Surface percolation for soil improvement by biocementation utilising in-situ enriched indigenous aerobic and anaerobic ureolytic soil microorganisms

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Running title: ground improvement utilising soil microorganisms

ABSTRACT

The use of bio-cementation via microbially induced carbonate precipitation (MICP) for improving the mechanical properties of weak soils in the laboratory has gained increased attention in recent years. This study proposes an approach for applying bio-cementation in-situ, by combining the surface percolation of nutrients and cementation solution (Urea/$CaCl_2$) with in-situ cultivation of indigenous soil urease positive microorganisms under non-sterile conditions. The enrichment of indigenous ureolytic soil bacteria was firstly tested at batch reactors. Using selective conditions (i.e. pH of 10 and urea concentrations of 0.17 M), highly active ureolytic microorganisms were enriched from four diverse soil samples under both oxygen limited (anoxic) and oxygen free (strictly anaerobic) conditions, providing final urease activities of more than 10 and 5 U/mL, respectively. The enrichment of indigenous ureolytic soil microorganisms was secondly tested in pure silica sand columns (300 mm and 1000 mm) for bio-cementation applications using the surface percolation approach. By applying the same selective conditions, the indigenous ureolytic soil microorganisms with high urease activity were also successfully enriched for both the fine and coarse sand columns. However, the in-situ enriched urease activity was highly related to the dissolved oxygen of the percolated growth medium. The results showed that the in-situ cultivated urease activity may produce non-clogging cementation over the entire 1000 mm columns with unconfined compressive strength varies between 850-1560 kPa (for coarse sand) and 150-700 kPa (for fine sand), after 10 subsequent applications of cementation solution. The typically observed loss of ureolytic activity during the repeated application of the cementation solution was recovered by providing more growth medium under selective enrichment conditions, enabling the in-situ enriched ureolytic microorganisms to increase in numbers and urease activity in such a way that continued cementation was possible.

KEYWORDS: Biocementation; in-situ cultivation; selective enrichment; ureolytic bacteria; ground improvement

INTRODUCTION

Injection of chemical grouting and deep mixing using chemical admixtures are commonly used soil improvement techniques (Solanki and Zaman 2010; Kazemian et al. 2015); however, these techniques are time consuming, expensive, and environmentally detrimental (DeJong et al. 2010). Therefore, continuing studies into finding alternative soil improvement methods are vital to achieve optimum performance, economic viability, and environmental sustainability. Improving the mechanical properties of soil by microbially induced carbonate precipitation (MICP) is currently of particular interest to engineers and microbiologists. MICP can be carried out in a number of ways, for example, by submersed sequential flushing of bacteria and cementation solution (van Paassen et al. 2010; Whiffin et al. 2007; DeJong et al. 2006) and surface percolation of cultivated bacteria and cementation solution (Cheng and Cord-Ruwisch 2014). These methods induce high concentrations of urease active bacteria positioned inside the soil prior to supplying the cementation solution. Once done, the introduced bacteria inside the soil pores usually face a number of challenges such as the reduction in population from predation and competition, stress from abiotic factors (such as pH, osmotic pressure, temperature, and availability of suitable nutrients), and bacteria capsulation by calcium carbonate crystal formation (Evans et al. 1993; van Elsas et al. 1991; van Paassen 2009). Gradually decreased in-situ urease activity is difficult to revert because it necessitates repeated injection of large quantities of ureolytic bacteria that are normally cultivated in an ex-situ reactor under sterilised conditions. The reinjection of bacteria becomes incrementally difficult due to the progressing calcite build-up with the flush of each cementation solution, leading to accumulation of bacteria around the injection end. This can eventually result in a surface or injection point blockage (Whiffin et al. 2007; Cheng and Cord-Ruwisch 2014). To overcome this problem apart from injecting grown bacteria,

65 Cunningham et al. (2009) has tested the in-situ establishment of pure *Sporosarcina pasteurii*
66 biofilm by introducing inoculum and growth medium together into sand columns, and
67 examined the recovery of biofilm by introducing additional growth medium.

68 Urease activity is widely distributed into soil, where it plays an essential role in nitrogen
69 metabolism (Ciurli et al. 1996). It can be attributed to the production of bacteria, yeasts,
70 filamentous fungi, and algae (Bekheet and Syrett 1977; Booth and Vishniac 1987). In a trial
71 of sampling six different soils, 17-30% of a cultivable bacterial population was urease
72 positive, including aerobes, micro-aerophiles and anaerobes (Lloyd and Sheaffe 1973). For
73 example, *Bacillus cereus*, which is widely distributed in soil and water, is a facultative
74 aerobic ureolytic bacterium (Vilain et al. 2006). Han et al. (2010) have successfully isolated a
75 urease positive, facultative anaerobic bacterium from soil collected in Melbourne, Australia.
76 Such facultative or anaerobic ureolytic bacteria might allow *in-situ* cultivation under oxygen-
77 limited conditions. However, due to the relatively low urease activity compared to that used
78 for MICP (Whiffin et al. 2007; Ciurli et al. 1996), indigenous soil urease existing in nature is
79 not suitable for a significant cementation to occur. Therefore, the cultivation or enrichment of
80 ureolytic microorganisms is essential.

81 The cultivation of urease active bacteria by traditional biotechnology production is very
82 costly which renders an economical large-scale application of biocementation. In an attempt
83 to produce high concentrations of urease active bacteria without the need for an expensive
84 sterile cultivation process, Burbank et al. (2011) has developed an approach for in-situ
85 cultivation of indigenous soil ureolytic bacteria prior to biocementation by adding an organic
86 carbon in the form of molasses and urea to soil at neutral pH. While an increase in the
87 ureolytic activity was not recorded, an increase in the copy number of the gene contributing
88 to urease synthesis was noticed. After adding solutions containing urea and calcium ions, it

was demonstrated (via cone penetration testing) that an increase in soil strength of up to two times was accomplished at a depth of around 1.2 m after about 25 days (note that the slow cementation process of a 25-day treatment might be due to low in-situ urease activity). Cheng and Cord-Ruwisch (2013) has also developed a selective enrichment technique that uses aerobic chemostat inoculated with an activated sludge for continued selective cultivation of highly active ureolytic bacteria culture (i.e. 60 U/mL) by applying high pH and urea/ammonia concentrations as the key selective conditions. As discussed above, indigenous soil urease positive microorganisms consist of aerobes, facultative aerobe and anaerobes, therefore, it is expected that in-situ enrichment of urease activity would strongly depend on the dissolved oxygen concentration in the environment. However, for MICP ground improvement, an investigation into in-situ enrichment of indigenous soil ureolytic microorganisms under various dissolved oxygen conditions and urease activity distribution along the soil depth has not yet been reported in the literature. The oxygen concentration in soil is usually decreased with depth, which is due to the oxygen depletion by the soil bacteria and plants roots (Hanslin et al. 2005) and also because of the air diffusion pathway being cut off by the pore solution within the soil matrix, especially in highly saturated soil regions.

In this paper, a method for selective in-situ cultivation of soil ureolytic bacteria is developed to investigate the distribution of in-situ enriched urease activity and examine whether or not sufficient urease activity can be created within a short period of time for successful biocementation of sand. In order to create different air diffusion capacities within bio-treated sand columns under free-draining conditions, two types of sand (fine and coarse) are used for investigation of in-situ urease activity enrichment and MICP.

MATERIALS AND METHODS

Indigenous Microorganisms

The soil used for bacteria extraction was obtained from Murdoch University Campus, Perth, Australia, using a hand shovel. Samples collected from 20 cm depth were suspended in deionized (DI) water with a weight ratio of 1 to 10. After 12 hours of continuous shaking, the liquid was filtered through 10 cm thick of a sand filter to remove any impurities or suspended solids. The filtered soil suspension containing indigenous soil microorganisms was stored at 4°C prior to use as inoculum. The optical density (OD_{600nm}) of the soil suspension was found to be around 0.1.

Batch Culture Enrichment of Ureolytic Bacteria

Enrichment of ureolytic bacteria under oxygen limitation conditions was carried out by cultivating soil microorganisms in a series of non-mixed glass flasks under non-sterile batch conditions. Specified concentrations of different growth ingredients (Table 1, Trials 1–6) were added into 100 mL of soil suspension (prepared as described above), which was placed into 250 mL flasks with cotton bungs. Soil suspension was then incubated at 28°C for 8 days.

