Guidance for Investigating Calcite Precipitation by Urea Hydrolysis for Geomaterials

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Abstract

Microbially Induced Calcite Precipitation (MICP) is a sustainable method of stabilizing (i.e., cementing) loose sandy deposits and/or to create an impervious barrier within the soil mass. MICP can occur through various biochemical pathways, among which 'Urea Hydrolysis (UH)' is considered to be the most efficient method of biochemically inducing calcite precipitation. To date, the geotechnical engineering community investigating MICP has tended to focus on the hydro-mechanical behaviour of the end product, i.e. MICP cemented sands; however, many biochemical factors that affect reaction-rate kinetics and MICP outcome have been understudied or neglected. This study investigated the kinetics of UH and compared different sources of urease enzyme: those microbially cultivated in the laboratory (i.e., *Sporosarcina pasteurii*) and those extracted from plants (i.e., Jack bean meal), to investigate the influence of urea concentration, buffer capacity, and cell harvesting method on UH. Through this study, an attempt has been made to arrive at an optimal concentration of urea, under the influence of the above mentioned parameters along with the buffering action of the soil, on urea hydrolysis. These results have implications towards optimising MICP and, in particular, for upscaling these methods to *in-situ* applications.

Keywords: soil stabilization, ground improvement, microbial activity, ureolytic activity, urea hydrolysis, calcite precipitation.

Introduction

For most civil engineering projects, in-situ soil conditions must meet the necessary technical (engineering or functional) requirements; as such, various ground improvement techniques mainly based on mechanical, chemical, thermal and vacuum treatments, either individually or involving a combination of techniques are employed. Such techniques include consolidation by preloading, vacuum assisted pre-consolidation, thermal treatment, stone/sand columns, excavation and replacement [1], dynamic compaction by heavy tamping [2], vibro-flotation [3] and deep mixing [4]. These techniques often consume large quantities of natural resources (i.e., soils, sands, weathered rocks, stones and lime) and/or manmade resources (i.e., cement, admixtures and chemicals), which can be both expensive and damaging to the environment to extract, produce and transport to project sites[5]. Hence, there is a need for alternative ground improvement techniques that are sustainable and satisfy performance expectations, and minimise environmental impact.

Biotechnology may offer such a technique through the process of biologically induced mineralisation wherein organisms secrete metabolites in their external environment, which in turn, react with ions or compounds to precipitate extracellular mineral phases [6]. One such 'bio-mineralisation' of interest to civil engineers is the microbially induced precipitation of calcium carbonate by urea hydrolysis by UH, having been proposed for use in bio-concrete, self-sealing and self-healing concrete, safeguarding heritage structures, mitigation of dust in construction sites [7; 8], and in particular, for ground improvement (see Table 1). Interestingly, all these studies considered coarse-grained materials (i.e., sands) due to the physical limitations associated with the migration of micro-organisms through the pores of fine-grained soils (i.e., those clayey and silty). For the application of microbially induced calcite precipitation (MICP) to finer-grained soils it is necessary to consider alternative

sources of urease enzyme (such as a plant source), as the urease enzyme is around 12nm in size compared to a few microns for the whole-cell of *S. pasteurii*.

Furthermore the effectiveness of MICP treatment (described in *Section 2* below) depends on (1) biochemical factors which affect the kinetics of UH, and (2) the soil/geomaterial specific parameters (i.e., porosity, particle size distribution, sorption properties, etc.). However, the optimal conditions/mix proportions of cementing reagents (i.e., urea and $CaCl_2$) reported in the previous studies (refer Table 1) for efficient MICP have been based on the improvement in engineering properties of the treated materials alone, and do not consider the kinetics of UH. The authors are of the opinion that engineers should seek to optimize the biochemical aspects and its influence on engineering behaviour of geomaterials, in order to select appropriate conditions for upscaling the growth and preparation of treatment fluids for in-situ applications.

This study aims to provide a fundamental understanding of the microbiological and biochemical influences on the kinetics of urea hydrolysis so that UH-based calcite precipitation may be optimized and better controlled. This is achieved through systematic investigation of the following: (1) bacterial cell harvesting method, (2) urea (substrate) concentration, (3) bacterial growth (nutrient) medium, (4) source of urease enzyme-microbial (*Sporosarcina pasteurii)* and plant (Jack bean meal extracts), and (5) pH in the presence of buffer medium.

Calcite Precipitation by Urea Hydrolysis

Enzymatic urea hydrolysis can be summarised by the following two chemical reactions [9]:

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

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

Urea (the substrate) is hydrolysed into ammonium (NH^{4+}) and carbonate (CO_3^{2-}) ions (reaction product) by utilising two molecules of water (H_2O) in the presence of urease enzyme, which catalyses the reaction. The hydrolysis progresses with time and depends on the concentration of urea (C_{urea}) and the urease enzyme (C_{urease}) in the system at any instant of time (t) and the rate of urea hydrolysis (UH). The hydrolysis of urea tends to increase the pH of the bulk fluid and once the buffer capacity of the UHM is reached, pH increases. As the pH approaches 9, calcium carbonate precipitates. Although a rise in pH is desirable and necessary, an instantaneous increase may not be preferred during its implementation in the field as this can lead to clogging of the soil matrix near the injection or inlet point by the rapid precipitation of calcium carbonate. This, in turn, restricts the extent of ground improvement around of the inlet point. However, a prolonged delay in precipitation might result in excess volumes of treatment fluids being injected into the soil matrix leading to increased costs as well as influencing the ground conditions in adjacent locations where the treatment is not mandated. Without proper optimisation, both of these situations could be detrimental in obtaining the desired results. Hence, in order to overcome this situation, controlling the time required for precipitation by regulating pH using a suitable buffer, which does not interfere either with the activity of the cells or with the reaction process, may be necessary. Previous researchers have attempted MICP by employing a combination of $NaHCO_3$ and NH_4Cl as a buffer (e.g. [10], [11]). However, it should be noted that prior to the introduction of urease enzyme in to the soil (either microbial or plant based form), $NaHCO_3$ and Ca^{2+} will react abiotically to form $CaCO_3$. This undesirable utilisation of calcium leads to reduced availability of calcium for MICP and the increased risk of clogging of soil pores during the injection stage.

Urease enzyme is derived from either plants or microorganisms. Among the various plant sources of urease enzyme such as jack bean, soybean, mulberry etc., urease from jack

bean is one of the most widely studied enzyme sources [12-15]. On the other hand, microorganisms such as *Sporosarcina pasteurii, Sporosarcina aquamarina, Bacillus subtillis, Sporosarcina ureae* etc. are known to be good sources of urease enzyme. The most widely used microorganism for calcite precipitation is *S. pasteurii* [13]. The enzyme from plants could be derived through their leaves and seeds, while the microorganisms secrete extracellular urease enzyme. Microbial cells need to be separated from the nutrient medium in which they were inoculated, commonly referred to as 'cell harvest', in order to avoid contamination of geoenvironment by these nutrient media. Different harvest methods affect cell yields and the sustenance of urease activity. Shear stresses, due to centrifugation or filtration, could lead to cell lysis, and hence, necessitate culturing larger volumes of microbial cells leading to an increased consumption of nutrient medium and increased cost of cell cultivation. Hence, the influence of cell harvest on the enzyme kinetics has also been investigated in this study, for the sake of completeness.

Experimental Investigations

The materials used and the process adopted in this study are depicted in Fig. 1 and described in the following sections.

SOURCE OF UREASE ENZYME

In this study, urease enzyme from two different sources were used: (i) microbial source (*Sporosarcina pasteurii*, DSM-33, procured from Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Germany) and (ii) plant source (urease-enzyme crude extract from Jack bean meal plant; Fisher Scientific Ltd., UK).

UREA (SUBSTRATE) AND BUFFER MEDIUM

Urea (99.9% purity, Fisher Scientific Ltd., UK) was used in the present study. Different buffers investigated include: (i) phosphate buffered saline (PBS), (ii) sodium bicarbonate/ ammonium chloride (2.12g/l $NaHCO_3$ + 10g/l NH_4Cl), (iii) Tris buffer

(Tris(hydroxymethyl)aminomethane, 10mM and 100mM), and (iv) sodium acetate (CH_3COONa, 0.1M and 1M). A stock solution of 2.2 M urea was prepared and diluted separately in each of the buffer mediums to obtain concentrations of urea ranging from 0.11 to 1.98 M. The stock solution of urea was diluted by mixing buffer medium and making up the volume to 18 ml, and 2 ml enzyme mixture was added to it. This 20 ml solution, herein after referred to as 'Urea Hydrolysis Mixture' (UHM), would have the desired target concentration of urea. Furthermore, as a control solution, urea was dissolved in sterilized tap water.

TESTING METHODOLOGY

The bacterial cell harvesting methods and the parameters influencing the kinetics of UH considered in this study are: (1) bacterial cell harvesting by centrifugation and vacuum filtration methods, (2) concentration of urea, (3) nutrient medium, (4) source of urease enzyme, and (5) pH buffer medium.

Harvesting microbial cells

Nutrient medium, either Brain-Heart Infusion (BHI) or Lysogeny Broth (LB), with 20 g/L urea, was used as the inoculation medium for the stock culture of *S. pasteurii* (0.1%, v/v). The inoculated medium was left on an orbital shaker (at 150 rpm) for 24hrs at a temperature of 30°C. Two different methods of harvesting were investigated: (a) centrifugation at 4600x g and (b) vacuum filtration (with a filter pore size of 0.22 microns). For the microbial cells harvested by centrifugation, the centrifugation speed of 4600x g, 8 minutes was determined to be appropriate for obtaining a stable cell pellet (i.e., remained minimally disturbed while the supernatant was decanted).

Harvested cells were then re-suspended in phosphate buffered solution (PBS) and OD was adjusted to 1.0, measured at 600nm wavelength using a spectrophotometer (UV-Vis Helios Zeta, Thermo Scientific). PBS is an isotonic solution, with an osmolaritiy similar to

cytoplasm, to maintain cell survival and activity. In case of jack bean meal, extracts of different concentrations were tested to identify a concentration that corresponded to similar urease activity as that of *Sporosarcina pasteurii* cells at OD_{600} of 1.0, and 2.7 g/l was found to be equivalent.

Ureolytic activity

Ureolytic activity in the UHM was determined based on the concentration of products formed and was determined through by measurement of electrical conductivity (EC) of the mixture at various time intervals for up to 60 minutes. As urea is hydrolysed, increase in EC of the UHM is directly proportional to the concentration of UH reaction products (*see* Eq. 1). By plotting the rates of urea hydrolysis (i.e., electrical conductivity change) against different concentrations of urea, the optimal concentration of urea, i.e., the concentration of urea beyond which had no further increase in rate of UH, could be identified.

However, due to the presence of calcium in the system, PBS cannot be used as a buffer medium during calcite precipitation owing to the affinity of calcium towards phosphate, which results in the formation of calcium phosphate instead of calcium carbonate. Hence, PBS is an indicator of ideal conditions for optimal ureolytic activity and not considered here as a suitable buffer for use in the full MICP process. Further, in order to verify the buffer's influence on the UH, the activity of the urease enzyme in different buffers, at the previously optimised substrate concentration, was also studied and compared.

Substrate-Dependent Enzyme Kinetics

To fully understand enzyme kinetics over a wide range of substrate conditions. Enzyme-mediated transformations were monitored over time to calculate reaction rates (R_{UH}); from a series of reaction rates (per substrate concentration, [S]), a Michaelis-Menten model represented in Eq.3 was developed by Johnson and Goody [16].

$$R_{UH} = \frac{R_{UHm}[S]}{k_m + [S]} \quad (3)$$

$$\frac{1}{R_{UH}} = \frac{k_m}{R_{UHm}} \times \frac{1}{[S]} + \frac{1}{R_{UHm}} \quad (4)$$

The maximum rates (typically V_{max}, here we use 'R_{UHm}') and the Michaelis-Menten constant (k_m), which is the concentration of the substrate required to produce half the maximum rate of the reaction, are the primary parameters defining the Michaelis-Menten model; they are calculated from Lineweaver Burk transformations (Fig. 4), which becomes a plot of $1/R_{UH}$ v/s 1/[S] and represented in the Eq. 4. By doing so, the Michaelis-Menten plot of R_{UH} v/s [S] is linearised; the intercept on y-axis becomes $1/R_{UHm}$, and the intercept on x-axis becomes $-1/k_m$. Although enzyme-substrate affinities (i.e., often represented by k_m) are not likely to be affected for a given combination of substrate and enzyme, k_m is presented in this study as a model parameter to help define the shape of the model curve. As such, the Michaelis-Menten model has been utilised to compare enzyme reactions.

