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Research Article

Assessing Indigenous Soil Ureolytic Bacteria as Potential Agents for Soil Stabilization

Abdulaziz Dardau Aliyu[1,2], Muskhazli Mustafa[1]*, Nor Azwady Abd Aziz[1], Yap Chee Kong[1], Najaatu Shehu Hadi[2]

1)Department of Biology, Faculty of Science, Universiti Putra Malaysia, Serdang 43400, Selangor Darul Ehsan, Malaysia.
2)Department of Microbiology, Faculty of Science, Federal University of Lafia, