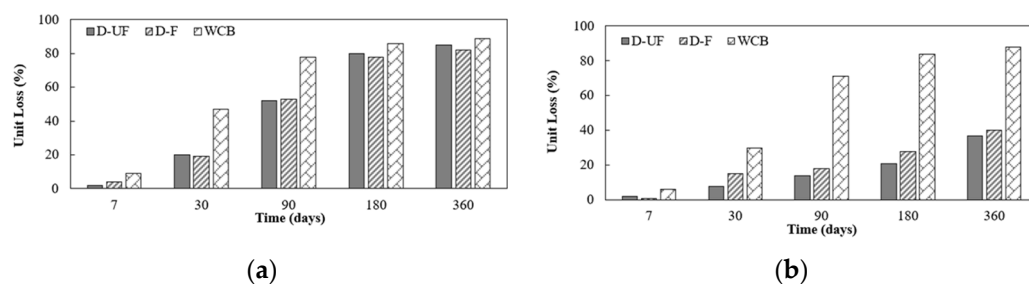


Figure 3. Percentage of unit loss of the three prepared crude extracts in solution at different storage times at (a) RT and (b) 4 °C. (D-UF) Dehusked Unfiltered, (D-F) Dehusked Filtered, (WCB) Whole Crushed Beans.

3.3. Storage Capability of Lyophilized Crude Extract

Freeze-drying (lyophilization) was performed on crude extract to study the effect of storage of crude urease extract after lyophilization. Freeze drying was conducted on crude extract from the three sources described above: (a) filtered dehusked beans; (b) unfiltered dehusked beans; and (c) whole crushed beans. The powder produced by lyophilization was stored at RT and 4 °C, rehydrated at various time intervals up to 360 days, and then subjected to activity measurement. Figure 4 presents the total units of urease for the rehydrated powders for all three extract sources. The results show remarkable stability for the lyophilized crude extract from all three sources. The percentage of total units' loss was less than 10% within each of the three groups for all three sources after 360 days of storage at both RT and 4 °C.

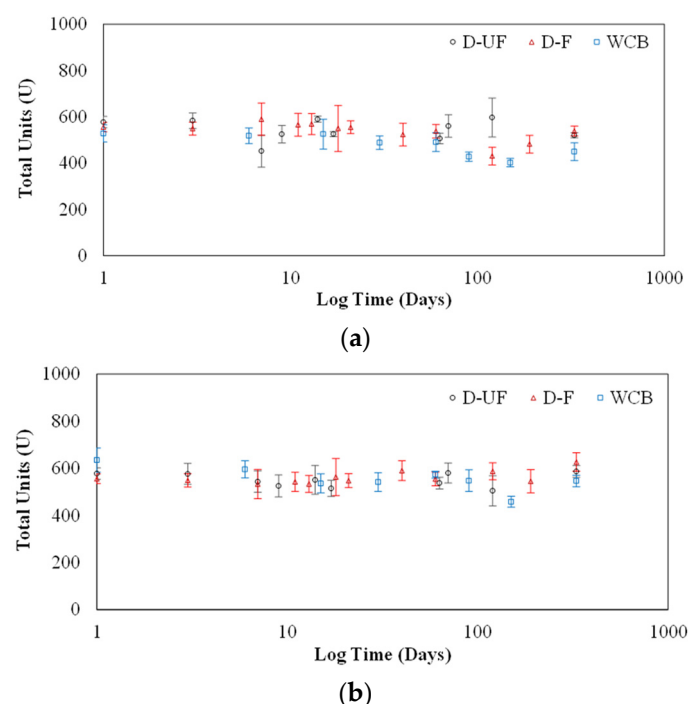


Figure 4. Urease activity measurements in units determined at different storage times for different sources of lyophilized prepared crude extract at (a) RT and (b) 4 °C. (D-UF) Dehusked Unfiltered, (D-F) Dehusked Filtered, (WCB) Whole Crushed Beans.

3.4. Storage Capability of the Crude Extracts vs. Commercial Enzymes

The stability of commercial enzymes after hydration (i.e., after preparation as an aqueous solution) was also evaluated for comparison to the stability of the crude extract both in aqueous solution and after lyophilization. Figure 5 presents the total units of urease for the hydrated solutions made from the two commercial enzymes (Sigma and Fisher) at storage times of up to 360 days at both RT and 4 °C. The results indicate that the activity of the two commercial enzymes reduced markedly with the storage time once hydrated into solution. The loss of activity was more much pronounced at RT than at 4 °C, as also observed for the crude extract solutions. After storage of the solubilized commercial enzyme for 1 year, the urease activity reduced by approximately 35% at 4 °C (Figure 5b) and by over 90% at RT (Figure 5a).