Selection of buffer medium

The harvested cells, as discussed previously, were re-suspended in different buffer solutions and in sterilised tap water (as control); the OD_{600} was adjusted to 1.0. Optimal concentration of urea, as derived through the process described in the previous section, was dissolved in different buffer media and pH of the system was monitored either for 60 minutes or until it reached pH 8.5. This was investigated to determine the selection of an appropriate buffer medium that would result in delayed calcite precipitation, which during in-situ practice could facilitate the distribution of solutions by increasing the time allowed for the reagents/grouts to flow through the pores.

Buffering capacity of soil

Whenever soil mass and a fluid interacts with each other, the pH of the fluid will be affected by its buffering action. As such, when UH is being implemented in the field, knowing the

buffering capacity of the soil mass is critical. To ascertain the effects of buffering action, five different soils whose pH ranges from 6.5 to 7.5 were considered in the study. These soils were interacted with de-ionised water for 24 hours and the supernatant was extracted by filtration. Further, the optimal concentration of urea derived through the process discussed previously), was dissolved in the supernatant and pH was monitored by adopting the methodology described for selection of buffer medium.

Results and Discussions

CELL HARVEST

The microbial cells harvested by centrifugation were re-suspended in buffer medium, and the OD_{600} of this suspension was adjusted by to 1.0. The total volume of the cultures, at the said concentrations, obtained was found to vary only by ±1%. However, filtration method decreased the total number of cells due to inherent constraints associated with the method (i.e., clogging of pores in the filter paper), yielding a volume 25% lower than the centrifugation method. The authors believe that this reduction could be due to the stresses generated on the cells during the filtration due to continuous application of vacuum. It was observed that centrifugation could be completed in a much shorter duration (12 minutes) than filtration, which may require 4 hours for filtration of 100 ml of cell culture.

SUBSTRATE-DEPENDENT ENZYME KINETICS

The non-linear relationship between rate of UH (R_{UH}, in terms of changes in electrical conductivity) with concentration of urea (substrate) were examined in the study. The individual R_{UH} (change in EC per time) are derived from Figs. 2 (a), (b), (c) and (d), which shows ureolytic activity of *S. pasteurii* cultured in BHI and harvested by centrifugation (Fig. 2a), *S. pasteurii* cultured in BHI and harvested by filtration (Fig. 2b), *S. pasteurii* cultured in LB (Fig. 2c), and Jack bean meal extracts (Fig. 2d). From these figures the rate of UH can be derived as described in the following: 1) the slope of the straight line portion of the trends

depicted in these figures represent the rate of formation of product (ammonium and carbonate ions) due to enzyme activity; 2) steeper trend which represent higher rates of urease activity, R_{UH}.

The variable reaction rates caused by harvest methods (i.e., centrifugation and filtration) for *S. pasteurii* (cultured in BHI) is depicted in Fig. 3 (a). Similarly, Fig. 3 (b) presents the variation in RUH for *S. pasteurii* cells cultivated in different media: BHI versus LB (harvested at 4600x g for 8 minutes). Further, Fig. 3 (c) compares the enzymatic activity of *S. pasteurii* cells (cultured in BHI and harvested by centrifugation) and that of Jack bean meal extracts.

The trends presented in these figures indicate that R_{UH} increases with increasing concentration of urea, but eventually attains a constant rate of UH (R_{UHm}). This observation is in-line with the Michaelis-Menten model of enzyme reactions, which suggests abundant enzyme molecules are available at lower substrate concentrations for the reaction to proceed. As the concentration of substrates increases the reaction velocity increases; however, the abundance of free enzymes decrease and reaches a critical point of saturation where any further increase in substrate concentration does not lead to an increase in reaction rates. At this critical concentration of the substrate (C_{crt}), the reactions are said to proceed at their maximum rates (R_{UHm}). The k_m and R_{UHm} values for all the conditions considered in the study are derived from the Lineweaver-Burk transformations (as depicted in Fig. 4) and the values are presented in Table 1