For the anaerobic growth and urease activity development, the soil suspension was incubated in the absence of oxygen (Table 1, Trial 7), and 50 mL of soil suspension containing 20 g/L YE and 0.17 M urea (starting pH = 10) was placed into 100 mL serum vial and purged with nitrogen gas for 5 minutes prior to incubation. The serum vial was sealed with a rubber bung and stored at 28°C, and the urease activity was measured daily.

In-situ Growth of Soil Ureolytic Microorganisms

After obtaining the optimum growth conditions for enrichment of the ureolytic microorganisms of the soil batch culture, the in-situ enrichment was tested for sand columns of 300 mm long and 45 mm in diameter. In order to generate soil of reproducible biological activity, cleaned and washed silicate sand (fine or coarse) premixed with the soil suspension described earlier was used using a weight ratio of 3 to 1. The 300 mm sand columns, packed with fine or coarse silica sand, were under continuous vibration to provide any even density resulting in an average dry density of approximately 1.63 g/cm^3 for both types of sand columns. The grain size distribution of the sand used (Figure 1) was obtained using the sieve analysis in accordance with the Australian Standards of AS 1289.3.6.1-2000 (2000). According to the water retention capacity under the free draining conditions, growth media (20 g/L YE, 0.17 M urea, and pH 10) of 180 mL and 80 mL, respectively, were percolated into the bottom-opened fine and coarse sand columns. The percolated sand columns were incubated at 28°C for 7 to 9 days. During the incubation period, the bottom of sand columns was closed and the top was covered with an aluminium foil to minimise evaporation.

It should be noted that in real world situations, a greater depth of more than 300 mm needs to be reached for most soil stabilisation and ground improvement projects. However, past experiments with longer columns typically resulted in an accumulation of bacteria towards the injection end by the filtering-out effect, as one would expect. This naturally leads to more calcite precipitation at the injection point of columns (Tobler et al. 2012). However, by growing bacteria in-situ, the effect of calcite accumulation at the injection point might be prevented. Thereby, additional sand columns of 1000 mm long and 55 mm in diameter were cemented and the effect of in-situ grown ureolytic bacteria for deeper cementation was investigated. The same procedure described above was conducted for the 1000 mm sand

columns, except for the volumes of the introduced growth medium, which were used to be 450 mL for the fine sand and 250 mL for the coarse sand.

Under free draining conditions, it is important to investigate the degree of saturation, which is the ratio between volume of water in the voids and the total volume of voids, because degree of saturation determines the amount of retained culture and cementation solution at different locations of the sand columns. The volume of water in the voids was obtained by collecting the partially saturated soil samples and drying them at 105 ^{o}C for 24 hours. It was found that the degree of saturation varied along the short 300 mm sand columns (Figure 2a). For fine sand, almost fully saturated condition of 95% was achieved along the entire column. In contrast, the short coarse sand column provided partially saturated condition in which the degree of saturation was about 10% at the top and gradually increased to about 80% at the bottom. For the 1000 mm sand columns, the profile of degree of saturation was different from that of the 300 mm short columns (Figure 2b). For the fine sand, the degree of saturation at the top was about 20 %, which gradually increased to more than 90% at a depth of 700 mm and remained to be almost fully saturated until reaching the bottom. However, for the coarse sand, 10% of degree of saturation was achieved from the top to the depth of 600 mm and then gradually increased with the increase in depth to about 75%. Overall, the variations of degree of saturation in the sand columns provided both fully saturated and partially saturated conditions for the in-situ enrichment of ureolytic bacteria and consequent biocementation.

Biocementation by Surface Percolation

The sand columns (300 and 1000 mm) with successfully established in-situ urease activity were treated with cementation solution of 1 M urea and 1 M $CaCl_2$ by the surface percolation method developed by Liang and Cord-Ruwisch (2014), followed by a reaction period varied

between 24 and 96 hours. For each sand column, the volume of cementation solution applied to one treatment was the same as that of the percolated growth medium (i.e. for fine sand: 180 mL and 450 mL for the 300 mm and 1000 mm columns, respectively; and for coarse sand: 80 mL and 250 mL for the 300 mm and 1000 mm columns, respectively). The sand columns were kept at the room temperature (25±1°C) during the biocementation process.

Urease Activity Measurement

The urease enzyme activity in the cultures was defined as the urease activity (U) present in 1 mL of culture (volumetric urease activity). One U is defined as the amount of enzyme that catalyses a conversion of 1 micromole of substrate per minute, which was endorsed by the Nomenclature Committee of the International Union of Biochemistry (N.C.o.t.I.U.o.B.) (1979) The in-situ urease activity of the sand columns was defined as the urease activity (U) present in 1 g of dry sand. All urease activity measurements were conducted at pH of 9 (25^{o}C and 1 atm). The determination of urease activity was determined through the following three steps:

1) For the culture urease activity, mix 1 mL of culture with 9 mL of urea solution to a final urea concentration of 1.5 M; For the in-situ urease activity measurement, sample about 10 g of sand from different depths of the sand columns and add to 10 mL of urea solution (1.5 M);
2) Incubate the above mixture for 1 h at 25°C to allow the urea hydrolysis reaction to happen; and

3) Determine the ammonia concentration before and after the urea hydrolysis reaction for calculation of urease activity.

After the in-situ urease activity was measured, the sand samples were rinsed with deionised (DI) water and dried at 105°C for 12 hours. The weight of dry sand samples was recorded and the corresponding urease activity was normalised per g of dry sand.

Unconfined Compressive Strength (UCS) Tests

After the MICP treatment, the treated sand samples were flushed with at least five void volumes of tap water so as to wash away all excess soluble salts prior to the UCS measurements. The UCS tests were then conducted on specimens with selected aspect diameter-to-height ratios of between 1:1.5 and 1:2. The axial load was applied at a constant rate of 1.0 mm/min.

Scanning Electron Microscopy (SEM) Analysis

SEM analysis (PHILIPS XL20 Scanning Electron Microscope, Eindhoven, the Netherlands) was conducted to demonstrate the immobilisation of the in-situ grown microorganisms and $CaCO_3$ crystal precipitation. Two identical fine sand columns of 300 mm long containing soil suspension were incubated at the optimum growth media for 5 days. Then, one of the sand columns was flushed with two times pore void volumes of DI water, to wash out any unattached bacteria. The other column was flushed with two times pore void volumes of DI water followed by 180 mL of cementation solution (12 hours of reaction). Sand samples

collected from the middle part of the two sand columns were dried at the room temperature for 2 days prior to SEM analysis.

RESULTS AND DISCUSSION

Optimum Growth Medium for Enrichment of Batch Culture Soil Ureolytic Microorganisms

In order to achieve an optimum growth medium, the soil used was tested under different growth conditions for both anoxic and anaerobic conditions (see Table 1). In previous enrichment of ureolytic bacteria from activated sludge, YE as carbon source resulted in a high level of urease activity (about 60 U/mL) (Cheng and Cord-Ruwisch 2014). In the current study, apart from YE, molasses was tested as a replacement substrate. This is because molasses is an inexpensive carbon source and has been used for enrichment of soil and marine bacteria in a number of processes including bioremediation (Boopathy et al. 1998); enrichment of indigenous ureolytic bacteria for soil consolidation (Burbank et al. 2011); and calcium removal from groundwater (Fujita et al. 2007). Apart from YE and molasses, acetate as a simple carbon source was also tested.

Anoxic (oxygen limited) Conditions

Figure 3(a) indicates the enrichment of urease activity soil sample under three different organic substances, as shown in Table 1. The dissolved oxygen (DO) concentration of all cultures dropped from about 8 to 0 mg/L within 1 day of incubation. The YE contained

culture resulted in urease activity of about 4 U/mL, which was about 10-fold higher than that obtained from the molasses or acetate-based medium (Figure 3a). This indicates that the cultivable urease positive microorganisms require protein-based media rather than carbohydrates, as reported for the aerobe *Bacillus pasteurii* (Mörsdorf and Kaltwasser 1989). Experiments of varying starting urea concentration and pH showed that a pH of 10 and the presence of urea were essential for the enrichment of highly urease active microorganisms (about 10 U/mL and OD_{600}=4.78) (Figure 3b). This is in agreement with previous findings by Cheng and Cord-Ruwisch (2013) for the aerobic cultures.

