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Experimental optimisation of various cultural conditions on urease activity for isolated *Sporosarcina pasteurii* strains and evaluation of their biocement potentials

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Abstract

Microbially induced carbonate precipitation (MICP) is a process that has emerged as an attractive alternative ground improvement technique in Geotechnical and Civil Engineering using ureolytic bacteria for soil strengthening and stabilisation. Locally isolated *Sporosarcina pasteurii* from limestone cave samples of Sarawak were found to have high urease-producing abilities. Optimisation of various cultural conditions (incubation temperature, initial pH medium, incubation period and urea concentration) were performed using conductivity and optical density measurements to determine the maximum specific urease activity. In addition, an *in vitro* biocement test was done to define the prospect of using these bacterial isolates in civil engineering work for the improvement of soils with inferior properties. The experimental results showed that urease activities were optimum at 25 to 30°C, pH 6.5 to 8.0, 24 hr incubation and 6 to 8% (w/v) urea concentration. It was also demonstrated that biocementation using the local ureolytic bacteria can improve the strength of poorly graded soils. However, the efficiency of the MICP process in improving the soil's strength varied among samples treated with different bacterial cultures.

Keywords: Microbial induced carbonate precipitation; *Sporosarcina pasteurii*; Urease activity; Bacterial optimisation; Biocementation

1. Introduction

Microbial induced carbonate precipitation (MICP) is a new ground improvement technique for soils which incorporates ureolytic bacteria such as *Sporosarcina pasteurii* (*S. pasteurii*) to induce carbonate precipitates via urea hydrolysis, thus filling the pores of loose soils and binding soil particles together (Rahim et al. 2015, Jiang et al. 2016). MICP is an effective and environmentally-friendly technology often used to solve various environmental problems such as soil instability and concrete crack (Anbu et al. 2016). During MICP process, ureolytic bacteria generate metabolic products (CO_3^{2}) which react with calcium ions (Ca^{2+}) in the microenvironment (Anbu et al. 2016), urease turns the urea molecule (non-conductive) into two charged ions: ammonium (NH_4^+, positively charged) and carbonates (CO_3^{2-}, negatively charged) (Cuzman et al. 2015). These biochemical reactions result in the precipitation of calcium carbonate ($CaCO_3$) minerals (Jiang et al. 2016).

MICP is highly desirable because of its natural availability, effectiveness and sustainability (Al-Thawadi 2008). Biocementation is one of the most reported methods of MICP in the literature. It uses a biochemical binding agent to improve the strength and stiffness properties of soils via microbial activity and its products (Ivanov and Chu 2008). Achal et al. (2009a) used a simplified MICP treatment technique for biocement production. The sand columns were injected with *S. pasteurii* (NCIM 2477) and cementation solution under gravimetric free flow direction for the duration of 120 hr. Their finding showed that the $CaCO_3$ contents precipitated in the sand columns were mostly deposited at the upper layer (40%) which led the reduction of soil porosity and permeability. A large-scale (100 m^3) biocement study by van Paassen et al. (2010) on poorly graded sands showed that when *S. pasteurii* (DSM 33) was injected into a sand

box container. They reported having unconfined compressive strength (UCS) results between 0.7 and 12.4 MP, with a nonhomogeneous $CaCO_3$ contents (12.6 to 27.3%) for their treated sample.

Another technique which is also used to treat loose sand is the surface percolation. Cheng and Cord-Ruwisch (2012) reported using this technique to treat Poly Vinyl Chloride (PVC) sand columns (length of 1 m). *Bacillus sphaericus* (DSM 23526) and cementation solution were poured alternatively from the top of the unsaturated sand columns in order to increase the chances of bacterial immobilisation. Their findings showed the UCS strength of the sand column was 390 kPa and there was no clogging at the injection point. They suggested the percolation technique produced higher strength than the submerged flow method previously reported by Whiffin (2004). The use of surface percolation technique was later tested on a larger container (2 m) by Cheng and Cord-Ruwisch (2014) as an attempt to minimise the clogging formation often found in small-scale columns around the injection points which often leads to un-even $CaCO_3$ content distribution. They reported having a relatively homogenous distribution of cement in the sand column, but the UCS results they obtained varied between 850 kPa and 2067 kPa for the coarse samples.

There are extensive reports on microorganisms capable of inducing $CaCO_3$ precipitates for various MICP applications. In a review by Sarayu, Iyer, and Murthy (2014), only a few of the tabulated microorganisms with abilities to induce $CaCO_3$ precipitate can produce urease enzyme and are non-pathogenic. Ureolytic microbes are preferred for MICP process because via urea hydrolysis, the mechanism is easily controlled with the capacity to induce a substantial amount of $CaCO_3$ within a reasonable time period (Dhami, Reddy, and Mukherjee 2014). *S. pasteurii* has been reported to be an ideal

ureolytic bacteria for biocement applications because of its high urease activity and inability to cause harmful diseases (Wei et al. 2015). In most reported studies, several type strains of *S. pasteurii* such as NCIM 2477, DSMZ 33, MTCC 1761, NCIMB 8841 and ATCC 11859 are purchased from different microorganism culture collection centres (Achal et al. 2009a, Wei et al. 2015, Abo-El-Enein et al. 2013, Achal et al. 2009b, Li, Cheng, and Guo 2013). Most locally isolated ureolytic bacteria are from water, soils and sludge samples (Mohammed 2013, Helmi et al. 2016, Arias, Cisternas, and Rivas 2017). However, exploitation on ureolytic bacteria isolated from limestone cave regions for effective biocement application is still very limited. This is because of the notion that caves are generally a region considered as extreme environments, unfavourable microbial growth (Tomczyk-Żak and Zielenkiewicz 2015).

Study on optimisation is essential in biotechnological processes because it provides optimum conditions for preferred microbial growth and proliferation (Karamba et al. 2016). Each microorganism has its own desired conditions for the maximum enzyme production (Kumar and Takagi 1999). Physical parameters such as temperature and pH play essential roles in enhancing microbial biomass production (Hamzah et al. 2012). Hence, it is necessary to optimise environmental conditions and bacterial growth which will aid large scale microbial production for field biocement application. It is evident that most studies utilise purchased ureolytic bacterial strains for biocement applications while limited studies have reported the use of locally isolated ureolytic bacteria. This present study investigated the effect of various parameters on urease activity for four locally isolated ureolytic bacteria (*S. pasteurii*). These parameters include temperature, initial pH medium, incubation period and urea concentration. A laboratory-scale test was also conducted, as well as a comparative analysis in terms of surface strength to

145 determine the possibility of using these locally isolated ureolytic bacteria for biocement
146 applications.

147 **2. Materials and Methods**

148 2.1. Bacterial strain and growth condition

149 Four ureolytic bacteria were isolated from limestone cave samples of Sarawak,
150 Malaysia using enrichment culture technique and Christensen's medium to isolate and
151 screen for ureolytic bacteria, previously described by Omoregie et al. (2016). These
152 ureolytic bacteria (NB33, LPB21, NB28 and NB30) were selected due to their high
153 specific urease activity (19.98, 23.97, 19.28, and 20.09 urea hydrolysed.min^{-1}.OD^{-1}
154 respectively) when compared to other isolated bacteria and the representative strain (*S.*
155 *pasteurii* DSM 33) (17.75 mM urea hydrolysed.min^{-1}.OD^{-1}) which served as a control
156 strain throughout this study. The bacterial cultures were grown in standard 250 mL
157 conical flasks containing sterile 125 mL of production medium. The growth medium
158 consisted of Nutrient broth (13.0 g.L^{-1}, HiMedia *Laboratories Pvt. Ltd*), supplemented
159 with urea (4% w/v, Bendosen *Laboratory Chemicals*). The initial pH of the media was
160 adjusted to 7.5 using 0.1 M NaOH or 0.1 M HCI before sterilisation (Reyes et al. 2009).
161 Sterile Urea substrate (by 0.45 µm filter sterilisation) was added post-autoclaving to
162 prevent chemical decomposition under autoclave condition. The cultures were
163 temporarily stored in the fridge at +4°C.

164 2.2. Monitoring methods

165 *2.2.1. Urease Measurement*

166 Conductivity (mS.cm^{-1}) method was used to determine the urease activity (mM urea
167 hydrolysed.min^{-1}) of the ureolytic bacteria. Conductivity measurement is an analytic

process used to determine the enzymatic rate of reaction because it is easy to operate and an inexpensive assay system (Al-Thawadi 2008). For urease assay, 1.0 mL of 24 hr cultivated bacterial cultures were inoculated into universal bottles containing 9.0 mL of 1.11 M urea solution and monitored for 5 min at 25±2^{o}C (Whiffin 2004) and the respective conductivity values were measured by immersing the probe of the conductivity meter (Walk LAB conductivity pro meter, Trans Instruments COMPRO) into the bacterial-urea solution. At the end of the assay, a graph was plotted using values from conductivity (mS.cm^{-1}) measurement against time (min). The conductivity variation rate (mS.cm^{-1}.min^{-1}) was acquired from the slope of the plotted graph, which was then multiplied by a dilution factor. This was taken as the ratio of the stock bacteria culture to the sampling bacteria culture before inoculating into the urea solution (Zhao et al. 2014). The specific urease activity (mM urea hydrolysed.min^{-1}.OD^{-1}) was derived by dividing the urease activity (mM urea hydrolysed.min^{-1}) by the bacterial biomass (OD600) (Whiffin 2004).

2.2.2. Optical density

Optical density (OD) was used as a biomass concentration indicator for the ureolytic bacteria based on the turbidity of the culture. Prior to measuring the OD of the bacterial cultures, a blank (un-inoculated medium) was used to calibrate the spectrophotometer readings. Subsequently, three millilitres (3 mL) of the aliquot was sampled from the bacterial culture and transferred into a cuvette (10 mm). The OD was measured by using a spectrophotometer (GENESYS^{TM} 20, Thermo Fisher Scientific) at a wavelength of 600 nm.

2.3. Effect of cultural conditions on urease activity

The protocol adopted for optimisation of cultural conditions aimed at evaluating the effect of individual condition using one-factor-at-a-time technique and integrate it as the standard level before optimising the parameter. Various cultural conditions affecting urease activity viz., incubation temperature (20-45°C), initial medium pH (6.0-8.5), incubation period (studied up to 96 hr with 24 hr sampling) and urea concentration (2-10%) were studied. The bacterial cultures were grown in nutrient broth medium (13.0 $g.L^{-1}$, HiMedia *Laboratories Pvt. Ltd*) and supplemented with urea (4% w/v, Bendosen *Laboratory Chemicals*) by 0.45 µm sterile syringe filter. The overnight grown bacteria were inoculated (2% v/v) into separate sterile conical flasks (containing 125 mL nutrient broth) under aerobic batch conditions with agitation at 130 rpm. The conductivity and OD_{600} were measured and used to determine the specific urease activity at the end of the cultivation period.

2.3.1. Effect of incubation temperature (°C)

The influence of different temperatures ranging from 20±2 to 45±2^{o}C with an interval of 5^{o}C was carried out by incubating the ureolytic bacteria cultures for 24 hr. The initial pH of the growth medium used was attuned to pH 7.5 with the use of 1 N NaOH and 1 N HCl. The temperature that promoted the highest enzyme activity was used for subsequent steps of the investigation.

2.3.2. Effect of initial medium pH

The effect of distinctive pH on the ureolytic activity from the selected isolates was determined by examining urease activity at different pH ranging from 6.0 to 8.5 with an

interval of 0.5. The initial medium pH that promoted the highest enzyme activity was used for subsequent steps of the investigation.

2.3.3. Effect of incubation period (hr)

The optimal incubation period was determined by incubating the ureolytic bacteria culture at different selected incubation periods ranging from 24 to 96 hr with an interval of 24 hr. The incubation period that promoted the highest enzyme activity was used for subsequent steps of the investigation.

2.3.4. Effect of urea concentration (%)

The influence of urea substrates with varied concentration for enzyme production was studied. Different urea concentration ranging from 2 to 10% (w/v) with an interval of 2% (w/v) was selected. The urea concentration that promoted the highest enzyme activity was used during bacterial cultivation for biocement experiment.

2.4. Small scale biocementation test

2.4.1. Bacterial culture and cementation solution

Each of the ureolytic bacterial cultures was grown in separate sterile shaking flasks (250 mL capacity) containing nutrient broth medium (13.0 $g.L^{-1}$, HiMedia *Laboratories Pvt. Ltd*). These bacterial cultures were then incubated using the previously optimised cultural conditions. The bacterial consortia used for biocement treatment was formulated by using a modified method of Hamzah, Phan, Abu Bakar, et al. (2013). The consortia consisted of an equal portion (1:1:1:1, v/v) of the isolates (NB33, LPB21, NB28 and NB30) by mixing 25 % (v/v) of each bacterial culture in shake flask. At the end of each incubation, the OD and enzyme activity of the bacterial cultures were measured immediately before each biocement treatments were performed. The

235 cementation solutions used to treat the sand columns consist of nutrient broth (13 $g.L^{-1}$,
236 HiMedia *Laboratories Pvt. Ltd*), urea (1 M, Bendosen *Laboratory Chemicals*), calcium
237 chloride (1 M, Sigma-Aldrich *Co. LLC*), sodium acetate (0.17 M, HiMedia
238 *Laboratories Pvt. Ltd*) and ammonium chloride (0.0125 M, HiMedia Laboratories Pvt.
239 Ltd) were adapted from Cheng et al. (2014) and Weaver et al. (2011). All the
240 cementation solution components were autoclaved except urea and $CaCl_2$, which were
241 added after the solution was autoclaved.

242 *2.4.2. Preparation of sand columns*

243 The sand used for the biocement test was classified as poorly graded medium sand in
244 accordance with British Standards, BS5930. The particle sizes selected ranged from fine
245 sand (0.075 mm) to fine gravel (4.75 mm). Re-informed paper tubes served as the
246 moulds used in this experiment having an internal diameter of 75 mm and length of 49
247 mm. Each column (mould) was packed with 294.73 g of autoclaved sand. All columns
248 were placed on flat surfaced polypropylene sheet; five holes were drilled on the surfaces
249 of the polypropylene sheets to allow the effluents of the cementation solution to pass
250 through. The polypropylene sheets containing drilled holes were later covered with
251 Whatman filter papers. A plastic container was placed below the polypropylene sheet to
252 accumulate the effluents. The top of each column was covered with a layer of scouring
253 pads (Scotch-Brite™) as filters to prevent disturbance on the surfaces of the sands
254 during biocement treatments. Seven sets of sand columns were prepared and each set
255 contained three replicates. The biocement test was done in triplicates per bacterial
256 treatment. As shown in Figure 6, Set 1 was treated with positive control (*S. pasteurii*
257 DSM 33), set 2 was treated with consortia, set 3 was treated with LPB21, set 4 was

treated with NB33, set 5 was treated with NB28 and set 6 was treated with NB33. Set 7 was the negative control (no bacteria) and was only treated with cementation solution.

2.4.3. Biocementation method

Prior to the beginning of the treatment, the sand samples were mixed with bacteria culture, calcium chloride and urea solution before being compacted into their respective columns. The sand columns were treated with the bacteria and cementation solutions using surface percolation technique. The sands were treated with separate isolates, bacterial consortia, positive control (*S. pasteurii* DSM 33) and negative control (sand specimen treated with cementation solution only). The bacterial cultures were harvested at their respective late exponential phases before being mixed with the air-dried sand specimens. The columns were treated twice daily with the 80 mL ureolytic bacteria culture and 80 mL cementation solution. However, the treatment was split into two series of treatment and added twice daily. The MICP treatment was performed by introducing 80 mL of bacterial culture and 80 mL of cementation solution into the sand specimens at an interval of 12 hr for a duration of 96 hr. The treatments of the sand columns were performed inside a fume hood (LabCraft, BASIX 52). Upon completion of the treatments, all the sand columns were cured at room temperature for a duration of 14 days before the treated sand were being removed from their respective mould.

2.4.4. Surface strength measurement

The surface strength measurements of the treated sand were obtained by using a pocket penetrometer (ELE International, 38-2695) as suggested by Al-Thawadi (2008). The penetrometer used had a reading scales from 0 to 700 psi (0 to 4.83 MPa). The pocket penetrometer was used to measure the surface strength by pushing the tip of the penetrometer into the soil to a depth of approximately 0.25 inches and three selected

surface regions were tested on each of the cemented sand. The readings of the loaded weight were recorded when the samples were completely penetrated (breakage).

2.5. Statistical analysis

A standard deviation for each experimental result obtained was calculated using the Excel spreadsheets available in the Microsoft Excel (version 2016). One-way analysis of variance (ANOVA) and post hoc Turkey's test was analysed using StatPlus program to indicate any significant difference between groups. The level of significance was set at 0.05.

3. Results and Discussion

The four ureolytic bacteria isolated from limestone cave samples of Sarawak showed high degrees of similarity (96-98%) to their respective closest bacterial species as shown in Table 1. The BLAST results suggested that the isolates were closely related to bacteria from the *S. pasteurii* group. Achal and Pan (2011) have previously reported isolating *S. pasteurii* group from an extreme environment. The microbial cell wall such as teichuronic-peptide play a role in the pH homoeostasis at alkaline pH, hence allowing for a survival in extreme environments such as caves (Aono, Ito, and Machida 1999).

3.1. The effect of incubation temperature (^{o}C)

The specific urease activity varied in the bacterial cultures grown at different temperatures (Figure 1). Isolate NB33 (25.32 mM urea hydrolysed.min^{-1}.OD^{-1}), isolate NB30 (41.98 mM urea hydrolysed.min^{-1}.OD^{-1}) and control strain (23.03 mM urea hydrolysed.min^{-1}.OD^{-1}) produced their respective maximum specific urease activity when incubated at 30^{o}C, while 25^{o}C was observed to be the maximum specific urease activity for isolate LPB21 (29.81 mM urea hydrolysed.min^{-1}.OD^{-1}) and isolate NB28

(26.26 mM urea hydrolysed.min^{-1}.OD^{-1}) when compared to other incubation temperature. Among the parameters that could affect urease activity, the temperature is a critical factor that needs to be controlled because it usually varies from one organism to another and can have a substantial effect on urease production (Coulon et al. 2004, Kumar and Takagi 1999, Soon et al. 2014). Hence, in this study, the temperature was first chosen as a parameter to optimised. The result presented in Figure 1 suggests that at 25^{o}C, the maximum specific urease activity for isolate LPB21 and NB30 were observed. On the other hand, at 30^{o}C, the maximum specific urease activity for isolate NB33, NB28 and the control strain were observed. It was obvious as shown in Figure 1 that these isolates were able to grow at the studied temperature range variation from 20^{o}C to 45^{o}C and could still display urease activities, which advocates the possibility of these isolates to mesophilic microorganisms (Willey, Sherwood, and Woolverton 2008). Statistical analysis using ANOVA with Tukey's procedure showed there were significant differences for the mean at 25^{o}C for isolate LPB21 (P-value = 1.74E-04, M= 29.81), as well as 30^{o}C for isolate NB33 (P-value = 4.06E-05, M= 25.32) and isolate NB30 (P-value = 8.91E-07, M= 41.98). Two separate temperatures (25^{o}C and 30^{o}C) were selected to cultivate the bacterial isolates, for the rest of the experiments in this study, and were performed in different incubation shakers. These two temperatures showed maximum specific urease activity for the different isolates. LPB21 and NB30, was cultivated at 25^{o}C, while 30^{o}C was used to cultivate isolate NB33 and NB28. The recommended temperature for cultivating the control strain is 30^{o}C.

3.2. The effect of initial medium pH

In Figure 2, the maximum specific urease activity was observed in the medium of pH 6.5 for isolate NB33 (23.71 mM urea hydrolysed.min^{-1}.OD^{-1}), whereas isolate LPB21

and control strain showed their respective maximum specific activity at pH 7.5 with 33.74 and 21.43 mM urea hydrolysed.min^{-1}.OD^{-1}, respectively. Isolate NB30 and isolate NB 28 showed their individual maximum specific urease activities at pH 8.0 with 30.92 and 34.51 mM urea hydrolysed.min^{-1}.OD^{-1}. The pH of a microorganism's growth medium plays an important role capable of inducing the morphological changes of the microbe, instigate its enzyme secretion and affect its stability (Sethi and Gupta 2014). *S. pasteurii* is known to grow well in medium ranging from 6.5 to 8.0 (Sakai and Imachi 2015), but the effect on urease productivity for isolated bacterial strains are limited. Hence, the effect of the initial medium pH (6.0 to 8.5) on urease activity for the bacteria cultures were studied. Analysis of variance with Tukey's procedure showed there were significant differences for the mean at pH 6.5 for isolate NB33 (P-value = 1.97E-05, M= 23.71), at pH 7.5 for control strain (P-value = 0.004, M= 21.43) and isolate LPB21 (P-value = 4.56E-04, M= 33.74), while at pH 8.0 for isolate NB28 (P-value = 1.84E-04, M= 34.51) and isolate NB30 (P-value = 2.24E-07, M= 30.92). The results illustrated in Figure 2 indicate that all the bacterial cultures had their respective maximum specific urease activity when incubated in growth medium with initial pH at 6.5, 7.5 and 8.0. A study by Gat et al. (2014) suggested that the pH values of *S. pasteurii* (DSM 33) for urea hydrolysis during incubation is at pH 7.39 after being incubation at 28 hr. During fermentation, urea hydrolysis is expected to lead to an increase in the pH value due to the production of ammonium (Gat et al. 2014). The enzyme activities at pH 7.5 and 8.0 in Figure 2 were the most noticeable values when compared to other pH values. On the other hand, when the bacterial cultures were inoculated into growth medium at pH 6.0 and 8.5, their respective specific urease activities were found to be lower than the aforementioned pH values. It has been reported in the literature that the optimum pH of

most microbial ureases is near neutral, while some alkali-tolerant bacteria such as *S. pasteurii*, *Bacillus sphaericus* and *Bacillus megaterium* have their optimum pH ranging between 7 to 9.5 (Soon et al. 2014).

3.3. The effect of incubation period (hr)

In Figure 3, the maximum specific urease activity was observed at 24 hr incubation period for all the bacteria; isolate LPB21 (25.98 mM urea hydrolysed.min^{-1}.OD^{-1}), isolate NB33 (27.93 mM urea hydrolysed.min^{-1}.OD^{-1}), isolate NB28 (25.54 mM urea hydrolysed.min^{-1}.OD^{-1}), isolate NB30 (29.70 mM urea hydrolysed.min^{-1}.OD^{-1}) and the control strain (22.08 mM urea hydrolysed.min^{-1}.OD^{-1}). Based on observations and reports from other studies (Qabany, Soga, and Santamarina 2012, Stocks-Fischer, Galinat, and Bang 1999), it was suggested that for higher urease activity, ureolytic bacterial cells have optical density (OD_{600}) values in the range of 0.8 to 1.2 (10^7cells⁄mL) which was achieved over a 24-hr incubation period. Hence, in this study, 24 hr was chosen as the starting incubation time, and the experiment was then monitored over a 24-hourly incubation interval, to assess the effect of incubation time on the specific urease activities of the isolates. Analysis of variance with Tukey's procedure suggested there were significant differences for the mean at 24 hr for the control (P-value = 8.70E-07, M= 22.08), isolate LPB21 (P-value = 3.88E-04, M= 25.98), isolate NB33 (P-value = 8.79E-07, M= 27.93), isolate NB30 (P-value= 3.15E-04, M= 29.70) and isolate NB28 (P-value = 1.10E-02, M= 25.54). The incubation period is an essential parameter for enzyme production (Gautam et al. 2011). To elucidate the effect of different incubation period on urease production and microbial growth, experiments were conducted at different selected incubation period from 24 to 96 hr. The data analysed showed that during fermentation period, the specific urease

activity for all the bacterial cultures reached their maximum values at 24 hr incubation period as presented in Figure 3. Further incubation (48 to 96 hr) of the cultures revealed that their specific urease activity reduced drastically. This finding is true only among the tested incubation period. A few possible reasons for this reduction of enzyme production might have occurred during the fermentation might be due to the reduction of essential nutrients, insufficient sugar contents in the medium, due to inhibitory metabolites (Ali et al. 2016) or absence of sufficient hydrolyzed urea as nitrogen for energy (Achal et al. 2009b, Burne and Chen 2000). In addition, the drastic reduction of specific urease activity observed in Figure 3 might be due to saturated active sites of the microbial enzyme by the substrate molecules (Robinson 2015) which occurs when ureolysis has been completely used up.

3.4. The effect of urea concentration (%)

In Figure 4, the maximum specific urease activity was observed at 6% of urea concentration for isolate LPB21 (32.36 mM urea hydrolysed.min^{-1}.OD^{-1}) and isolate NB28 (25.98 mM urea hydrolysed.min^{-1}.OD^{-1}), while 8% urea concentration was observed to show the maximum specific activity for isolate NB33 (33.95 mM urea hydrolysed.min^{-1}.OD^{-1}), isolate NB30 (39.21 mM urea hydrolysed.min^{-1}.OD^{-1}) and control strain (24.66 mM urea hydrolysed.min^{-1}.OD^{-1}). Bacteria need a source of nitrogen to support their maximal growth because nitrogen is a key building block of protein, enzymes and nucleic acids (Hamzah, Phan, Abu-Bakar, et al. 2013). ANOVA with Tukey's procedure showed there were significant differences for the mean at 8% for control strain (P-value = 0.00, M= 24.66), and isolate NB30 (P-value= 2.62E-04, M= 39.21), while at 6% for isolate LPB21 (P-value = 6.43E-04, M= 32.36) and isolate NB28 (P-value = 3.00E-02, M= 25.98). The result illustrated in Figure 4 suggests that

401 urea concentration at 6% (w/v) and 8% (w/v) were most favourable for the specific
402 urease activities of the bacteria isolates and control. Ammonia, a nitrogen source for
403 most bacteria can be detrimental or toxic to their environment when present in high
404 concentration due to cytotoxic effect (Hess et al. 2006). However, a higher
405 concentration of ammonia can be advantageous to ureolytic bacteria by assisting their
406 Adenosine triphosphate (ATP) generation which can supply more metabolic and
407 enzymatic activities (Mempin et al. 2013). On the other hand, increasing the urea
408 concentration can result in a decrease of the bacteria's biomass production (Cheng and
409 Cord-Ruwisch 2013). This is because the energy utilised by ATP for microbial
410 replication and growth would be reduced and then be used to generate and supply
411 energy enzymatic reactions (Mempin et al. 2013). The finding also showed that these
412 isolates can grow and produce substantial urease when 4% (w/v) and 10% (w/v) of urea
413 were supplemented into their respective growth medium, but when 2% (w/v) of urea
414 was used to grow the bacterial cultures, their specific urease activities were significantly
415 low. Hence, it is recommended that higher concentration of urea be used to cultivate
416 these ureolytic bacteria for MICP process.

417 3.5. Biocementation treatment

418 In Figure 5 (a), there was no visual observation of calcium carbonate precipitates at the
419 top layer of the columns during the initial period of immersion of the bacterial culture
420 and cementation, however on the third day of inoculation, white precipitates were seen
421 on all triplicate samples of the columns containing bacterial cultures (Figure 5 (b)). On
422 the other hand, none of the columns containing the negative control displayed any
423 visible precipitation on their respective top layers. The white precipitates on the top
424 layers of the biocemented sand shown in Figure 5 and Figure 6 were also reported by

Zhao et al. (2014) and Chu, Stabnikov, and Ivanov (2012), indicating the presence of nucleation sites for MICP due to addition of more bacterial solution in order to promote more urease enzyme. The influence of microbial cementation on granular behaviour is dependent on the ability of the bacteria to move freely throughout the pore spaces of the sand and on sufficient particle-particle contact per unit volumes at which cementation will occur. Hence, this quicker formation of calcium carbonate precipitates at injection points of the bacteria and cementation solution prevents more precipitates from flowing freely down the columns and causing a non-uniformity of calcium carbonate precipitates (Achal et al. 2009a, b). The columns holding the biocemented sands were carefully removed at the end of the curing period as shown in Figure 6. All the biocemented sand samples appeared to remain intact after removal from the columns. It was also observed that the scouring pads (Scotch-Brite^{TM}) which were used to prevent any disturbance of the column's top surfaces was not very productive during injection of the cementation solution. However, the hardness of the biocemented sands was not affected. The negative control contained only the cementation solution was done to rule out the possibility that precipitates found in the sand columns were only as results microbial urea hydrolysis and not any other process. Surface percolation method was used for biocement treatment on the sand samples because with the help of gravity and capillarity, the bacterial culture and cementation reagents were easily transported into the sand particles (Cheng and Shahin 2016). The main merit of utilising this biocement method is that it does not require heavy machinery (Mujah, Shahin, and Cheng 2016), making it cost effective, simple and a practical approach due to the free movement of the liquid used. Typically, during biocementation process using percolation method, the solutions are alternatively oozed onto the surface of the sands while the sand column

was kept in a vertical position (Cheng and Cord-Ruwisch 2014, Dhami, Reddy, and Mukherjee 2016) with the top and bottom of the column fully unsealed as shown in Figure 5. Since the objective of this study was to exploit the capability of using locally isolated ureolytic bacteria to improve the strength of loose soils under simple biocement treatment method, the downward vertical flushing using percolation method was selected rather than using other soil treatment methods via injection (Rowshanbakht et al. 2016, Liang, Mohamed, and Donovan 2016) or pressurized flow (Soon et al. 2014, Martinez et al. 2013). These methods often require hydraulic injection of the cementation reagents and physical removal of the effluents (Cheng and Cord-Ruwisch 2014). It is vital to take note that even though percolation method feasible, it must be performed cautiously so as not distort the surface of the sand column. This distortion can be seen in Figure 6 for one of the sand columns treated with isolate NB28 which occurred during the treatment process Cheng and Cord-Ruwisch (2012) reported that based on MICP using ureolytic bacteria, percolation treatment method does well in saturated and unsaturated sands and produces higher strengths than submerged flow method used by van Paassen et al. (2010) and Whiffin, van Paassen, and Harkes (2007) in waterlogged soils.

