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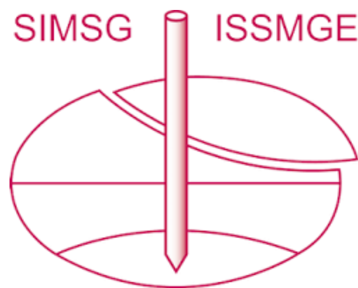

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Towards a monitoring tool to quantify urease during biocementation treatment at microscale

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ABSTRACT

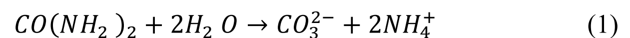
Biocementation consists in using urease enzyme and a solution rich in urea and in calcium to precipitate calcium carbonate (biocement). When applying this treatment in soils, the biocement minerals bond the grains improving overall soil's hydro-mechanical properties. For the practical use of this technique, it is necessary to be able to predict the properties of the treated soil after following specific protocols, by preference avoiding non-destructive testing such as those performed on samples extracted after the treatment. The amount of biocement precipitated depends on the amount of urease enzyme, urea and calcium. This idea has inspired the development of one magnetoresistive biosensor to detect urease, to be used as a non-destructive monitoring tool during the treatment. A magnetoresistive platform was used to quantify the signal, which is related to the urease concentration through a calibration curve. The sensor was tested to measure the enzyme present in the inflow and outflow fluids used to treat cylindrical soil samples (2.5 cm diameter and 2.0 cm height), prepared with a uniform grading size sand (D_{50}=0.3 mm). Purified urease from *Canavalia ensiformis*, was used. The improvement of the biocemented sand samples was quantified through measuring the calcium carbonate content of the soil after the treatment and the values were related with the amount of enzyme retained by the soil, determined using the sensor readings. This work found, for the first time, the relationship between the measured concentration of urease retained by soil and the calcium carbonate content precipitated. This relationship is an important tool for monitoring the treatment, without the need to use destructive tests or even stop the treatment.

Keywords: Biocement; urease; biosensor; monitoring.

1. Introduction

Nowadays, sustainability is one of the major concerns of engineering design, being the environmental impact as important as safety and costs. It is necessary to study and give credibility to environmentally friendly techniques in engineering. This is the case of biocementation techniques, which have a lower carbon footprint when compared with the traditional techniques usually used to improve soils for engineering purposes, such as Portland cement or polymers. In fact, cement production consumes energy during extraction and production processes such as heat, grind, and transport (Habert et al. 2010). For example, C8/10 concrete releases 75.6 kg CO_2 eq/ton, while biocement releases between 11.6 – 24.4 kg CO_2 eq/ton of greenhouse gas emissions (Røyne 2017). Therefore, the use of biocementation has the potential to reduce, considerably, the production of greenhouse gas emissions in a range of 70-85%.

Biocementation is a soil improvement technique where biological agents such as bacteria or enzyme are added to the soil to produce calcium carbonate (biocement). The biocement bonds the grains and clogs the soil pores, improving the hydro-mechanical properties of this porous medium. Biocementation occurs when the urease enzyme, urea ($CO(NH_2)_2$) and a calcium source (Ca^{2+}) are combined. The urease enzyme catalyzes the hydrolysis of urea (Eq. (1)), and calcium carbonate precipitates due to the combination of calcium and carbonate ions present in the treatment solution (Eq. (2)) (Stocks-Fischer, Galinat, and Bang 1999).



$$CO(NH_2)_2 + 2H_2O \rightarrow CO_3^{2-} + 2NH_4^+ \quad (1)$$

$$CO_3^{2-} + Ca^{2+} \rightarrow CaCO_3 \downarrow \quad (2)$$

The treatment is known as microbially induced calcite precipitation (MICP) when the urease is produced by a microorganism such as non-pathogenic bacteria (for example *Bacillus pasteurii*) or enzyme induced carbonate precipitation (EICP) when using enzyme urease (from *Canavalia ensiformis* (jack bean), for example). The advantages of using the enzyme instead of bacteria are mainly that it is easier to produce large quantities of the enzyme as it is extracted from plants, is commercially available in powder and the enzyme concentration added to the soil is known. The disadvantages are mainly the cost of production.