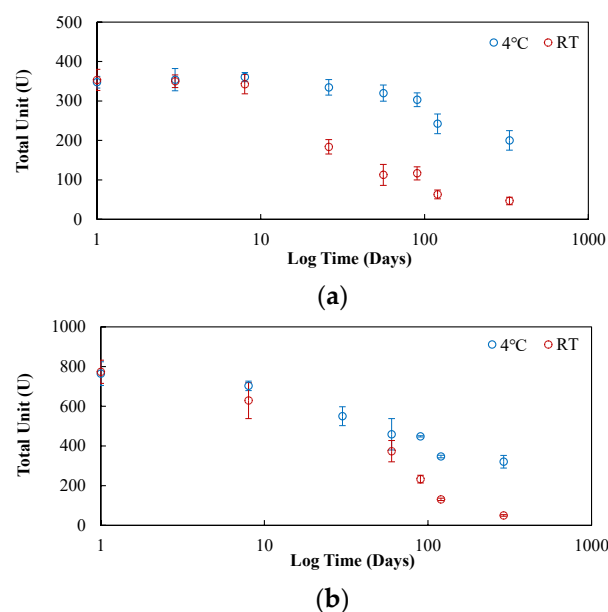


Figure 5. Urease activity measurements when stored at RT and 4 °C at different times for (a) Fisher Scientific and (b) Sigma Aldrich aqueous solutions.

In Figure 6, the stability of the crude extract from the unfiltered dehusked jack beans when stored in solution and in powder form (i.e., after lyophilization) is compared to the stability of the two commercial enzymes in solution. For the case of the crude extract solution, the unfiltered crude extract from the dehusked beans was used for this comparison because, as shown in Figure 2, eliminating the filtration step did not adversely affect urease activity and removing the jack bean husks enhanced urease stability in the crude extract. Note that the activity and stability of all the three sources of the lyophilized crude extract were similar. The results presented in Figure 6 indicate that lyophilized crude extract maintained remarkable urease activity over time regardless of storage temperature. For example, at room temperature the loss of activity for the lyophilized crude extract was less than 10% over 360 days.

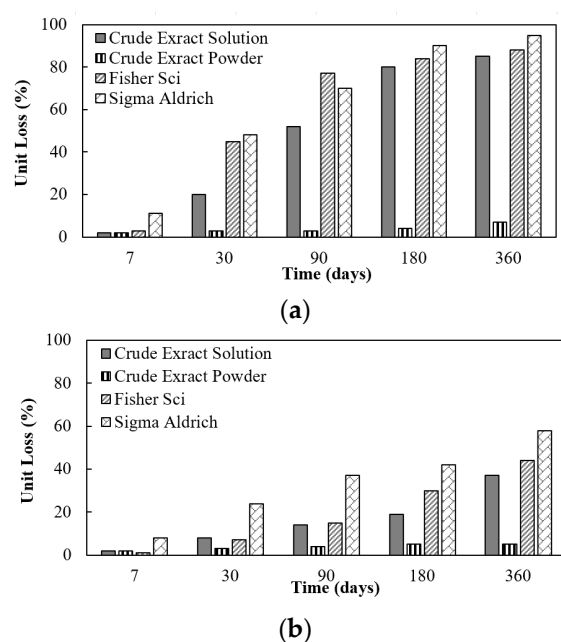


Figure 6. Percentage of unit loss of the jack bean crude extract, Fisher Scientific, and Sigma Aldrich at different storage times at (a) RT and (b) 4 °C.

3.5. Storage Capability of Crude Extract Powder with Dextran and Sucrose

Because the lyophilized crude extract did not show a difference in stability when stored at RT and at 4 °C, the stability of the lyophilized crude extract with the addition of dextran and sucrose was studied at RT only. The effect of adding dextran (mol wt. = 40,000) at 0%, 2%, and 4% (*w/v*) on the storage stability of the lyophilized crude extract at RT is presented in Figure 7. It can be seen from Figure 7 that the use of 2% dextran extract enhanced the total units of urease by 40% at the early stages of storage, e.g., over a period of up to 15 days. At longer storage times, while there is some scatter in the data, ultimately 2% dextran proved to be most effective at inhibiting activity loss, with about a 20% reduction in activity after 360 days. While the 2% dextran extract had a higher initial number of units than the 0% crude and 4% dextran extracts, the differences in number of total units in the crude extract with and without dextran becomes relatively insignificant after about 15 days. This suggests there is no real benefit to adding dextran alone prior to lyophilization, as the lyophilized crude extract without dextran also lost less than 20% of its activity after 360 days.