Anaerobic Conditions

In order to test the possibility of enriching soil ureolytic microorganisms under anaerobic conditions, soil suspension containing 20 g/L YE and 0.17 M urea was prepared and incubated in nitrogen degassed, sealed serum vials. After about 150 hours of incubation, bacterial growth and urease activity were developed but with 75% less biomass (OD_{600}=1.08) and 50% less urease activity (5.4 U/mL) than those produced under the oxygen-limited conditions (Figure 3b). However, the specific urease activity (urease activity per biomass) produced was about 2-fold higher than that of the anaerobic conditions.

The ureolytic bacteria have a unique mechanism for the formation of adenosine triphosphate (ATP), which involves coupling of ATP generation with urea hydrolysis and ammonium gradient (Mobley and Hausinger 1989). Ammonia has been described to be toxic to bacteria when presented in high concentration due to its well-known cytotoxic effects (Hess et al. 2006; Tobler et al. 2011). While high concentration of ammonia is detrimental to most cells, it may be an advantage for specific ureolytic bacteria that have a unique

mechanism for the ATP formation involving a coupling of ATP generation with urea hydrolysis and developing an ammonium gradient (Mobley and Hausinger 1989). Such characteristics allow the selection of enrichment of ureolytic bacteria against urease negative bacteria under extreme conditions of high starting pH of 10 and urea/ammonia concentration of 0.17 M. Such conditions allow the ureolytic soil communities to grow and become ureolytically active within about 72 hours. In contrast, Tobler et al. (2011) reported that several weeks were required for the ureolytic groundwater communities to become ureolytically active with molasses addition as food source at neutral pH condition. This may be explained by the more favourable growth condition for ureolytic bacteria associated with higher concentration of the substrate applied in the current study compared to that applied in the study conducted by Tobler et al. (2011) (i.e., pH 10 with 20 g/L YE compared to pH 7.5 with 1 g/L molasses). For cost minimisation at the industrial scale, the expensive protein-rich YE medium could be replaced by industry organic wastes (Achal et al. 2009).

Enrichment of Urease Activity for Various Soil Samples under Non-sterile Conditions

Under the optimum growth medium of 20 g/L YE, 0.17 M urea and pH 10, three other soil samples collected from different locations in Perth, Australia, were also tested and all indicated positive urease activities (Table 2; the soil sample denoted as *S*-1 is the one used in the above study, whereas the soil samples denoted as *S*-2 to *S*-4 are those investigated in this section). However, the maximum urease activities of these four soil samples were different, which was possibly due to the variation of soil characteristics such as the organic content, pH, bacterial community (Table 2). It is also interesting to note that the optimum growth medium works for marine sediment, producing a maximum urease activity of about half of

that obtained for *S*-1 (data not shown). This provides a high potential for MICP to be used in applications such as mitigation of submarine sediment liquefaction and prevention of beach sand erosion and cliffs scouring, as proposed by Cheng et al. (2014) and Ivanov et al. (2015).

In-situ Enrichment of Soil Ureolytic Microorganisms

Previous experiments (Figure 3, Table 2) have demonstrated an optimum growth medium for selective enrichment of highly urease active bacteria under oxygen-limited (anoxic) and oxygen-free (anaerobic) conditions. These results are used herein to attempt enrichment of urease activity directly in-situ. The four soil samples tested above (i.e. *S*-1 to *S*-4) showed various maximum urease activities after enrichment. Generally speaking, the efficiency of MICP (i.e. urea conversion) increases with the increase in the urease activity. In the current study, the outcomes of MICP in the presence of the lowest urease activity may represent the worst case scenario of ground improvement. Therefore, the soil sample of the lowest level of enriched activity (i.e. *S*-1 in Table 2) was selected for the in-situ enrichment of soil ureolytic microorganisms as presented below.

Development of Urease Activity in Coarse and Fine Sand Columns

A series of coarse and fine sand columns was premixed with soil suspension, followed by percolation of the above tested optimum growth medium (i.e. pH 10, 0.17 M urea and 20 g/L YE) and incubation at 28^{o}C. According to the profile of in-situ dissolved oxygen (DO) concentration (Figure 4), it was suggested that the surface percolation method creates anoxic and anaerobic conditions inside of the coarse and fine sand columns.

330 The in-situ urease activity in both the coarse and fine sand columns significantly increased
331 during the time period of incubation and reached a maximum level of about 5 to 7 days
332 (Figure 5). The distribution of urease activity for the fine sand columns was relatively
333 uniform (Figure 5b), whereas it varied with the depth for the coarse sand columns (Figure
334 5a). It should be noted that after 9 days of incubation, the pH of in-situ cultivated culture
335 dropped from 10 to 8.8 ± 0.1 in all tests, which was similar to the pH level of normal
336 ureolytic bacterial culture used for biocementation by Whiffin et al. (2007).
337 The in-situ urease activity (U/g sand) at a specific location was determined by the volume
338 of culture present (pore liquid content) (mL/g sand), which was determined by drying the
339 partially saturated soil sample obtained from different locations at 105^{o}C for 24 hours, and
340 the volumetric urease activity of bacterial culture (urease activity per volume of culture,
341 U/mL). For the coarse sand columns, the in-situ urease activity increased with depth, from
342 the top to 200 mm deed (Figure 5a). This is probably due to the increased pore liquid content
343 with depth (Table 3). However, at locations deeper than 200 mm, the lower volumetric urease
344 activity resulted in lower in-situ urease activity (Table 3, Figure 5a) possibly because of
345 oxygen limitation as indicated in Figure 4. In contrast, the pore culture content and
346 volumetric urease activity were relatively homogeneous along the short fine sand columns
347 (Table 3), resulting in a relatively constant in-situ urease activity (Figure 5b).
348 The fact that higher volumetric urease activity was developed in the coarse sand than in the
349 fine sand may be caused by the more oxygen availability. In the fine sand columns, more
350 than 80% of pores were filled with water under free draining conditions, which limits the
351 oxygen supply compared to the coarse sand columns in which less than 30% of pores were
352 filled with water. In fact, the oxygen level dropped to 0 mg/L after 1 day of incubation in the
353 fine sand columns while it lasted for more than 5 days in the coarse sand columns (Figure 4).

354 The inhibitory effect of the lack of oxygen on the volumetric urease activity production of
355 soil bacteria was evident by the anaerobic batch experiment presented above (Figure 3b).
356 The anaerobic enrichment and cultivation of ureolytic bacteria with high urease activity is
357 potentially novel as the existing highly urease active bacteria are largely confined to aerobic
358 genus of *Bacillus pasteurii*. The isolation of pure cultures could be a worthwhile research aim
359 for future development of the current study. In contrast to the pure silica sand used in the
360 current study (initial soil pH was neutral), other soils may have different pH values ranging
361 from acidic to alkaline, with varying amounts of buffer capacity. Therefore, the optimum
362 enrichment condition that requires high pH of 10 might not be applicable to all soils such as
363 the acidic soils and soils with strong buffer capacity.
364
365 *Immobilisation of In-situ Grown Ureolytic Microorganisms*
366
367 The above described high in-situ urease activity provides promising environmental
368 conditions for precipitation of $CaCO_3$ crystals. However, biocementation can only be
369 efficiently operated when the ureolytic bacteria are immobilised and prevented from being
370 washed out by the cementation solution. Scanning electron microscopy (SEM) images of the
371 incubated fine sand columns (after 5 days) showed that the bacterial cells were successfully
372 immobilised on the sand surface (Figure 6a). The bacterial cells had rod shape with sizes of
373 up to 2 μm. Apart from attached bacterial cells bacterial spores were also observed (Figure
374 6a). The SEM images taken after flushing with the cementation solution showed that typical
375 rhombohedral crystals of calcite biocementation, similar to the published results by Al-
376 Thawadi et al. (2012), have formed and precipitated on the sand grain surface with sizes of
377 up to 10 μm. Bacterial cells were also observed around the crystals (Figure 6b). By

comparing the urease activity immobilised in the sand columns with that of the effluent after flushing with 2 times pore void volumes of DI water, the percentage of immobilised urease activity was determined. Approximately 85% and 75%, respectively, of the total in-situ urease activity were immobilised in the coarse and fine sand columns (300 mm long).