Figure 3 (a) compares vacuum filtration versus centrifugation cell-harvest methods, and it is observed that the R_{UH} of cells harvested by vacuum filtration appears to be higher than those that were centrifuged. It is not likely that the centrifugation is affecting substrate-enzyme affinity as the model k_m, is decreased by 5-fold when centrifuged. On the other hand, R_{UHm} for BHI- filtration is found to be 20% higher that BHI- centrifugation. It is noticed that

the pseudo-first order reaction rates (represented by ration of R_{UHm}/k_m) is approximately 4 times higher for BHI- centrifugation as against BHI-filtration. This implies that centrifugation speed of 4600x g for 8 minutes is more optimal condition than filtration (Fig. 3a). A possible reason for low k_m could be the built up cellular stresses during filtration, ultimately impacting enzyme affinity and hence the efficiency of the UH. Furthermore, Fig. 3(a) indicates that using a $C_{Urea}>C_{crt.}$ does not benefit in increasing urease activity.

Further, the rate of UH of *S. pasteurii* cells grown in different media as shown in Fig. 3 (b) indicates an increase in k_m and reduction in R_{UHm} when LB nutrient was used. Consequently, the maximum rates that can be achieved in practice by using cells grown in LB is less than half of that using BHI. Furthermore, the pseudo first-order rates of reaction for UH by cells grown in LB is approximately 1/3 of BHI- centrifugation. For such a scenario, time required and efficiency for UH would be impacted.

In the case of Jack Bean meal (JBM) extracts, though the urease activity (measured over a 5 minute period where there is an abundant supply of urea) is selected such that it is comparable to that of cells grown in BHI and harvested by centrifugation, the urease curves shift slightly between k_m and R_{UHm} (Fig. 3c), with R_{UH} for *S. pasteurii* initially, at low substrate concentrations, being higher, but becoming lower than JBM at high concentrations. When comparing pseudo first-order rates, means rates appear twice as large for *S. pasteurii* cells as Jack bean meal extracts. However, microbial cells provide a constant source of urease enzyme (while cells are active); the urease activity can be sustained for a prolonged period. Urease extracted from Jack bean meal is dissolved and freely available to encounter the substrate (urea), whilst, in order for urea to be hydrolysed by bacterial urease, the urea must first be transported through the microbial cell membrane and thus there is a delay evident as a difference in R_{UHm}. Ultimately, *S. pasteurii*, when cultivated, are less expensive than commercially available JBM. These factors, suggest the choice of microbial cells (i.e., *S.*

pasteurii) for a sustainable and efficient calcite precipitation system, the use of sources like Jack bean meal extracts would suit the treatment of soils with pore sizes comparatively smaller than the bacterial cell i.e., the fine-grained soils.

A relative performance matrix of k_m and R_{UHm} for all the UHM conditions discussed above are presented in Table 3. The relative performance of the UHM listed in the top row of the matrix is compared with respect to the UHM listed in the left column of the matrix. For example, in the k_m matrix, a negative value of element k_{m12} represents the decrease in k_m of Jack bean meal extracts with respect to *S. pasteurii* cultures grown in LB nutrient medium. The R_{UHm} matrix is similar to the k_m matrix in representation. The higher the negative value in the k_m matrix, the better the performance of the respective UHM over the other. On the other hand, in R_{UHm} matrix, the higher the positive value, the better is the performance of the respective UHM over the other. With respect to k_m matrix, it can observed that cultures of *S. pasteurii* grown in BHI and harvested by centrifugation has relatively better k_m by varying degrees with respect to all other combinations of UHMs considered in the study. However, in the case of R_{UHm} comparison, Jack bean meal extracts perform relatively better with respect to other UHMs considered in the study. In addition, a further measure of comparison between the UHMs is the ratio of R_{UHm} to k_m as presented in the Table 2. The ratio of to R_{UHm} to k_m is required to be higher for an efficient and economical UHM. With this criteria, it is clear that *S. pasteurii* cultures grown in BHI nutrient medium and harvested by centrifugation are better compared to all other UHMs considered. It can also be noted that the urease activity of *S. pasteurii* cells, harvested by centrifugation, is greater when they are grown in BHI as compared to those grown in LB.