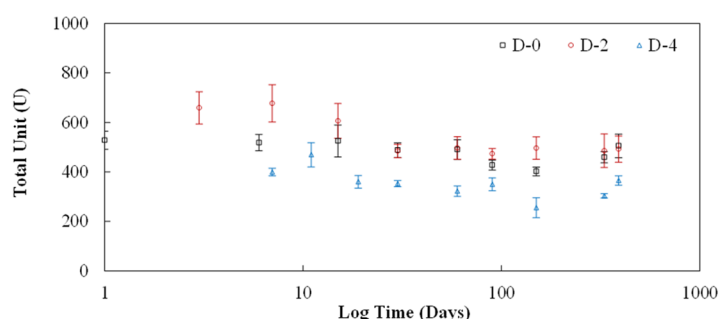


Figure 7. Urease activity measurement in total units for 0%, 2%, and 4% of dextran (D-0, D-2, and D-4) added during lyophilization of crude extract and rehydration and storage at different times at RT.

Figure 8 shows the measurements of total urease units versus time for jack bean crude extract after lyophilization in the presence of different concentrations of dextran and sucrose at room temperature for concentrations up to 4.75% (*w/v*). The relative proportion of dextran and sucrose were 1:1 (*w/w*). The results do not suggest any benefit for any of the concentrations tested and decreasing effectiveness at the highest concentrations used in this study. The total units of urease enzyme appear to have slightly increased after lyophilization and rehydration in the presence of 2.37% dextran and sucrose at early stages of storage (although this could be simply due to natural variability in the specimens). Increasing the percentage of dextran and sucrose from 2.37% to 3.56% and 4.75% reduced the urease activity of lyophilized crude extract following storage and rehydration.

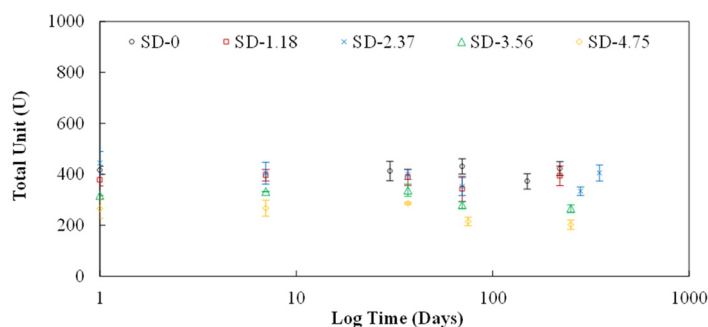


Figure 8. Urease activity measurement in total units for different concentrations (0%, 1.18%, 2.37%, 3.56%, and 4.75%) of sucrose and dextran (SD) added during lyophilization of crude extract after rehydration and storage at different times at RT.

4. Conclusions

The storage capability of urease extracted from jack beans through a simple extraction procedure was investigated for three different extraction solutions, at two different temperatures, before and after lyophilization. The simple extraction process requires only a kitchen blender, muslin cloth, and glass wool, uses tap water rather than a buffer solution, and does not require centrifugation. The stability of the jack bean crude extract stored at room temperature and 4 °C in both solution and upon rehydration following lyophilization was studied by measuring the urease activity at storage times of up to one year (360 days). The key findings of this study can be summarized as follows:

- The effect of the storage on the whole jack beans prior to extraction when stored at room temperature and 4 °C was insignificant.
- The stability (storage capability) of the crude extract improved by storing it at 4 °C rather than room temperature.
- Filtration and dehusking steps are not required as part of the crude extraction process, further simplifying the process; however, the dehusking step was effective for maintaining the stability of the urease in the crude extract, particularly when stored at 4 °C.
- The jack bean crude extract both in solution and when lyophilized showed better stability (longer storage capability) compared to the commercialized enzymes in solution.
- Lyophilization of the crude extract was found to be the most effective approach to prolong the storage capability of the enzyme. After storing the lyophilized crude extract for 365 days, the loss of urease activity following rehydration was less than 10% regardless of the storage temperature.
- The addition of 2% dextran into lyophilized crude extract provided no significant benefit in maintaining stability of urease.
- The use of hybrid dextran-sucrose at 2.37% enhanced the storage capability of the lyophilized crude extract, resulting in little to no loss of activity over 365 days of storage.

This demonstrates that the crude extract can be stored after production followed by lyophilization for periods in excess of one year without a significant loss of activity offers the potential for a significant reduction in the cost of applying enzyme induced carbonate precipitation (EICP) in engineering practice by mass production of the enzyme.

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