MICP with In-situ Enriched Soil Ureolytic Bacteria

After concluding that in-situ enriched and grown ureolytic bacteria were adequately immobilised in sand, a cementation trial was carried out by repeatedly adding the cementation solution as presented below.

Recovery of In-situ Urease Activity during Biocementation by Initiating Renewed In-situ Growth

It is generally observed in MICP with immobilised ureolytic bacteria that the urease activity diminished with repeated treatments by the cementation solution (e.g. urea and $CaCl_2$) (Van Paassen et al. 2010; Cheng and Cord-Ruwisch 2014). This can also occur in the current trial. In both sand columns, the immobilised urease activity decreased with repeated treatments and cementation solution in such a way that after 5 treatments the in-situ urease activity has dropped to 30 % and 10 % of its original value in the coarse and fine sands, respectively (Figure 7a). The loss of in-situ urease activity has been explained by the compounded effects of biological degradation and chemical reaction (Van Paassen 2009). When the in-situ urease activity decreases, provision of an additional urease activity into the sand is necessary to enable the biocementation reaction to continue. This is traditionally achieved by injecting

402 further volumes of ureolytic bacteria. Figure 7 shows that the addition of further selective
403 medium and incubation for another 5 days allowed the ureolytic bacteria to regrown in-situ,
404 resulting in a recovery of the urease activity in the sand column (Figure 7a).

405 The ability to enrich ureolytic bacteria in-situ at large scale (in the order of meters) is
406 important for upscaling biocementation in the field. Similar to the observations in the short
407 columns, it can be seen from Figure 7b that the in-situ urease activity was developed for the
408 1000 mm columns by incubation but the activity was also decreased during the cementation
409 solution treatment, and the stagnant activity was recovered by re-incubation with fresh
410 growth medium. The overall in-situ urease activity in the long sand column indicated lower
411 value to that obtained in the short sand column, and this may be attributed to the lack of
412 dissolved oxygen, which decreases with the depth of sand columns.

413

414 **$CaCO_3$ and UCS**

415

416 After being treated with the cementation solution for a total of ten times, the short sand
417 columns were prepared to the UCS and $CaCO_3$ content measurements. The ammonium
418 concentration, UCS and $CaCO_3$ content were determined as described previously by Cheng et
419 al. (2013). The total amounts of precipitated $CaCO_3$ in the coarse and fine sand columns were
420 65 and 78 g, which represent about 81% and 43%, respectively, of the conversion of injected
421 cementation solution. The UCS for the coarse sand (q_{ucs}: 920-1250 kPa; ε: 1.48-1.85%) was
422 twice as high as the fine sand (q_{ucs}: 410-570 kPa; ε: 1.03-1.64%), although similar levels of
423 calcite content were formed (about 0.06 g/g calcite) in both tests (Figure 8).

424 Figure 9 shows that significant amount of crystals were formed at the surface of grain
425 particles in the fine sand column, while the crystals formed clear “bridges” at the connecting

426 points of grain particles of the coarse sand column. This is consistent with previous
427 observations (Cheng et al. 2013), in which the percolated sand columns produced effective
428 bonding crystals at the low degree of saturation zones leading to more strength per calcite.
429
430 **Front End Blockage Testing with Longer Sand Columns**
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432 As mentioned earlier, in real world cementation, greater depths than 300 mm need to be
433 reached, which may lead to accumulation of bacteria towards the injection point end causing
434 blockage. To test the effect of in-situ grown ureolytic bacteria for deeper cementation, sand
435 columns of 1000 mm long were cemented and tested. In the fine sand column, the $CaCO_3$
436 content was increased with depth (Figure 10a), which was contrary to the normal
437 biocementation treatment of long fine sand columns where the highest amount of $CaCO_3$ is
438 generally obtained near the injection point, eventually leading to blockage (Whiffin et al.
439 2007; Cheng and Cord-Ruwisch 2014). This is because in dry or partially saturated
440 conditions, MICP solution introduced by surface percolation will be retained and
441 accumulated at the connection points as a meniscus shape at the top layers due to capillary
442 force, and the excess of MICP solution moves deeper into the soil pores. The gradient of
443 saturation degree along the 1000 mm column also indicates less pore-liquid content at the
444 upper location and more pore-liquid content at the lower location, resulting in higher amount
445 of $CaCO_3$ were formed at deeper soil, far away from the injection point.
446 For the coarse sand column, the $CaCO_3$ content was relatively constant from the top of the
447 column to approximately 600 mm deep, with strength of about 1560 kPa (Figure 10b). It is
448 interesting to note that increased calcium carbonate content lead to a decreased UCS in the
449 sand column close to the bottom. It has been reported that only crystals that precipitate at the

450 connecting points can provide enough bonding force among the adjacent soil grains (Cheng
451 et al. 2013; Martinez et al. 2013; Al Qabany et al. 2012). In the top layer of sand column, the
452 residual water after drainage is known to accumulate largely at the contact points between
453 sand grains as menisci due to capillary forces (Lu and Likos 2004). Hence the crystals
454 precipitation will be restricted to these precise locations, at what could be considered the
455 optimum position for bridging of sand grains and developing strength. Crystals at junctions
456 are likely to be strength providing crystals, while crystals that form on the sand grain or in the
457 pore space, as expected at higher water content (i.e. bottom layers) cannot be seen to add to
458 strength (Cheng and Cord-Ruwisch 2012). The high strength produced here is useful and
459 sufficient for several geotechnical engineering applications, for example, prevention of soil
460 liquefaction (Andrus and Chung 1995). The fact that the calcite build-up in the coarse sand
461 column was similar to that of the fine column proves that anaerobic in-situ enrichment was
462 achieved as successfully as anoxic.
463 The final hydraulic conductivity values of the 1-meter treated fine and coarse sand
464 columns were 2.9×10^{-5} and 22×10^{-5} m/s, respectively, showing a good draining quality for
465 biocemented soils. The cementation induced by the in-situ enriched, grown and re-fed soil
466 ureolytic microorganisms can successfully avoid injection end blocking that can occur when
467 injecting externally large quantities of grown bacteria into soil, and achieve relatively
468 homogeneous cementation. This may be due firstly to avoiding urease activity filtering-out
469 effect at the injection end and allowing in-situ urease activity (U/g sand) increased with depth
470 according to the distribution of retained pore culture content; and secondly the low urease
471 activity in the top layers enabled more chemical reagents (urea/$CaCl_2$) to transport to the
472 bottom layers; and thirdly the high urease activity ensured biocementation reaction was
473 completed at the bottom within a short period. The fact that the calcite precipitation increased

474 with depth is remarkable as the soil pore clogging and more calcite precipitation near the
475 injection point have been reported in many previous reports (Stocks-Fischer et al. 1999). It
476 should be also noted that the chemical precipitation of $Ca(OH)_2$ during the cementation
477 solution injection will not occur in the current pH environment (i.e. pH =8.8 after
478 enrichment), according to the solubility product constant (Kps) of $Ca(OH)_2$ (i.e. 5.5×10^{-6} at
479 25^{o}C). And also surface percolation method diminished internal mixing of the in-situ
480 enriched culture and the injected cementation solution, which could also prevent a strong
481 chemical precipitation from happening
482
483 **Potential Advantages and Applications**
484
485 The approach of enriching urease activity in-situ can overcome several problems encountered
486 when introducing ureolytic bacteria into soil for the purpose of biocementation by: (1)
487 minimising the perceived environmental impact on local soil as the injection of exogenous
488 ureolytic bacteria would fall under close legislative scrutiny in some countries; (2) enabling
489 urease activity to reach deeper soils by avoiding the filtering-out effect at the injection end;
490 and (3) saving costs by avoiding external production, transport and injection of large
491 quantities of bacterial culture.
492 The approach of increasing the quantity of urease-producing microorganisms from soil
493 under both aerobic and anaerobic conditions provides technical feasibility of in-situ growing
494 ureolytic bacteria at both unsaturated and saturated soils such as waterlogged areas. The
495 surface percolation can be applied, for example, by spraying, irrigating or trickling into the
496 soils. These simple methods are expected to decrease the cost of MICP via avoiding
497 construction of solution injection systems. If required, the surface percolation can also be

applied in case of water-saturated soils after lowering the underground water table, which will, however, lead to extra cost. A practical application of this technology can be the stabilisation of subgrade, which has a potential to reduce the cost of excavation and replacement of unsuitable soil by allowing engineers to stabilise and strengthen the existing soils. Subgrade strengthened by MICP may also eliminate the need for other base layers provided that biocementation creates sufficient strength. It should be noted that the surface percolation method may not be applicable for saturated soil (i.e. water logging areas), therefore, in such cases submersed the flow method can be an alternative option.

Although this paper presented a promising technology (i.e. in-situ enrichment of soil ureolytic microorganisms), the laboratory test of enriching ureolytic microorganisms from local soil samples is recommended before any field applications as the soil microbes might be varied at different locations. Furthermore, in field applications, the subsoil temperature may be different from that tested here, resulting in different growth rate of ureolytic bacteria and the period required for bacteria enrichment. Generally speaking, low environmental temperature will lead to a slow growth of bacteria. Therefore, in order to obtain a successful MICP treatment it is recommended to extent the enrichment period for low temperature soil to gain sufficient ureolytic bacteria and urease activity.

CONCLUSIONS

Based on the results obtained from the laboratory tests of the current study, it was demonstrated that bacteria from four different soil samples may be enriched to increase the quantity of ureolytic microorganisms at both oxygen-limited and oxygen-free conditions. The most effective enrichment medium was similar to that used for the aerobic ureolytic bacteria

522 in the Chemostat, containing 20 g/L YE as carbon source 0.17 M urea as nitrogen source and
523 high starting pH of 10. The tests conducted on the in-situ sand columns, using the soil
524 sample with the lowest enriched activity, showed that the process for enrichment of ureolytic
525 bacteria may be applied in both the fine and coarse sand columns. The strength and $CaCO_3$
526 content of the biocemented sand columns showed that biocementation using in-situ cultivated
527 indigenous soil bacteria is feasible. Although the common obstacle of surface clogging was
528 avoided in the long fine sand columns, it is still necessary to optimise the proposed method of
529 supplying the cementation solution, especially for soils of low permeability, to obtain deep
530 cementation.
531
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533
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536 assistance in conducting the grain size distribution and UCS tests.
537
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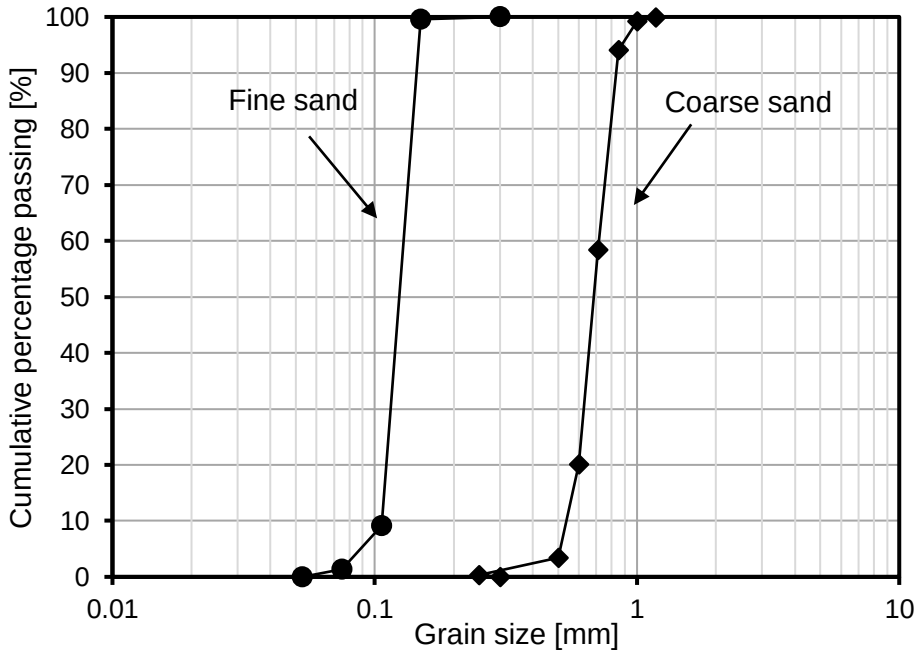
640 **Figure 1**



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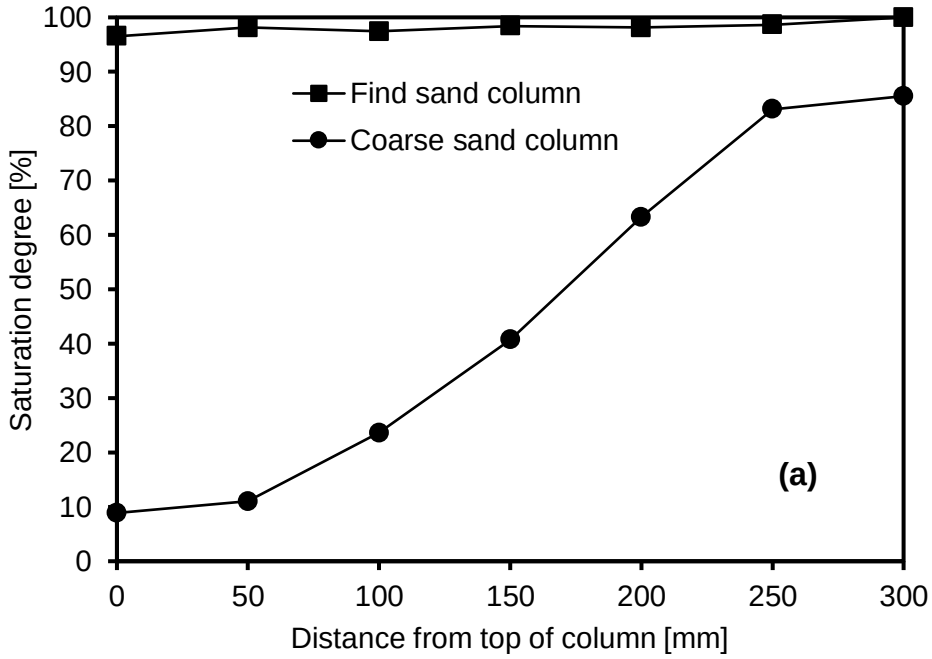
644 **Figure 2**



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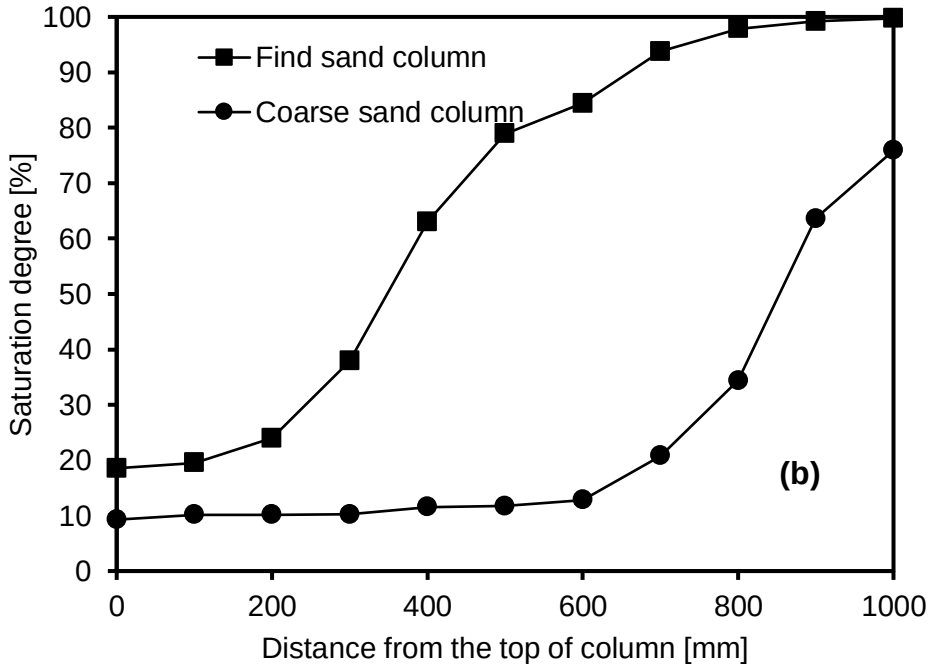

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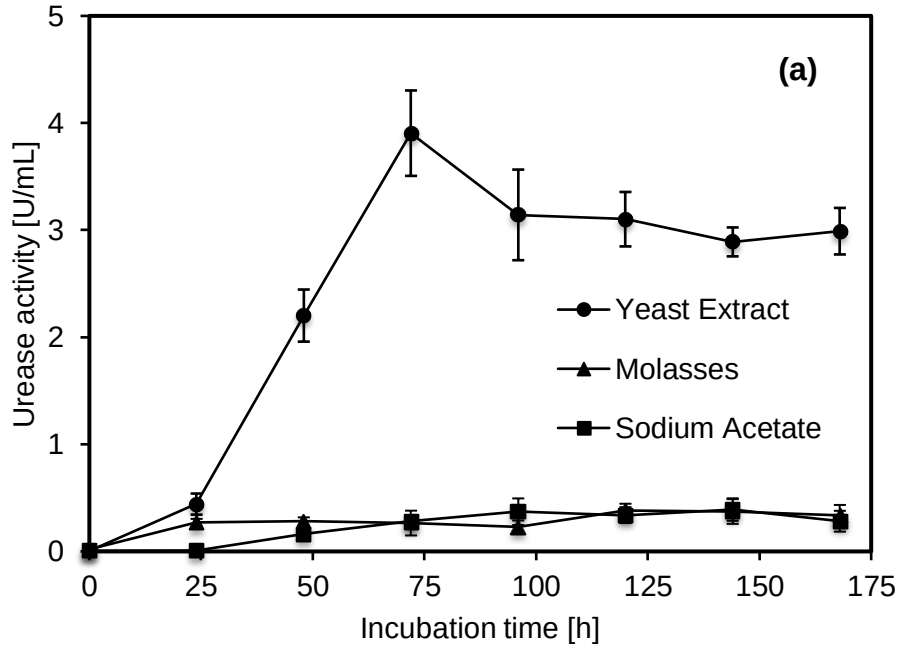
649 **Figure 3**



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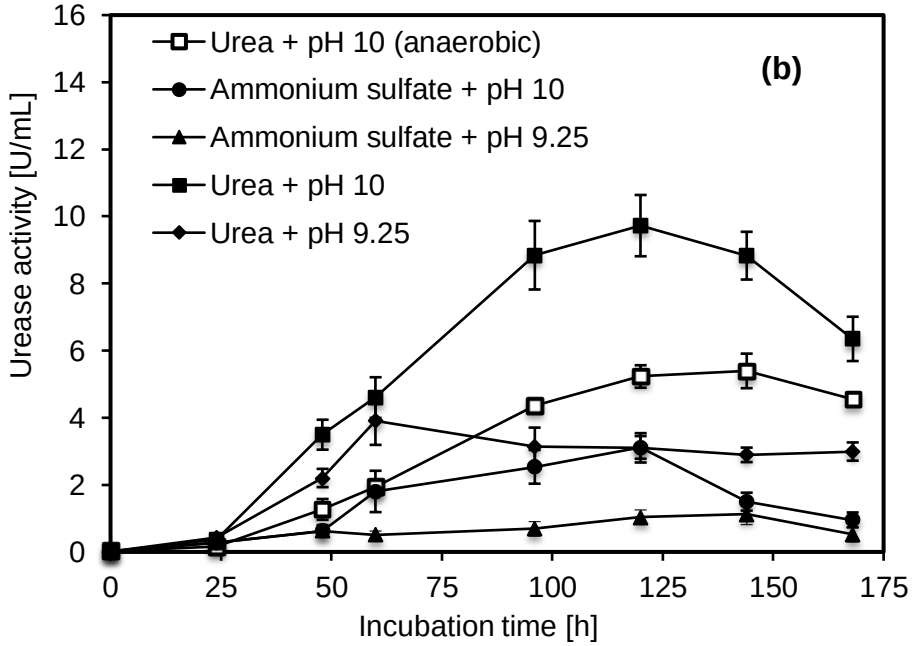

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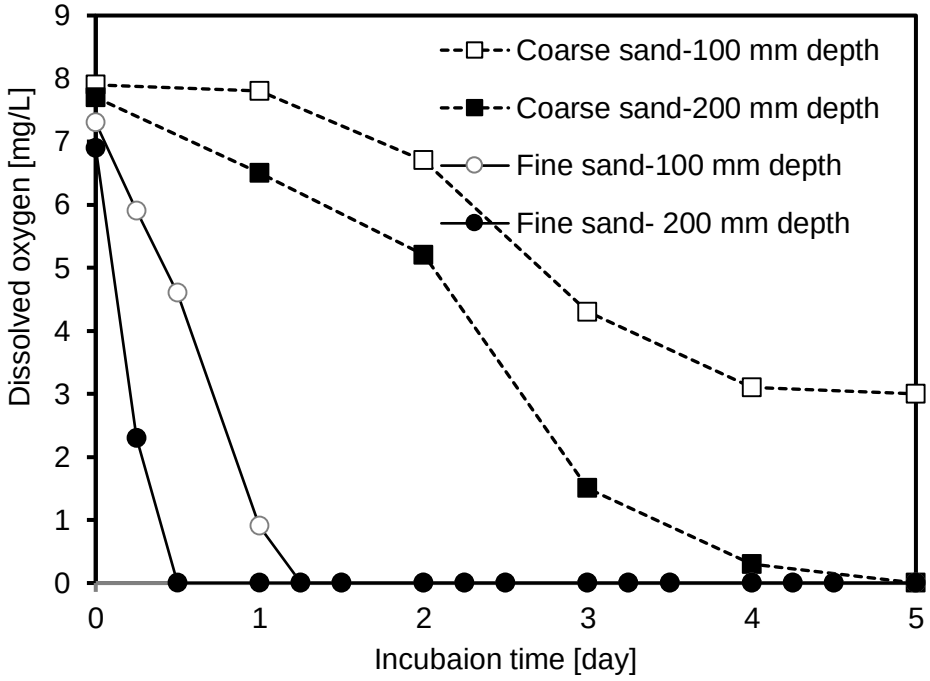
653 **Figure 4**



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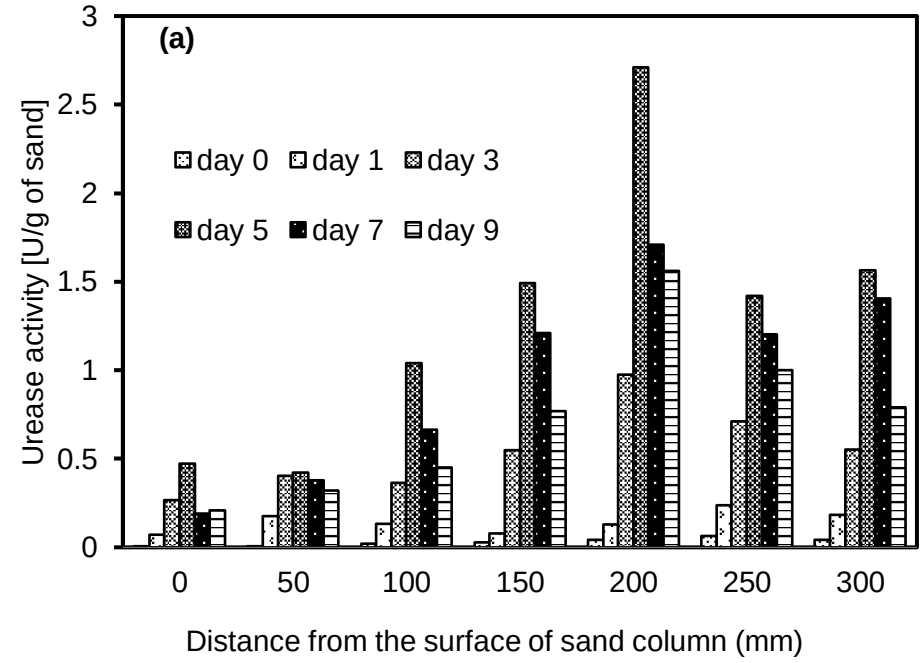
657 **Figure 5**



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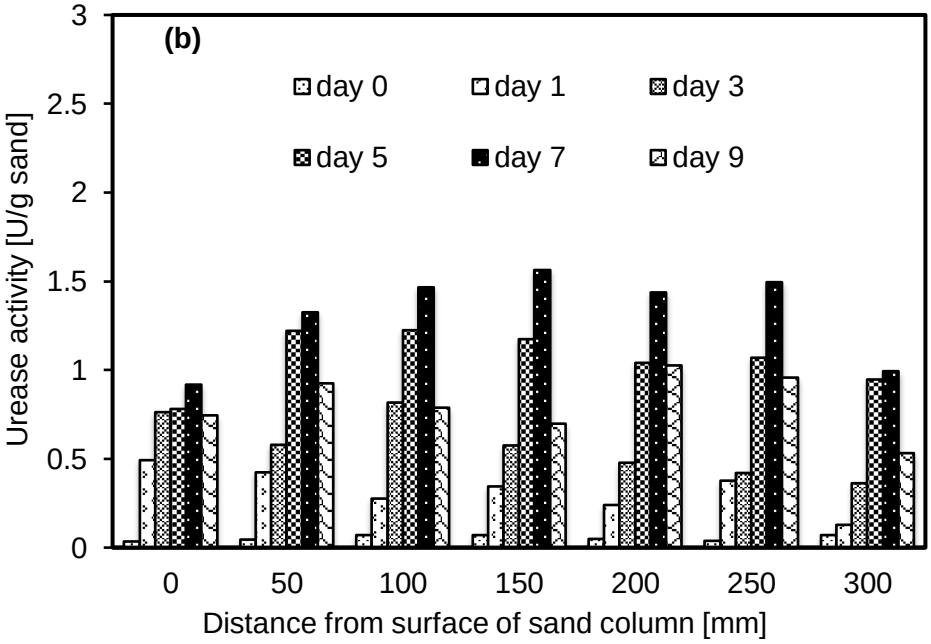

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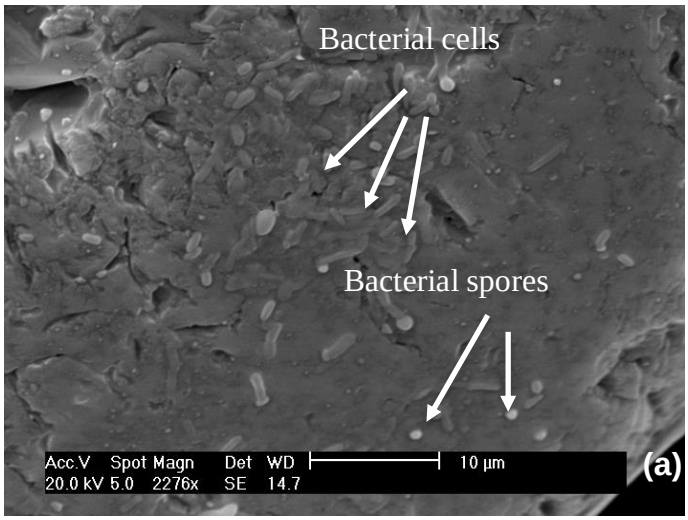
662 **Figure 6**



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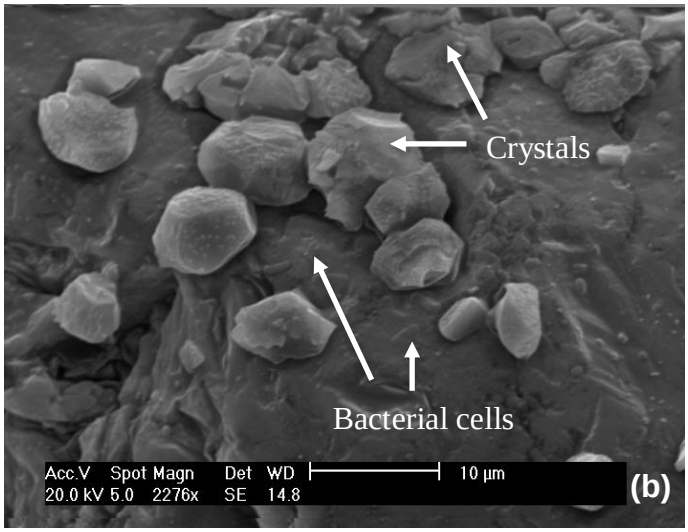

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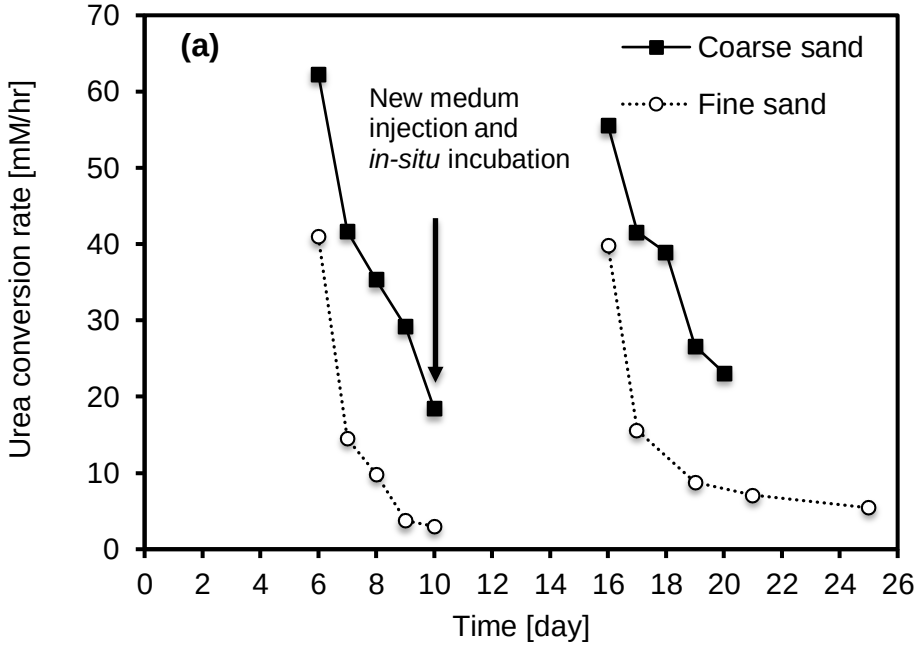
667 **Figure 7**



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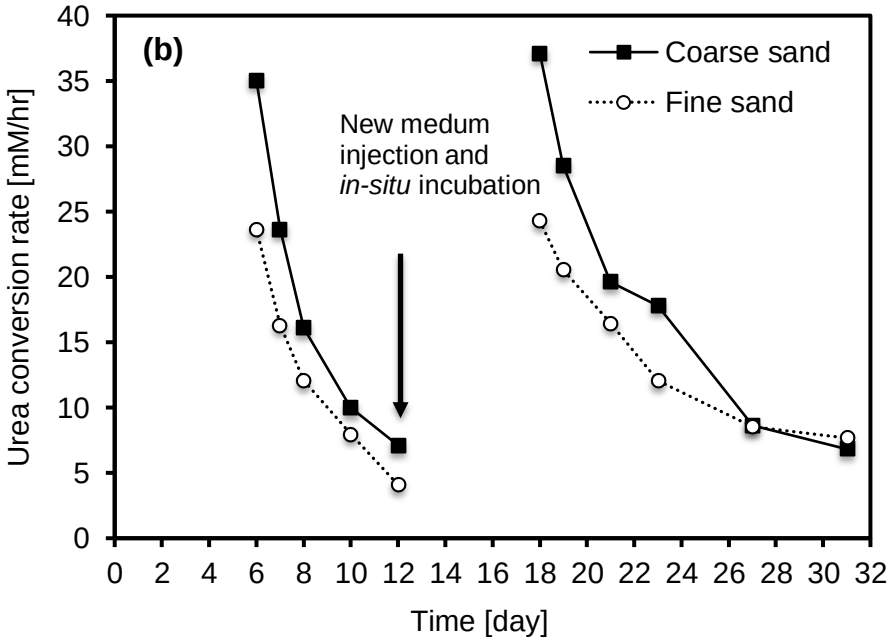