MEDIA CONDITIONS AND CONCENTRATION

In order to arrive at an optimal concentration of urea for the UHMs used in a study, for practical applications, authors define a critical concentration of the urea (C_{Crt}), beyond

which the R_{UH} does not change more than 15%. In other words, C_{Crt} is that concentration of urea which produces an R_{UH} that is 85% of R_{UHm}. The corresponding C_{crt}s for the various conditions are presented in Table 2. While the range of C_{crt} varies from 0.8M to 4.25M, the C_{Crt} is lowest in case of *S. pasteurii* cells, cultured in BHI harvested by centrifugation (0.8M) and, the C_{crt} is highest in case of *S. pasteurii* cells grown in BHI and harvested by vacuum filtration (4.25M). An optimal concentration of urea would be equal to or less than C_{Crt} and from Fig. 3 (a), (b) and (c), it can be observed that the UH appears to be stable at 1M urea for all cases except for *S. pasteurii* cells harvested by vacuum filtration, where the optimal concentration appears to be at 1.75M. Further optimisation or the process is to be carried out based on the factors discussed earlier i.e., time required for $CaCO_3$ precipitation and the extent of precipitation to be achieved.

However, it should be realized that treating large volumes of soil, in real life situations, would be extremely expensive when BHI/LB/any other commercially available nutrient media, that are normally employed for laboratory-scale experiments. Hence, an alternate source of nutrients for culturing microbial cells in large volumes without compromising on their urease activity should be explored. It should also be borne in mind that such alternate nutrient media should be environmentally friendly, directly injectable into the soil.

SELECTION OF THE APPROPRIATE pH BUFFER

Using previously optimized conditions, urease activity of microbial cells and variation of pH of the UHM were monitored over time in different buffer media: combination of ammonium chloride and sodium bicarbonate, sodium acetate and Tris [tris(hydroxymethyl)aminomethane], an organic buffer solution (Fig. 5).

The urease activities were measured in the form of EC (Fig. 5b) in different buffer media. The orders of timing, by which reactions reached pH 8.5, were as follows: tap water, acetate (0.1M), PBS, Tris (0.01M), acetate (1M), ammonium chloride and sodium bicarbonate, and Tris (0.1M). The first two buffers had elevated pH at start of reactions, which may have contributing to their timings, but still have relatively immediate effect (based on experience). Interestingly, increasing the buffering capacity of the system, especially with organic buffer (e.g., Tris), delays the onset of calcite precipitation by increasing the amount of urea that must be hydrolysed before there is a sufficient pH increase to trigger $CaCO_3$ supersaturation.

Incidentally, the most suitable buffer will be selected based on the desired time for pH to become 8.5, as depicted in Fig. 5 (a), which highlights the amount of time for reactions to reach critical pH 8.5. As mentioned previously (in the introduction), treatment time depends on the permeability and volume of soil to be treated. However, it should be reminded that the absence of buffer causes instantaneous rise in pH of the UHM, which leads to instantaneous calcite precipitation and/or clogging of flow paths. In the presence of a buffer, care should be taken so that the rise in pH does not get too delayed, which might result in the use of excessive reagents. By varying the buffer used, the time for precipitation of $CaCO_3$ to occur could be controlled from 16 minutes (10mM Tris) to 80mins (100mM Tris) for a UHM of 1M Urea and 1.0 OD_{600} bacteria. This indicates that the MICP process can be tailored to site specific and application specific scenarios.

Further, it should be realized that when any fluid interacts with the soil, its pH varies depending upon the chemical characteristics, and in particular pH, of the soil, as depicted in Fig. 6. It is observed that UHM gets buffered by the soil extracts unlike the tap water where no buffering action was observed. However, buffering of pH, due to influence of soil, does not provide any significant delay in the rise in pH unlike most other buffers considered in this

study. Among the five soils considered, the critical pH, equal to 8.5, was attained in all the cases within 15 minutes, a time which is less than the time taken by the other buffers except for 0.1M CH_3COONa. This indicates that even though the soil buffering action influences the rise in pH of the UHM, the significance of such a buffering action from a practical point of view is debatable.

From a field application perspective, cementing reagents (the mixture of urea and calcium source) could be mixed with the buffer medium instead of water and injected in to the ground. Another way of achieving this, but, with limited efficiency, would be to inject a known volume of buffer in to the ground prior to injection of the bacterial suspension or the cementing reagents. However, such an exercise needs to be simulated in the laboratory conditions prior to field implementation.