3.6. Soil surface strength

In Figure 7, the strength measured for the biocemented sand treated with different ureolytic bacteria are 582.33 psi for isolate LPB21, 626.67 psi for isolate NB33, 573.33 psi for isolate NB30, 700 psi for isolate NB28, 533.33 psi for bacterial consortia and 563.33 for the positive control strain. However, the negative control was too soft to measure and could not yield any result. The highest strength measured was 700 psi for biocemented sand treated with isolate NB28 while the lowest strength measured was

533.33 psi for consortia. One of the factors which may have affected the biomass synergy of the bacterial consortia could be attributed to insufficient oxygen in the microenvironment (Hamzah, Phan, Abu-Bakar, et al. 2013). It is also suggested bacterial consortia construction with varied biomass concentration (v/v %) be optimised to achieve a highly-desired biomass production and urease activity (Hamzah et al. 2016). Among all the biocemented sand, the sample treated with isolate NB28 reached the maximum reading of the penetrometer and none of its samples cracked during this surface strength test, unlike other samples. It was also observed that the sand treated with bacterial cultures and cementation solutions were slightly more cemented in areas closest to the point of injection regions. Visual observation after the strength test also indicated that there were much more precipitates on the surface of the biocemented sands than other areas. The bacterial consortia might not have performed better than the individual bacterial isolates because of a low number of the bacteria cells. This led to the sand that was treated with the bacterial consortia to have the lowest strength result because of low biomass and urease production. Statistical analysis suggested there were no significant differences among the surface strengths treated with local isolates and the control. These local bacteria have potential applications for soil surface treatment using MICP technology, especially in tropical rainforest climate such as Malaysia which has abundant loose sands, posing challenges to engineers during the early stage of construction. Some of these Malaysians soils often experience further soil softening due to an extreme and prolonged downpours, which can be problematic for engineers hence MICP treatment is strongly needed (Soon 2013).

4. Conclusions

In this study, a series of experimental tests were conducted to determine the optimised specific urease activities for four locally isolated ureolytic bacteria (*S. pasteurii*) using different parameters: temperature, pH, incubation period and urea concentration. In addition, an *in vitro* laboratory biocement test was performed using MICP process to investigate the feasibility of using these four isolates for strengthening and stabilisation of poorly graded soil. The following conclusions can be drawn from this report:

(1). Based on the one-factor-at-a-time method used to investigate the effect of different parameters on specific urease activities, the optimum incubation temperatures were 25^{o}C (for isolates LPB21 and NB28) and 30^{o}C (for isolates NB30 and NB33). The optimum initial pH medium was pH 6.5 (for NB33), pH 7.5 (for LPB21) and pH 8.0 for (NB30 and NB28). Isolates LPB21 and NB28 showed their optimum urea concentration at 6% (w/v), while isolates NB30 and NB33 showed their optimum urea concentration at 8% (w/v). Moreover, all four locally isolated ureolytic bacteria showed their respective optimum incubation period at 24 hr. These findings suggest that urease activity for the isolates were favoured by mesophilic temperature, alkaline pH, short incubation period and high urea concentration.

(2). The use of percolation method as an inexpensive and simple MICP process to treat low rigidity soils was effective. The results showed that local ureolytic bacteria were able to induce calcium carbonate precipitates comparable to the performance of the control strain (*S. pasteurii* DSM 33). However, the efficiency of the MICP process in improving the soil's strength varied among the samples which were treated with different isolates, bacterial consortia, and the control strain.

(3). Local strength of the treated sand columns obtained from penetrometer test showed that among all the bacteria used during the treatment via MICP process, isolate

NB28 (700 psi) had the highest surface strength, while the bacterial consortia (533.33 psi) had the lowest surface strength. This could be due to different bacterial cell concentration and urease produced by the microbes.

The test results attained from this present study suggest these isolates can serve as alternative ureolytic bacteria for improvement of soil strength via MICP process as a sustainable technology. Although, this study only investigated the use of percolation method for these four isolates, further works are currently being conducted using these ureolytic bacteria employing other alternative treatment methods in attempts to improve the distribution of calcium carbonate contents in soil particles, reduce formation of clogging at regions close to treatment points and enhance the soil's strength. Large-scale bacterial production using these isolates are ongoing for biocement field application.

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744 (ANOVA) with Tukey's procedure was used to compare the variance between
745 different groups with the variability within each of the groups. The level of
746 significance was set at 0.05 (*****).

747 **Figure 2:** The effect of different pH on urease activity. Cultivation of ureolytic
748 bacteria in NB-medium in 250 mL conical flasks incubated for 24 hr. Vertical error
749 bars indicate standard deviation. The analysis of variance (ANOVA) with Tukey's
750 procedure was used to compare the variance between different groups with the
751 variability within each of the groups. The level of significance was set at 0.05 (*).

752 **Figure 3:** The effect of different incubation period on urease activity. Cultivation of
753 ureolytic bacteria in NB-medium in 250 mL conical flasks incubated for 24 hr.
754 Vertical error bars indicate standard deviation. The analysis of variance (ANOVA)
755 with Tukey's procedure was used to compare the variance between different groups
756 with the variability within each of the groups. The level of significance was set at
757 0.05 (*).

758 **Figure 4:** The effect of different urea concentration on urease activity. Cultivation of
759 ureolytic bacteria in NB-medium in 250 mL conical flasks incubated for 24 hr.
760 Vertical error bars indicate standard deviation. The analysis of variance (ANOVA)

761 with Tukey's procedure was used to compare the variance between different groups
762 with the variability within each of the groups. The level of significance was set at
763 0.05 (*).

764 **Figure 5:** Treatment of sand column using locally isolated bacteria, consortia,
765 positive and negative controls. (a) setup of sand columns before treated with
766 ureolytic bacteria and cementation solution (Left). (b) sand columns during treatment
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769 specimens were left to cure for 14 days before being removed from their respective
770 moulds.

