

Soil Biocementation via Enzyme Induced Carbonate Precipitation (EICP) Method Employing Soybeans as a Source of Cheap Enzyme

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In this work, the soil improvement technique via Enzyme Induced Carbonate Precipitation (EICP) was investigated by employing, as an alternative to expensive pure enzymes, enzymes extracted from agro-food wastes (tomato, apple, and soybean) such that the process is economically viable and fully embraces the concept of the circular economy. The feasibility of the process was evaluated by monitoring calcium carbonate precipitation in a sand sample. The effect of selected operative parameters was investigated during the injection into different grain size sand samples. The optimal operating conditions in terms of sand grain size, temperature, Urea/Calcium concentration were found. Results demonstrated the effectiveness of this alternative solution for EICP method in term of acquired material strength and the possibility to operate sand consolidation through an economically sustainable process.

1. Introduction

Various strategies have been employed to improve the performance of sandy and poorly consolidated soils in such a way as to make them suitable for various purposes, e.g., from foundations for lightweight structures such as pavements, riverbanks. Most of these soil improvement methods rely on replacing the soil or adding binders such as cement or lime to strengthen the soil. Because of the significant carbon dioxide (CO₂) emissions during cement production, more sustainable approaches to improving soils without using cement are being sought. Induced calcite precipitation has been studied as a viable solution for soil improvement among other options. There are several techniques used to induce calcite precipitation primarily by microbes (MICP) and enzyme-induced calcite precipitation (EICP) (Rohy et al., 2019).

The enzyme urease initiates the hydrolysis of urea, which is the starting point of MICP and EICP processes. A cytosolic enzyme called urease oversees the breakdown of urea into ammonia and carbon dioxide, Eq. (1). Reacting with water, hydroxide ions are formed, which means an increase in pH.

Due to the rise in pH, carbon dioxide simultaneously dissolves in water and produces bicarbonate and hydrogen ions; carbonates are produced by the reaction of bicarbonate and hydroxide ions. Due to its low solubility, calcium carbonate develops and precipitates when there are calcium ions present, as stated in Eq. (2).



Urease is responsible for the hydrolysis of urea, whether it is produced by bacteria or used as a free enzyme. Consequently, the source is different between MICP and EICP methodologies. Urease-producing bacteria are the basis of MICP approach, and a regulated growth environment is required (Almajed et al., 2021).

Calcification solution, consisting of urea and calcium chloride, is added to the microbial solution after being mixed with sand. The enzyme promotes hydrolysis of urea to produce carbonate ions, and Ca²⁺ ions bind to the hydrolysis products to induce CaCO₃ precipitation. Sand consolidation is the final product.

Although the microbiological technique is environmentally friendly and has other benefits, it is an expensive procedure that accounts for about 90% of the overall cost (Almajed et al., 2021).

Furthermore, there is concern about inadequate adaptability to different soils (Almajed et al., 2021). Because MICP is effective only in the subsoil, it is possible that other areas of the soil may not allow adequate bacterial growth.

Due to the bacteria's inability to settle on the relatively smaller pores, this technique does not produce results that are acceptable when applied to exceedingly fine soils (Almajed et al., 2021).

As stated by some authors (Almajed et al., 2021), after treatment, the approach under consideration leaves bacteria in the soils, necessitating the approval of the relevant authorities and routine inspection to guarantee the existence of the microbial is harmless (Chen et al., 2021).

In addition, MICP oversees the generation of NH_4^+ , a by-product that should be treated because it is detrimental to the environment. Additionally, this will raise pH levels, potentially causing corrosion and additional chloride pollution of groundwater after CaCO_3 precipitation (Chen et al., 2021).

Problems in solidifying sandy soil using the urease bacteria of the soil itself are investigated in Chen et al., 2021. Primary screening, rescreening, strain identification, and other procedures are required, and the chosen strain's urease activity may not be enough.

The uncontrollability of enzymatic activity and the difficulty of growing urease-producing bacteria for the required technical skills are further reasons limiting the implementation of MICP (Zhao et al., 2021).

As a result of all these issues, this study focuses on EICP, the most replicable brand of these stabilization technologies. This is because the process begins with the free enzyme and solves the first step without the need for strong expertise in microorganism cultivation, so the experimental setup can be much simpler and cheaper and is able to work even on finer soils.