TIME REQUIRED FOR COMPLETE UREA HYDROLYSIS

Urea hydrolysis decreases with time exponentially, reaching a residual activity level, which remains constant as the reaction progresses. This residual activity continues so long as urea remains present in the system. The time required for complete hydrolysis of urea is of prime importance in applying this methodology for geomaterials either in the laboratory or in the field. An optimal time interval between successive treatments to the geomaterials would be the time required for complete hydrolysis of urea, as complete utilisation of urea is key to reduce or minimise the intermixing of urea with the ground water in the geoenvironment. Any additional time interval is unwarranted for as it delays the process with no progress towards the desired outcome. The residual activity for 1 OD_{600} bacteria in case of 1M concentration of urea is 2.5mM/min/OD. Considering this as the rate of the reaction for complete hydrolysis of urea, the time required would be 6 hours.

However, when a geomaterial is being treated, the amount of microbial cells retained in the matrix of geomaterial plays an important role on the time required for full utilization of

urea. The retention of microbial cells inside a porous media depends on several other factors such as the advective force of the fluids flowing through the matrix, the sorption characteristics of geomaterials and microbial cells, the physiology of the cells when it comes in contact with the geomaterials and so on. Considering various retention percentages within a control volume, a theoretical estimate of the time required for complete urea hydrolysis has been presented in Fig. 7. Further studies are necessary to understand the phenomena on microbial retention in a porous media.

Conclusions

Calcite precipitation by urea hydrolysis through urease enzyme, a biochemical process, offers a technique for improving the engineering properties of soils and other construction materials. Though several studies reported this technique as a promising technique for sustainable construction, materials and technologies, there is an absence of a standard protocol for selection of urease source, growth of microbial cells, harvesting of microbial cells, and for mix proportioning and performance assessment of calcite precipitation. To date geotechnical studies have focused on reporting geomaterial and porous media characteristics, rather than the biochemical and microbiology influences.

Crossing disciplinary borders to delineate the biochemical influence of geomaterial process, this study presents a strategy for optimising urea hydrolysis. Ureolytic activity has been shown to be affected by the nutrient media in which the cells have been cultured. While the study highlights that methodology employed for harvesting of cells does not affect the ureolytic activity of the cells, the yield of active cells from filtration is about 25% less than compared to centrifugation, as explained in this paper, and can significantly affect the cost efficiency of the project. Further, optimal urea concentration was found to vary from 1M to 1.75M for the cases considered in this study. It should be noticed that, any further increase in urea concentration would not result in better ureolytic activity. These optimal parameters, though, they serve as a guiding formula for calcite precipitation studies, need to be further validated with a larger spectrum of variables involved during employing urea hydrolysis in calcite precipitation systems.

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List of Tables

TABLE 1 *A summary of MICP laboratory studies*.

Reference	Parameter(s) considered	Type of material(s) used	Remarks
[17]	Urease activity	Artificial ground water and strontium as contaminant	Effect of temperature and presence of strontium contaminant on rate of urea hydrolysis.
[18]	Geotechnical parameters	Itterbeck fine sand	Reflects on improvements in geotechnical parameters of the treated sands but does not discuss any biochemical factors affecting urea hydrolysis.
[19]	Urease activity Geotechnical parameters	Fine sand	• Unilaterally uses 1:1 equimolar urea and calcium chloride as cementing reagents. • Urease activity measured in soil column with different porosities. • Reflects on improvements in geotechnical parameters.
[20]	Urease activity	Natural ground water	Stimulated microbial growth in the aquifer to enhance *ureC* gene, responsible for urease activity, to promote contaminant sequestration and reduction in permeability.
[10], [11], [21]	Geotechnical parameters	Ottawa sand (50-70)	Reflects on improvements in geotechnical parameters of the treated sands but does not discuss any biochemical factors affecting urea hydrolysis.
[22]	Microbial retention in porous media	Itterbeck sand	Reflects on the urease activity due to various levels of bacterial fixation in a porous media.
[23]	Urease activity	Artificial and natural ground waters	Reflects on the importance of urease activity and the concentration of microbial cells in calcite precipitation in rocks under oxic and anoxic conditions through microcosm studies.