771 **Figure 7:** Surface strength of the biocemented sand samples. A pocket penetrometer
772 (ELE International, 38-2695) was used to test the surface strength. Vertical error bars
773 indicate standard deviation.
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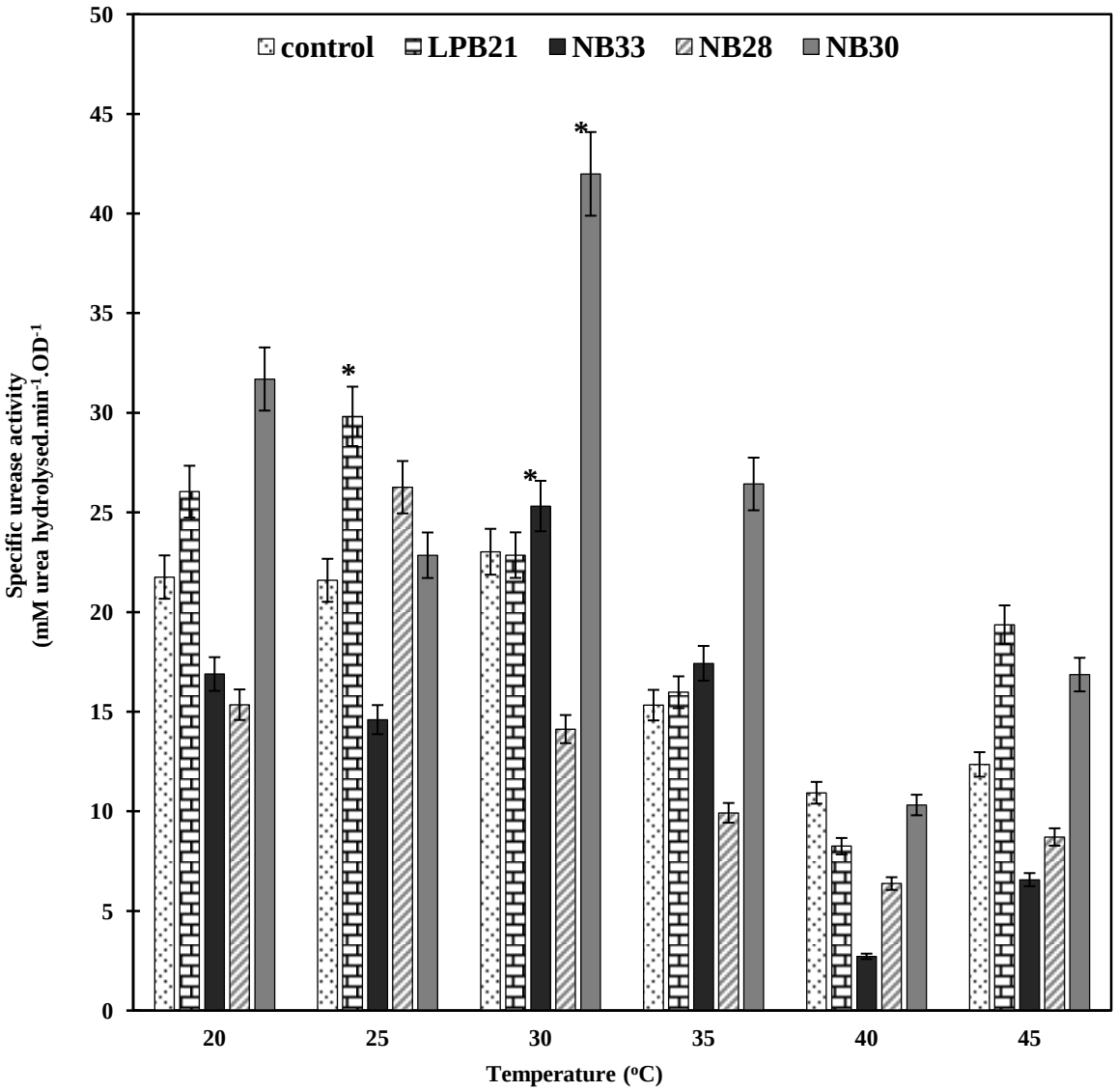
Table 1: Bacteria identification based on 16S rRNA sequencing

Isolate ID	Accession number	Closest match	Base pair	Similarity
NB33	KX212190	*Sporosarcina pasteurii* WJ-4 [KC211296]	1198	97%
LPB21	KX212191	*Sporosarcina pasteurii* fwzy14 [KF208477]	1385	97%
NB28	KX212192	*Sporosarcina pasteurii* WJ-5[KC211297]	1280	96%
NB30	KX212196	*Sporosarcina pasteurii* fwzy14 [KF208477]	1279	98%



805

806 **Figure 1:** The effect of different temperature on urease activity. Cultivation of ureolytic
807 bacteria in NB-medium in 250 mL conical flasks incubated at 20 to 45^{o}C for 24 hr.
808 Vertical error bars indicate standard deviation. The analysis of variance (ANOVA) with
809 Tukey's procedure was used to compare the variance between different groups with the
810 variability within each of the groups. The level of significance was set at 0.05 (*).

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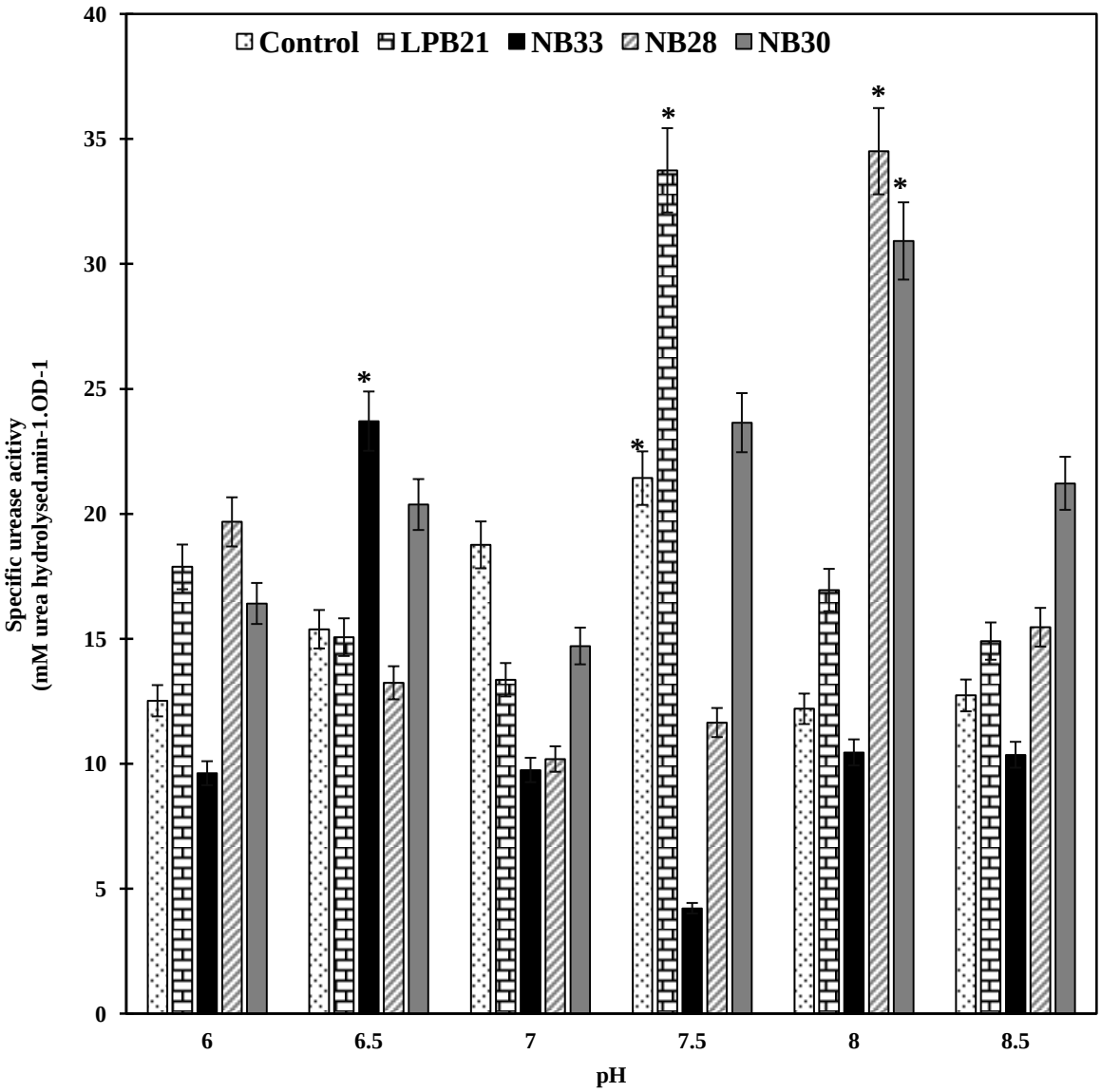
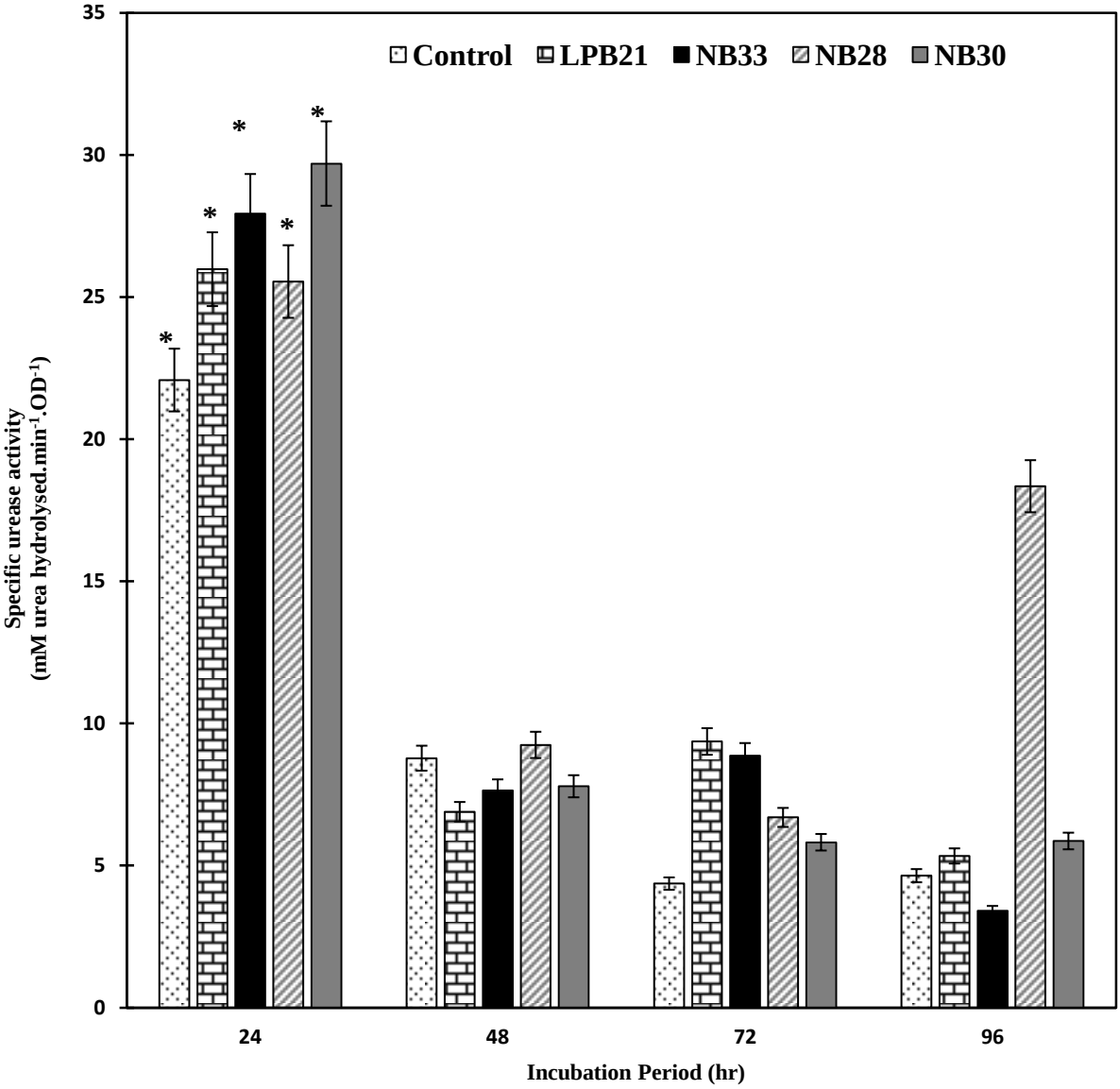


Figure 2: The effect of different pH on urease activity. Cultivation of ureolytic bacteria in NB-medium in 250 mL conical flasks incubated for 24 hr. Vertical error bars indicate standard deviation. The analysis of variance (ANOVA) with Tukey's procedure was used to compare the variance between different groups with the variability within each of the groups. The level of significance was set at 0.05 (*).



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820 **Figure 3:** The effect of different incubation period on urease activity. Cultivation of
821 ureolytic bacteria in NB-medium in 250 mL conical flasks incubated for 24 hr. Vertical error
822 bars indicate standard deviation. The analysis of variance (ANOVA) with Tukey's
823 procedure was used to compare the variance between different groups with the variability
824 within each of the groups. The level of significance was set at 0.05 (*).

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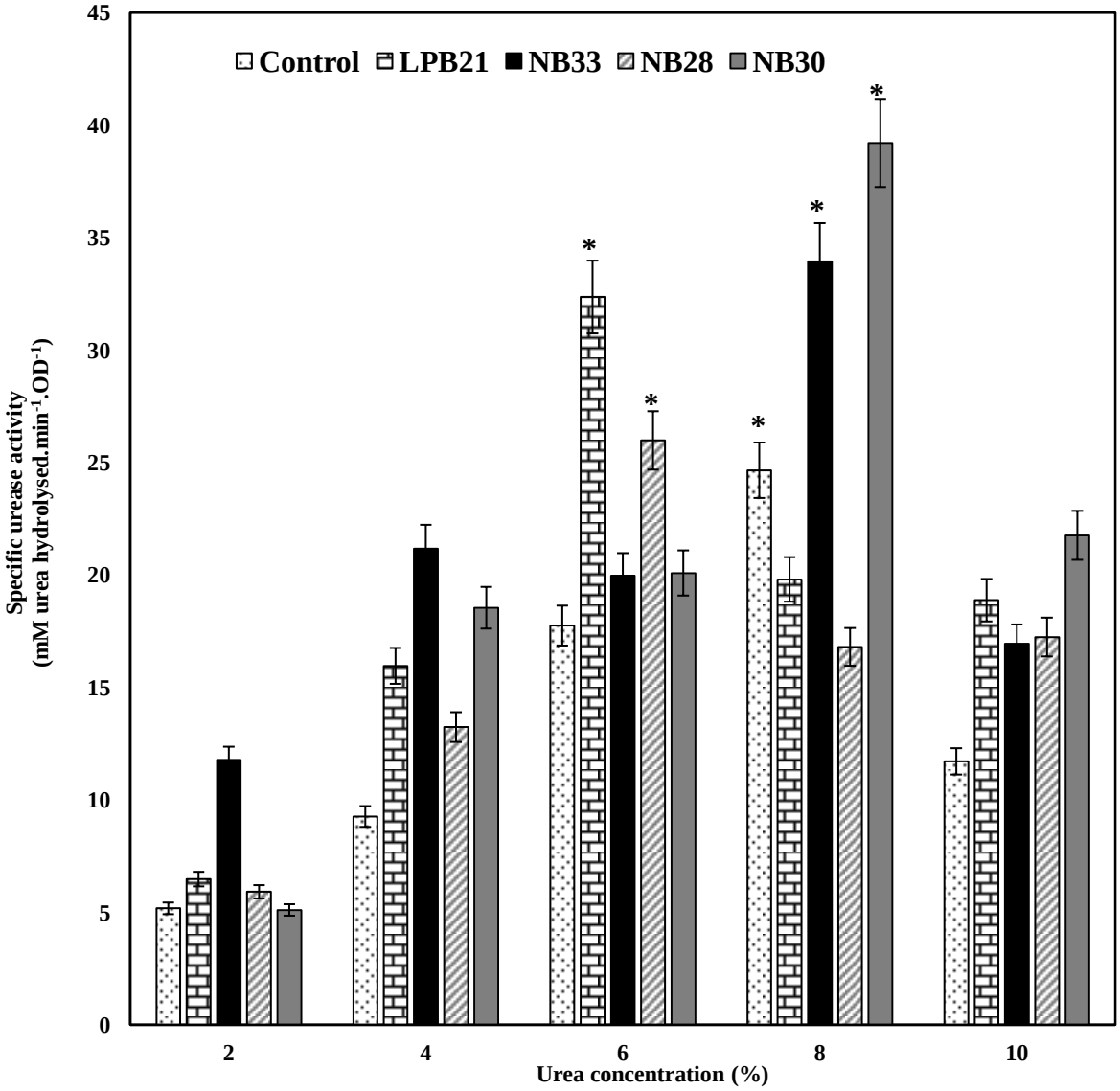

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828

829 **Figure 4:** The effect of different urea concentration on urease activity. Cultivation of
830 ureolytic bacteria in NB-medium in 250 mL conical flasks incubated for 24 hr. Vertical
831 error bars indicate standard deviation. The analysis of variance (ANOVA) with Tukey's
832 procedure was used to compare the variance between different groups with the variability
833 within each of the groups. The level of significance was set at 0.05 (*).