The huge disadvantage of this approach is that free urease, like almost all enzymes, is very expensive; on a common site, it can be found for 17 €/g and can account for as much as 80 % of the total cost of reagents (Ahenkorah et al., 2021). To overcome this problem, an alternative source of urease enzyme can be employed, such as from plant seeds, particularly, as a new possibility, from soybeans.

2. Materials and methods

2.1 Chemicals

Urea ($\text{CO}(\text{NH}_2)_2$, Carlo Erba, >95%) and anhydrous calcium chloride (CaCl_2 , Carlo Erba, >95%) were used for the calcifying solution (CS) without further purification, Magnesium chloride (MgCl_2 Carlo Erba, >95%) was added in some cases. Soybeans (Soia gialla biologica Biostock distributed by Probios S.p.A.) were used for the enzymatic solution (ES). Demineralized water was used as a solvent for both solutions.

2.2 Enzymatic solution (ES): urease extraction

Several sources of urease enzyme were tested as derived as agro-food waste: tomato seeds, watermelon seeds, and soybean seeds (the latter expired and therefore not usable for food purposes).

Seeds were dried (5 g), finely ground with a blender for 5 min, placed in 100 mL of demineralized water (50 g seeds/L), and kept under constant stirring at 500 rpm for 1 h at room temperature. The solution was centrifuged twice at 7,000 rpm for 5 minutes to remove the solids residue from the enzyme-enriched liquid.

2.3 Calcifying solution (CS)

Urea and CaCl_2 -based calcifying solutions were prepared at a fixed concentration of 0.5 mol/L as it was considered the optimal concentration since excessive amounts of reagents could inhibit enzymatic activity. To evaluate the effect of Mg/Ca ratio, different concentrations of MgCl_2 solutions were prepared and added to CaCl_2 according to the ratio summarized below in Table 1:

Table 1: Calcifying solutions with different Mg/Ca ratio

Urea (mol/L)	CaCl_2 (mol/L)	MgCl_2 (mol/L)	Mg/Ca
0.50	0.50	0	0
0.50	0.50	0.06	0.12
0.50	0.50	0.25	0.50
0.50	0.50	0.50	1.00

2.4 Urease activity verification and evaluation: CaCO₃ production optimization

To check urease activity, 22 mL of ES were mixed with 22 mL of CS in constant stirring at 400 rpm for 72 hours. The resulting suspension was centrifuged at 7000 rpm for 5 min to recover the solid phase which was dried in an oven at 60 °C overnight, ground in a mortar and analyzed by IR.

2.5 Urease activity evaluation: CaCO₃ production optimization

To evaluate the urea hydrolysis, a series of experiments were conducted in polypropylene tubes where urease and calcification solutions directly were mixed. In detail, 22 mL of ES and 22 mL of CS were mixed, after selected times (from 0 to 14 days) the samples were centrifuged and the solid was recovered from the liquid, dried at 60 °C overnight and weighed. Tests were performed by varying parameters such as temperature (20-40 °C), pH, and Mg/Ca ratio to see how urease activity was affected to optimize CaCO₃ production. The conversion efficiency at different reagent concentrations (0.125- 2 mol/L) was also evaluated according to the equation given below.

$$\text{Conversion efficiency } (\eta) = \frac{\text{moles CaCl}_2}{\text{moles CaCO}_3} * 100 \quad (3)$$

Further verifications were performed using Nessler's reagent on the supernatant to detect and quantify the ammonia produced by spectrophotometer measurements as described in paragraph 2.4.

2.6 Consolidation tests

Consolidation tests were carried out on sand specimens of different grain sizes (0.25- 2 mm), treating them with the EICP method and administering the ES and CS solutions (8 mL of each solution for each injection). At the end of the treatment (performed at different numbers and with different injection methods) the consolidation rate was measured according to the following equation:

$$\text{Consolidation rate} = \frac{\text{Consolidated sand (g)}}{\text{Total sand (g)}} * 100 \quad (4)$$

Sand specimens were prepared as follows: 50 g of dried sand were placed in a 50-mL PVC cylinder with measurements of diameter 3 cm and high 7 cm and gently compacted. The cylindrical reactor was punched, closed on the top with a cap. The solution was provided through a tube placed at the top of the column and the liquid residue from the bottom. Different strategies of consolidation were followed: the addition of the solution obtained from the mixing of urease and calcification solutions was operated from the top of the column (every 48 h) and letting the solution flow by gravity (percolation) or by operating the mixing on the top of the column through a Y-joint with two different derived from different tanks, each containing a different process solution (ES and CS) and the solutions were filled every 48 h at a flow rate modulated by a peristaltic pump (semi-batch injection).