[24], [25]	Urease activity Geotechnical parameters	Fine sand	Urease activity at 1.5M urea concentration, measured at different flow conditions in the sand column.
[26]	Geotechnical parameters	Clayey sand	Optimised the cementing reagent concentration based on improvements in geotechnical parameters. Tests performed at different concentrations of urea in the presence of a high OD_{600} (3.3) bacterial suspension
[27]	Geotechnical parameters	Fine sand	Optimised cementing reagent concentration based on improvements in geotechnical parameters. Tests performed by varying frequency of injection and different concentrations of cementing reagents in to the sand column
[25]	Geotechnical parameters	Coarse sand Fine sand	Measured urease activity of 1 OD_{600} bacteria at 1M urea concentration and used the same for treating the sands
[28], [29]	Geotechnical parameters	Sandy silt	Reflects on improvements in geotechnical parameters and does not discuss any biochemical factors affecting urea hydrolysis.
[30]	Geotechnical parameters	Ottawa 20-30 and F-60 sand	Reflects on feasibility of plant source of urease enzyme for cementation of sands by calcite precipitation.
[31]	Urease activity Geotechnical parameters	Borosilicate bead	Reflects on the urease activity of *Canavalia ensiformis*, a plant source of the enzyme. Further, the distribution of calcium carbonate precipitated in the beads column has been studied.
[32]	Urease activity	Field soils	Investigates urease activity of soils from various parts of India to assess the availability of nitrogen for optimisation of fertilizer use in agricultural practices.
[33]	Geotechnical parameters	Field investigation	Investigates the potential and performance of MICP for practical field-scale applications.
[34]	Calcite precipitation and geotechnical parameters	Glass beads and sandstone	Investigates the kinetics of calcite precipitation and permeability of enzymatically treated specimens

TABLE 2- k_m *and* R_{UHm} *(analogous to* V_{max}*) from Lineweaver-Burk transformation plots. The ratio* R_{UHm}/k_m *represent pseudo first-order rate kinetics at low substrate concentrations.*

	k_m[a], M	**R_{UHm}[a], mS/cm/min ($x10^{-4}$)**	**R_{UHm}/k_m**
BHI-centrifugation	0.151 (± 0.036)	5.57 (± 0.28)	3.8
BHI-filtration	0.880 (± 0.249)	7.7 (± 0.99)	0.9
LB-centrifugation	0.273 (± 0.038)	3.52 (± 0.14)	1.3
JBM	0.42 (± 0.045)	7.10 (± 0.33)	1.9

[a]values in parenthesis indicates the statistical range

TABLE 3- *Relative performance matrix of different UHMs used in the study.*

k_m				
	LB	**JBM**	**BHI-Fil**	**BHI-Cent**
LB	-	50.75	166.01	-48.37
JBM	--	-	76.46	-65.75
BHI-Fil	-	-	-	-80.59
BHI-Cent	-	-	-	-
R_{UHm}				
	LB	**JBM**	**BHI-Fil**	**BHI-Cent**
LB	-	131.87	95.57	55.06
JBM	-	-	-15.65	-33.13
BHI-Fil	-	-	-	-20.72
BHI-Cent	-	-	-	-

Figure Captions

FIG. 1- Different parameters considered for urea hydrolysis.

FIG. 2- Urease activity of UHM (a) BHI-centrifugation, (b) BHI-filtration, (c) LB, (d) Jack Bean meal extracts.

FIG. 3- Rate of UH (R_{UH}) of (a) Sporosarcina pasteurii cells cultured in BHI, (b) Sporosarcina pasteurii cells, cultured in BHI and LB nutrient medium, both harvested by centrifugation, (c) Sporosarcina pasteurii cells (BHI/centrifuged) and Jack bean meal extracts.

FIG. 4- Lineweaver-Burk plots for different UHMs considered in the study.

FIG. 5- Variation in pH (a) and EC (b) duringurea hydrolysis with different buffers (C_{urea} = 1M).

FIG. 6- Variation in pH during urea hydrolysis with different soil extracts (C_{urea} = 1M).

FIG. 7- *Time required for complete urea hydrolysis with varying bacterial retention.*