Biocementation technique has several potential applications in geotechnical engineering, such as (i) enhancing stability for retaining walls, embankments, slopes and dams (Borges et al. 2020); (ii) reducing the

permeability in dams and dykes (Chu et al. 2013); (iii) increasing the bearing capacity of foundations and underground constructions (Van Paassen 2011); (iv) protecting for wind erosion by binding of the dust particles on exposed surfaces (Gomez et al. 2015); (v) strengthening slopes to prevent water erosion and failure in coastal areas and rivers (Fernández Rodríguez and Cardoso 2022; Salifu et al. 2016); (vi) reducing the liquefaction potential of soil; (vii) increasing the resistance to petroleum and natural gas extraction borehole degradation during drilling and extraction. There are successful fieldworks performed by companies such as Soletanche-Bachy (Esnault Filet et al. 2020) and Medusoil (Dimitrios et al. 2020).

To promote this soil improvement technique as an alternative to traditional techniques it is important to develop design procedures, monitoring tools and evidence on the durability of the treatment. The study presented in this paper focuses on the development of monitoring tools able to be used during the treatment, to ensure that the biological reactions are in progress. These reactions are strongly dependent on environmental conditions and therefore monitoring, either in real-time or in time-lapse, brings the advantage of earning trust that the treatment is occurring.

A usual way to monitor biological reactions is by performing chemical analysis of the collected fluids, to detect the presence and concentration of ions in direct or indirect ways. In case of biocementation, it is usual to measure pH, and ammonium (NH^{4+}) and chlorides concentrations (Whiffin et al. 2007). An alternative is measuring urease activity, which is a method where the quantity of urea hydrolyzed per unit of time is computed through changes in the electrical conductivity of the fluids along time immediately after adding urea to the fluid (Harkes et al. 2010). These are indirect methods with some limitations. As an alternative, it is possible to measure urease activity directly by standard enzymatic assays such as spectrophotometry, fluorimetry, potentiometry and amperometry. However, these traditional assays are mostly time-consuming, require extensive sample pre-treatment, and are unsuitable for *in situ* implementation (Narayanasamy 2011). For this reason, a magnetoresistive biosensor was developed to detect enzyme urease in effluent fluids. These types of devices are highly sensitive and selective to their target, rapid processing, user-friendly, easy to implement, and cost-beneficial (Salek-Maghsoudi et al. 2018). This sensor is presented in this paper, as well as an example of its application using fluid from the treatment of a real soil.

2. Biosensor used

2.1. Biosensor to detect enzyme urease

A biosensor is an analytical device capable of detecting an analyte (substance or chemical constituent interesting from an analytical point of view) in a large variety of biological samples such as body fluids, food samples, cell cultures and environmental samples. The analytes can be, for example, nucleic acids, natural products, antibodies, enzymes, cell receptors, organelles, microorganisms, tissues, etc., so biosensors are very versatile. As illustrated in Figure 1, their working principle combines a biological entity, that recognizes the sample analyte, with a transducer or a detector element, that transforms the signal resulting from the interaction of the analyte with the biological entity into a signal that can easily be measured and quantified. Transducers in biosensors may be optical, electrochemical, micromechanical, piezoelectric, magnetic, or thermometric, each producing either discrete or continuous electronic signals proportional to the quantity of analyte present in a sample.

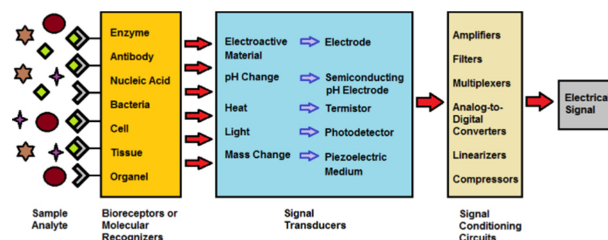


Figure 1. Biosensor operating principle: main subsystems (Vargas-Bernal et al. 2012).