2.7 Methods

The DHS pH 80+ benchtop instrument with the standard DHS electrode, capable of 0.01 pH accuracy, was used for pH measurement. The chemical composition of the samples has been studied by Fourier-transform infrared (FT-IR) analysis. Infrared measurements were carried out with a Bruker Vertex 70 spectrometer (Bruker Optik GmbH) equipped with a single reflection Diamond ATR cell. Spectra were recorded with a 3 cm⁻¹ spectral resolution in the mid infrared range (400–4000 cm⁻¹) using 512 scans.

3. Results and discussions

3.1 Urease sources

Before the consolidation tests, the presence of urease from different seeds: soybean, apple, and tomato were evaluated by monitoring the production of CaCO₃. The result of the IR analysis is shown in Figure 1. A higher purity of CaCO₃ derived from the urease extracted from soybeans was found. As for the other seeds, additional peaks appeared (Figure 1) not attributed to calcium carbonate (the result of IR analysis for tomato seeds was not reported) and indicative of a lower purity of the product of interest. In addition, the production of calcium carbonate from 22 mL of ES with 22 mL of CS after 72 h was far greater for the enzyme extracted from soybean seed (0.96 g of solid was synthesized), whereas 0.10 g and 0.21 g were produced for the enzyme from apple and tomato seeds, respectively. The presence of enzyme activity in these plant wastes was then tested, which could be used for this purpose in the circular economy perspective as waste products from the agri-food chain, but given the results, subsequent experiments were conducted with soybean seeds, which showed higher calcium carbonate production. However, the use of these waste materials as a source of urease enzyme is only

possible if they do not undergo heat treatment above 70 °C, a temperature above which the enzyme would denature (Dilrukshi et al., 2018).

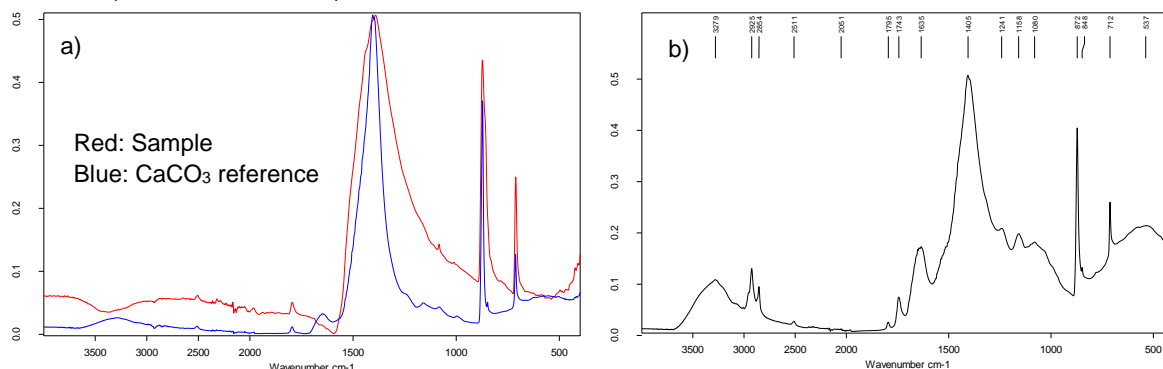


Figure 1: Infrared spectra (IR) from solids obtained after 72 h after mixing ES and CS of equal volume, centrifuged and dried, using soybean (a) and apple (b) as urease sources.

3.2 pH, Mg/Ca ratio and CS concentration effect

CaCO₃ precipitation tests were conducted at selected initial pH, Mg/Ca ratio and CS reagent concentration. The results are shown in Figure 2.

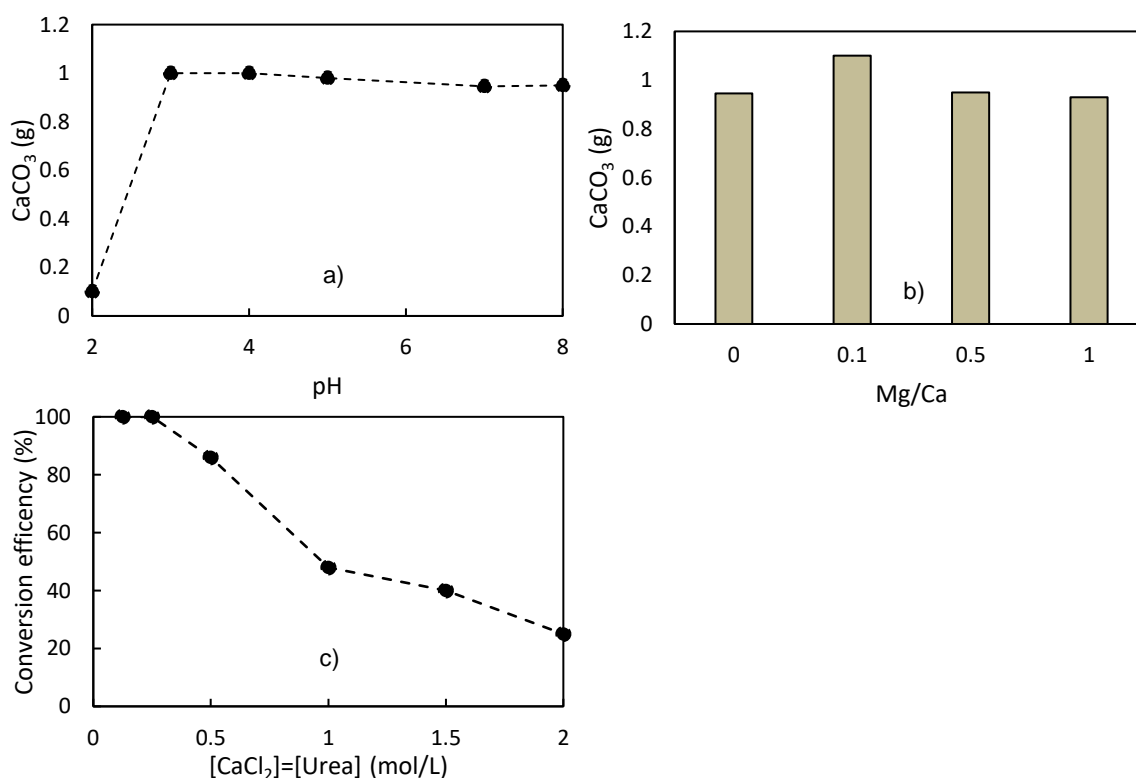


Figure 2: Grams of CaCO₃ formed at room temperature after 48 hours after mixing 22 mL of ES and 22 mL of CS, varying for CS the initial pH (a), the Mg/Ca ratio (b), and the concentration of urea and CaCl₂ (c) for which conversion efficiency was reported (moles of calcium carbonate produced versus moles of calcium chloride initially).

The results of the tests performed at different initial pH of CS (Fig. 2a) show that for a wide pH range (from 3 to 8) the enzyme did not change its activity and CaCO₃ production remained constant. In all cases, a sudden increase in pH to about 8.5 was observed because of ammonia in solution and its buffering power.

A drastic reduction in activity was observed at pH 2, probably at a pH that was too acidic, and the enzyme was denatured irreparably (Miklos et al., 2011);

The effect of Mg was investigated. The tests shown in Fig. 2b show that the optimal Mg/Ca ratio for enhancing enzyme activity was 0.11, that agrees with other literature findings (Imran et al., 2021). High amounts of Mg did not provide any benefit. The effect of the Ca^{2+} and Urea reagents was tested: from the results reported in Figure 2c, it was noticed that as their concentration increased, an inhibitory effect on enzyme activity was observed (Neupane et al., 2013), thus resulting in a decrease in the conversion efficiency of CaCl_2 to CaCO_3 . Considering these results, the 0.5 mol/L concentration was chosen for further testing as it demonstrated high yields (85% conversion) and a satisfactory CaCO_3 mass production.