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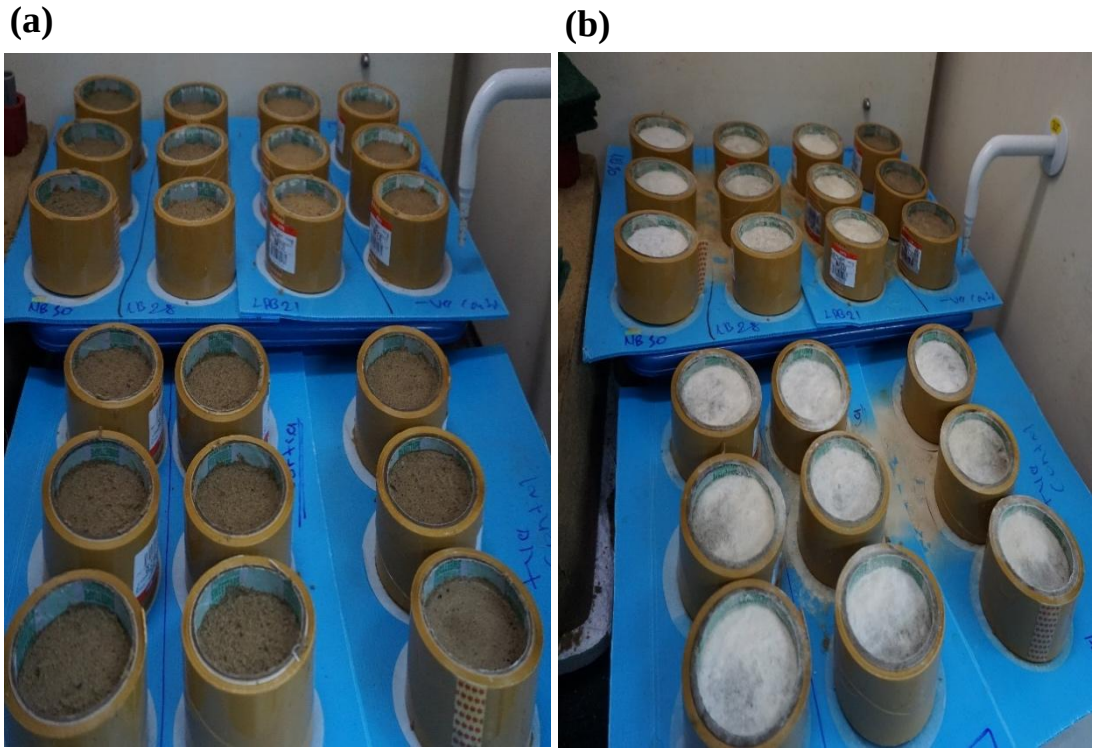

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838 **Figure 5:** Treatment of sand column using locally isolated bacteria, consortia,
839 positive and negative controls. (a) setup of sand columns before treatment with
840 ureolytic bacteria and cementation solution (Left). (b) sand columns during
841 treatment with bacteria and cementation solution (right).

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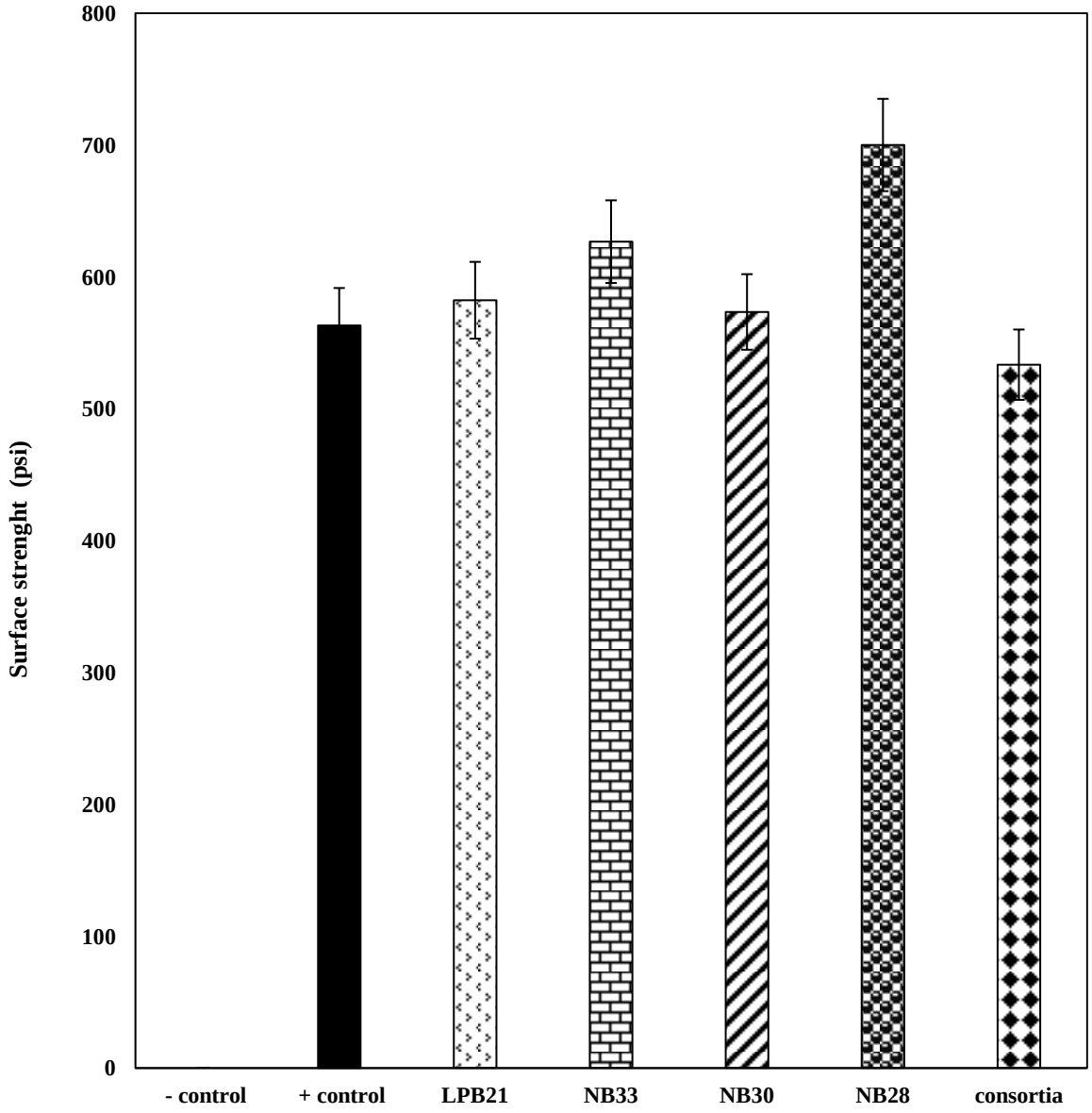
Set 1: +ve control **Set 2: Consortia** **Set 3: LPB21** **Set 4: NB33** **Set 5: NB28** **Set 6: NB33**

Figure 6: Treated sand removed from their respective columns. The biocement specimens were left to cure for 14 days before being removed from their respective moulds.



861 **Figure 7:** Surface strength of the biocemented sand samples. A pocket penetrometer (ELE
862 International, 38-2695) was used to test the surface strength. Vertical error bars indicate
863 standard deviation.

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