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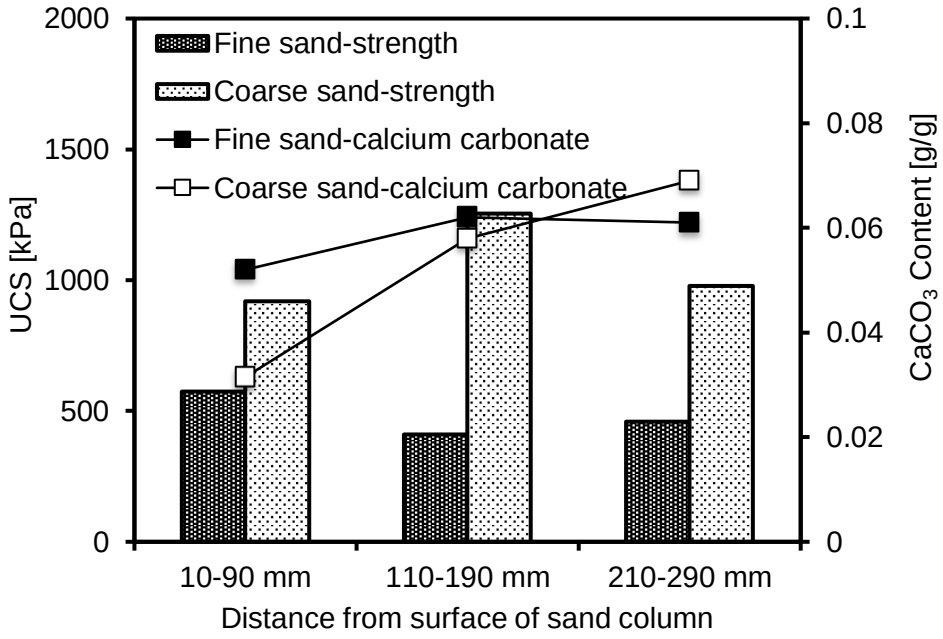

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674 **Figure 9**

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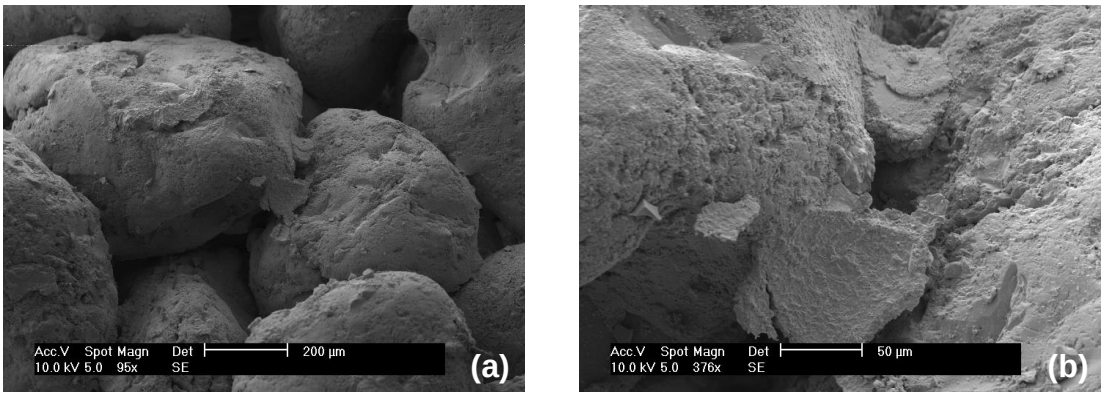

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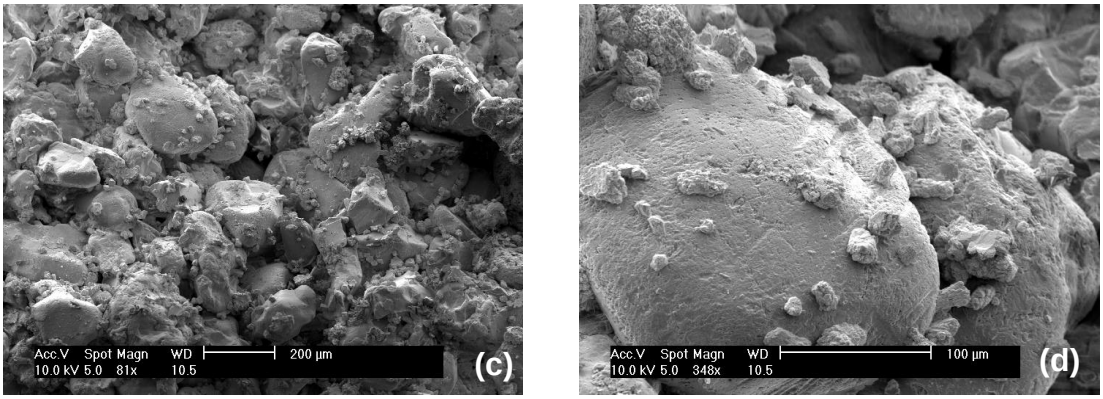

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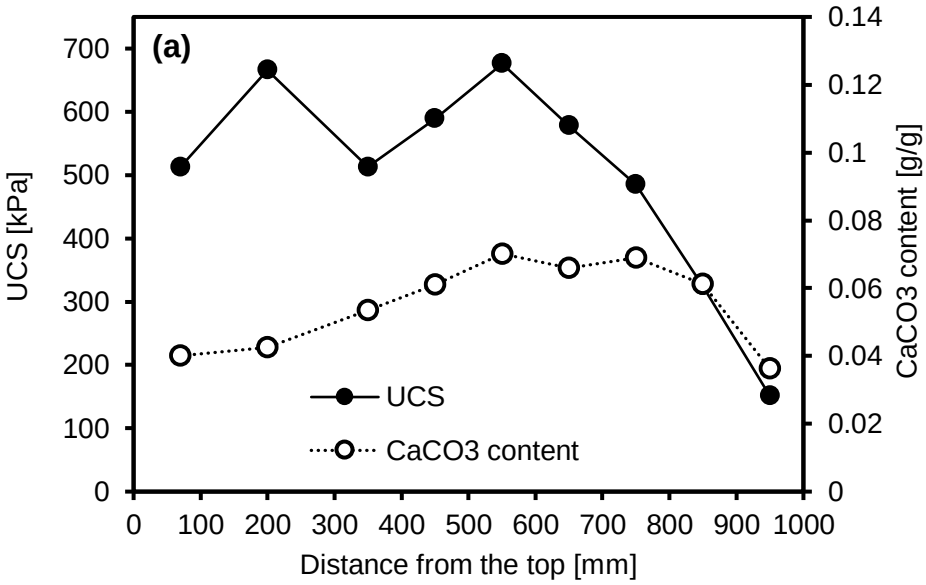
678 **Figure 10**



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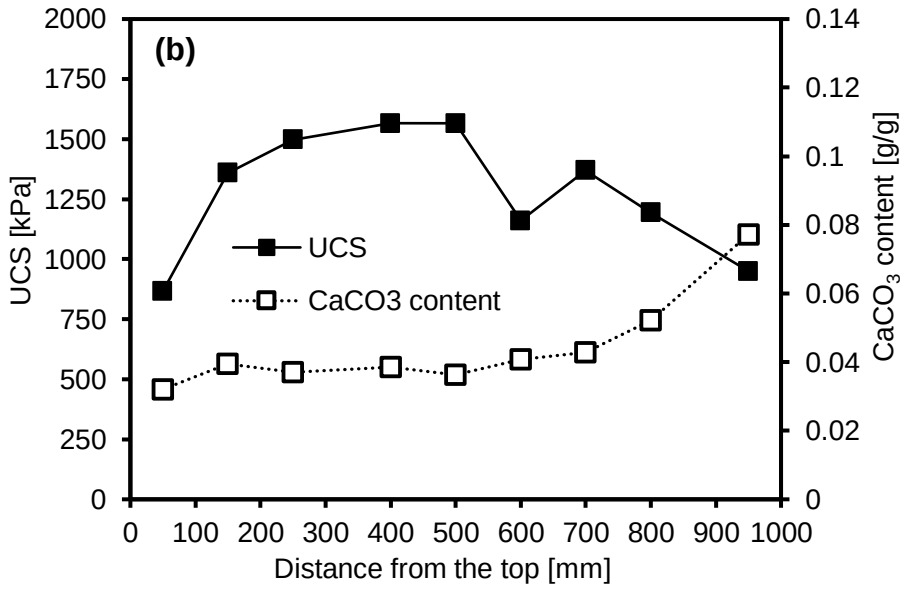

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