3.3 Effect of Temperature and contact time

To assess the optimal temperature for the development of the EICP process, experimental tests were carried out at 20, 30 and 40 °C evaluating the reactivity of urease through the production of CaCO_3 deposits after mixing 22 mL of CS, consisting of Urea (0.5M) and CaCl_2 (0.5M), and 22 mL of ES. The reaction was monitored for 14 days. No significant differences were observed in the production of CaCO_3 (Figure 3), in the investigated temperature range, thus suggesting that the CaCO_3 production reaction (Eq. 2) is mainly influenced by the availability of Ca^{2+} and HCO_3^- , and the precipitation of CaCO_3 occurred in 2 days and no increase in the amount of precipitate was detected until 14 days. This suggests that, during sand stabilization tests, 2 days are required and beyond which EICP solutions need to be replaced.

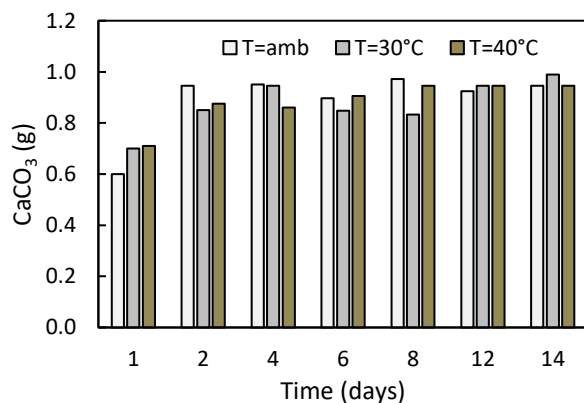


Figure 3: Precipitation analysis of CaCO_3 obtained by mixing 22 mL of ES and 22 mL of CS (0.5 mol/L) by weighing the resulting dried powders after a curing time from 1 to 14 days at 3 different temperatures.

3.4 Consolidation tests

Table 2 shows the results of the consolidation tests performed at the optimal conditions: 14 percolation injections were performed every 48 hours to allow the carbonate to precipitate totally, at room temperature, with CS 0.5 mol/L.

Table 2: List of consolidation tests performed by percolation injection, 8 mL of ES and 8 mL of CS 0.5 mol/L were injected 14 times every 48 hours at room temperature.

Mg/Ca	Sand diameter (mm)	Sand content (g)	Stabilization (%)
0	0.25-0.50	77	0
0	0.50-1	77	3
0	1-2	85	39
0.1	0.5-1	69	4
0.1	1-2	55	27
0.5	0.5-1	70	1
0.5	1-2	70	76
1	0.5-1	74	0
1	1-2	61	53

Based on the data reported in Table 2, it resulted that the factor that most influenced the occurrence of bio-cementation by giving a high rate of stabilization (76 %) was the size of the sand grains. The specimens with sand of diameter 1-2 mm were, in fact, more consolidated than those with sand with smaller grain size. In particular, sand of size 0.25-0.50 mm did not show satisfactory results, probably because the percolation of the cementing solution was hindered by the compactness of the soil. Therefore, the injection mode turns out to be a key parameter to be optimized to allow the cementing solution to homogeneously fill all soil voids.

The injection by percolation, although allowing to achieve a stabilization rate higher than 50 %, was not found to be a reproducible method, being strongly influenced by soil composition. To overcome this issue, 2 injections (CS 0.5 mol/L, Mg/Ca=0, room temperature) were conducted every 48 hours on a 1-2 mm diameter sand specimen by the semi-batch method: with this method, a stabilization rate of 40 % was achieved solution was homogeneously arranged in the reactor with the help of the peristaltic pump instead of mainly disposing on the sample surface, given the sand's resistance to percolation of the solution. Therefore, although more complex, this injection mode was better than percolation injection for the same number of injections.

4. Conclusions

The possibility of extracting urease from agri-food waste could be an important advance in CO₂ emission reduction policy and circular economy. This study shows how it was possible to extract urease enzyme from plant seeds (agro-food waste). The crude extract from soy seeds showed a higher purity and quantity of the precipitated product than the extracted from apples and tomatoes. It was found that the the breakdown of urea into ammonia and carbon dioxide completely occurred within 48 hours; temperature did not significantly affect the enzyme activity, in the investigated range (20 to 40 °C). The enzyme appeared to be unaffected by pH in the range of pH 3-8, but its activity dramatically decreased at pH 2. Gravity injection reactors yielded the best results, with a maximum consolidation rate achieved of 76 % at room temperature. Finally, the semi-batch system resulted in 40 % of stabilization. Injection mode and soil particle size variability proved to greatly affect the consolidation rate.

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