Since in this work we aim to detect a protein, the bioreceptors used were antibodies (the "Y" shape molecules in Figure 1) that specifically bind to urease and that are immobilized on the sensor/magnetic labels surface.

The recognition strategy developed in this work is similar to the one employed for ELISA (enzyme-linked immunosorbent assay) immunoassays which rely on antibodies/antigens to detect a target molecule using highly specific antibody-antigen interactions to produce a measurable result. These assays are the most used, reliable, and sensitive methods for quantifying enzymes.

Immunoassays can have different immobilization and capture strategies. Among these we have the direct assay, where no immobilization antibody is used on the sensor surface, with urease attaching directly to it; and sandwich assay, where the biosensor is coated with a known concentration of antibody specific to urease, only allowing the immobilization of urease protein on the surface.

Besides sensor functionalization, streptavidin-coated magnetic nanoparticles (MNPs) are also functionalized with antibodies that are then used to capture the target in solution. This MNP-target complex is then passed over the sensors. If there is urease present in the sample, the sensor will change its resistance, otherwise the MNPs will not bind to the sensor, with no change in sensor signal.

2.2. Magnetoresistive sensor

The fabrication of the magnetoresistive biochip (MR-biochip) comprises several steps of microfabrication, as described in Martins et al. (2009). Each MR sensor consists of two spin-valve (two magnetic layers separated by a non-magnetic spacer) with dimensions 46.6 x 2.6 μm^2. The sensors were microfabricated with the stack Si/Al2O3 100/Ta 1.5/NiFe 2.8 /CoFe 2.8 /Cu 2.7 /CoFe

3.3 /MnIr 7.5 /Ta 5 (thicknesses in nm). In the end of the microfabrication process, the sensors were coated with a gold film layer (Cr 5nm /Au 40nm) for biological immobilization purposes. The spin-valve sensors used were characterized, showing a minimum resistance of 730-850 Ω and a magnetoresistance of ~5.5 %.

2.3. Magnetoresistive biochips detection

The LoC device used consists of a magnetoresistive platform using biochips with magnetoresistive sensors. Previous work was done to establish a calibration curve using a direct immunoassay (Albuquerque et al. 2019). However, this is not the best approach when using complex samples (e.g. soil samples), since it allows for greater non-specific adsorption to the sensor surface, diminishing specific binding of urease, and thus increasing false negatives.

The electronic read-out set-up used in this work was the same as that reported by (Martins et al. 2010). The measurement conditions used were 1 mA DC for sensors biasing, an in-plane transverse external AC excitation magnetic field of 1.1 kA/m rms (211 Hz) and a DC field of -3.2 kA/m for MNP magnetization.

After biochip insertion in the platform, an U-shaped microfluidic channel made of PDMS, and prepared as described in (Dias et al. 2016), was used in order to transport the sample over the sensing area of the chip. The experiment started by acquiring a baseline of the sensors, then, the sample was pumped until the PDMS channel was filled. The target solution was left to settle down over the sensing area for 20 minutes. Finally, it was performed a washing step to remove the unbound MNPs and it was record the binding signal. The difference between the baseline voltage and the binding voltage results in a voltage variation (ΔV) which corresponds to the detection signal that is then normalized by the sensor output (ΔV/V) (Figure 2).

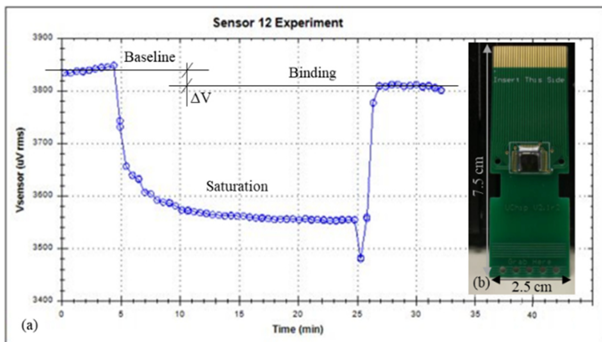


Figure 2. The output of the electrical potential of the biosensor with time (a) and the biochip used (b).

2.4. Calibration curve

The calibration curve to estimate urease concentration of the protocol for the sensor is presented in Figure 3, where a function was adjusted to the data with a coefficient of determination R^2=0.98. The sensor has a detection range between 10 and 30 mg/ml of urease concentration. The blank measured was - 0.03% ± 0.05, so a signal measured equal to or under 0.02% is considered as indetectable, with a urease concentration of 0 mg/ml. Urease concentrations detected over 30 mg/ml are considered over the range. Eq. 3 estimates the urease concentration (uc) in mg/ml, with a signal in %.

$$uc = 6.51 \ln(signal\ (\%)) + 20.48 \quad (mg/ml) \qquad (3)$$

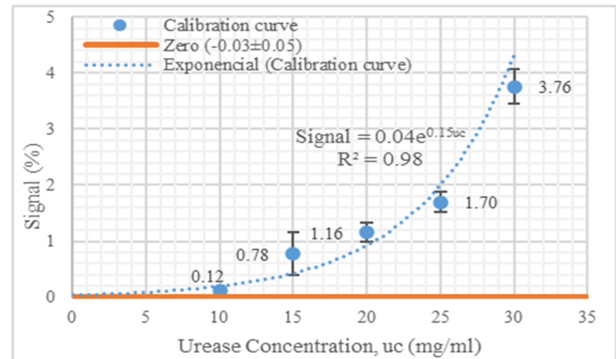


Figure 3. Calibration curve of the sensor using the magnetoresistive platform.

3. Materials and methods

3.1. Soil and samples preparation

The soil tested is a silty sand with uniform grading size distribution (D_{50}=0.3mm, C_c=0.94 and C_u=3.18 (Figure 4). The minerals present were mainly silica with solid particle density Gs= 2.70.

The samples were prepared in cylindrical plastic molds (Figure 5), with 2.5 cm diameter and 2.0 cm height, by carefully placing the dry material with the final dry volumetric weight of 15 kN/m^3, which corresponds to void ratio of 0.78 and void volume of 4.3 cm^3. The plastic molds were set over a plastic grid with 5 mm of height and filter paper, to allow the drainage of the treatment fluids and prevent particles from dragging through the specimen bottom, respectively.

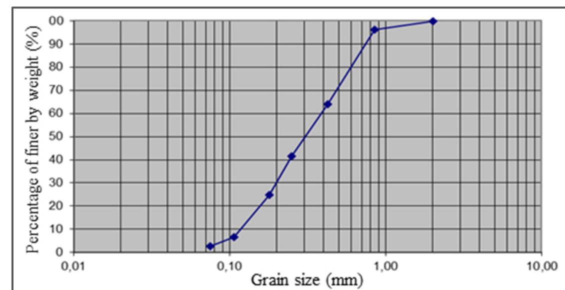


Figure 4. Grading size distribution curve of the sand.

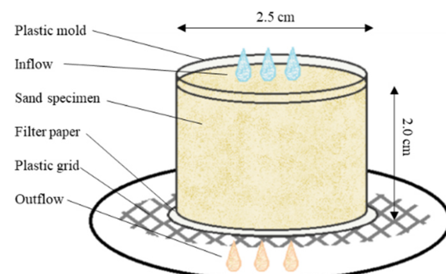


Figure 5. Set up to prepare biocemented treated soil samples.

3.2. Treatment protocol

The urease solutions (US) were prepared in phosphate buffer 0.1 M pH 7.4 (PB), to keep the pH stable. Urease *C. ensiformis* (Jack bean), was used to prepare the treatment fluids with the concentrations 10, 20 and 30 mg/mL. The feeding solution (FS) was prepared using urea and calcium chloride (source of calcium), each at 0.5 M and prepared with distilled water. The solutions were irrigated in the soil on the top of the sample (inflow), using a syringe to distribute drops homogeneously in the entire area. The total volumes used were multiples of the void volume Vv (Vv=4.3 cm^3) and left on the top of the sample infiltrating just by gravity. The specimens were saturated with distilled water, then they were injected with 1 Vv of US and 4 Vv of FS, they were left overnight at room temperature, and then they were washed with 2 Vv of distilled water from the top of the specimen. In total, 9 samples were prepared and saturated with different fluids: 1 with distilled water (dH2O), 2 with feeding solution and no urease (US0FS), 2 with urease solution at 10 mg/ml (US10FS), 2 with urease solution at 20 mg/ml (US20FS) and 2 with urease solution at 30 mg/ml (US30FS).

3.3. Liquid samples for the biosensor

The collection of liquid samples (outflow or analyte) after passing through the soil to measure the urease concentration was made at the bottom of the samples in the space left by the grid. The above was done with the intention to collect only US after passing through the soil, so the collection was made after the injection of the US and before that of FS. To avoid cross-contamination the grid was cleaned before the collection. The collection of the outflow in the bottom was made right after adding some drops of FS on the top of the soil. These outflow samples were kept at 4 ºC overnight and filtered using a #0.2 µm filter before being used in the immunoassay.

3.4. Calcium carbonate content

The calcium carbonate content (CCC%) was determined using a washing method adapted from NP E-196. The dry mass of the sand sample was measured using an oven at 105°C for 24 hours before the test (m_1) and then the soil was placed in hydrochloric acid (0.5 M) until the reaction was complete. Then it was washed with distilled water, filtered and dried again in an oven at 105°C for 24 hours to measure the final mass (m_2) The calcium carbonate content is the ratio between the dry mass lost after the test (mass of biocement which is acid soluble) and the initial dry mass of the sample, calculated according to Eq. (3).

$$CCC\% = \frac{m_1 - m_2}{m_1} \times 100 \quad (4)$$

4. Results and discussion

Figure 6 shows the calcium carbonate content determined for all the soil specimens prepared considering the amount of enzyme added in the treatment fluid (inflow). The blue dashed line marks the calcium carbonate content of the sample to which only water was added (dH2O), so it sets a reference for the samples with treatment.

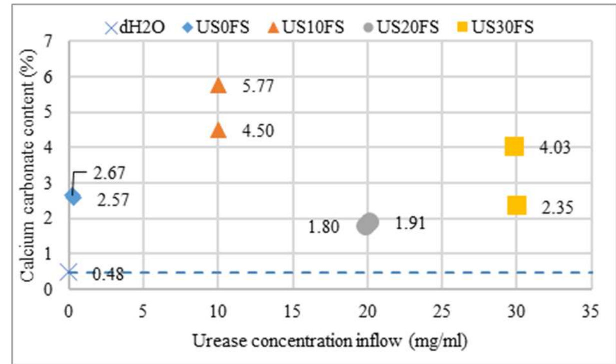


Figure 6. Calcium carbonate content vs urease concentration of the inflow.

The results in Figure 6 show that all samples are above the reference line, which indicates that there was precipitation of biocement caused by the treatment. The presence of biocement in the sample to which only feeding solution was added (US0FS) is explained by the activity of indigenous bacteria, because the soil was not sterilized before the treatment. There is no correlation between the amount of enzyme added and the amount of calcium carbonate precipitated.

The biosensor was used to measure the urease concentration of the outflow solution ($uc_{outflow}$), which indicates the amount that was not retained by the soil. By knowing the urease concentration of the prepared urease solutions (uc_{inflow}) added to the soil specimens, it is possible to compute the amount left inside the specimen (uc_{inside}) using Eq. 5.

$$uc_{inside} = uc_{inflow} - uc_{outflow} \quad (5)$$

The information from the tests performed on the soil samples concerning calcium content measurements, and the measurements from the biosensor considering already the urease concentration fixed by the soil (urease inside the soil, computed using Eq. 5) are in Table 1. The values found for the soil samples to which enzyme was added are plotted in Figure 7, where it can be seen now a good correlation between the amount of enzyme fixed by the soil and the amount of calcium carbonate precipitated.

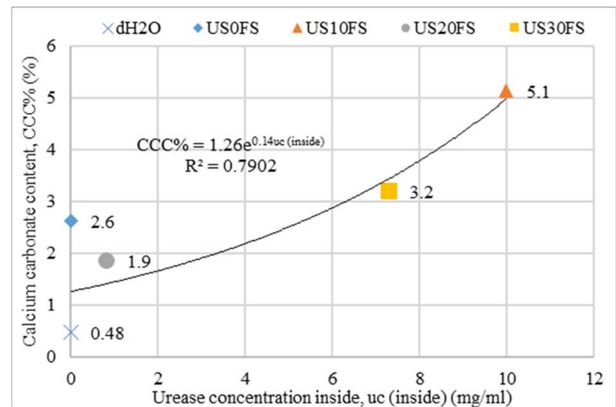


Figure 7. Calcium carbonate content plotted vs urease concentration left fixed by the soil (inside the soil).

Table 1. Calcium carbonate content and urease concentration inside the sand samples

	Calcium carbonate content (%)	Urease concentration inside (mg/ml)
dH2O	**0.5***	**not measured**
US0FS	2.7 2.6	not measured
	2.6*	**not measured**
US10FS	5.8 4.5	10.0
	5.1*	**10.0***
US20FS	1.8 1.9	2.8 0.1 0.0
	1.9*	**0.8***
US30FS	4.0 2.4	14.0 3.6 4.3
	3.2*	**7.3***

*average

As it can be seen in Figure 7, calcium carbonate content increases with the increment of urease concentration fixed inside the specimen and can be adjusted with an exponential function (Eq. 6). Although there is some error, this result improve compared to when the urease added was considered (Figure 7). This is because only the urease inside the soil can produce calcium carbonate inside the soil. Therefore, Eq. 5 defined knowing the urease concentration inside the sample in mg/ml (uc_{inside}) determined by the biosensor, can be used to estimate the calcium carbonate content (CCC, in percentage).

$$CCC\ (\%) = 1.26\ e^{0.14\ uc_{inside}\ (mg/ml)} \quad (6)$$

Although this equation is specific to this work, a new curve can be easily fitted using data from different soils or treatment protocols with the calibration curve defined for the biosensor. This strategy of monitoring can be prepared in the laboratory to be used *in situ* as a nondestructive test. However, the reduced size of the samples for the biosensor implies the need to define sampling protocols to be representative of the large volumes of treated soil.

5. Conclusions

In this paper, sand samples were treated with the EICP technique and the outflow fluids were collected to detect urease using the biosensor. The calcium carbonate content of all the biocemented sand specimens were also measured.

Concerning the biosensor, the protocol to detect purified urease from *Canavalia ensiformis* was optimized, using a magnetoresistive platform to perform sandwich immunoassay. The calibration curve of the biosensor was defined, with a range between 10 and 30 mg/ml of urease.

Using the data from the sensor it was possible to estimate the urease concentration fixed inside the sample, i.e., the urease that was not washed when the feeding solution was added. Data found allowed to understand that the amount of enzyme fixed was smaller than that added to the soil, which suggests the need to investigate ways to fix the enzyme and improve the efficiency of the treatment.

The information about the amount of enzyme fixed in the soil was related with that measured in the soil samples to define a relationship between the urease concentration inside the sample and the calcium carbonate content precipitated. This relationship can be used in the future to predict the amount of biocement in a sand sample treated with the EICP technique in similar conditions. Relationship such as the one found can be determined in the laboratory for other types of soils or treatment protocols using the same biosensor protocol and its calibration curve.

The biosensor optimized has strong potential to be used as a monitoring tool during the biocementation treatment, which will allow to estimate the effects of the treatment and therefore help designing biocementation treatment for different engineering geotechnical solutions.

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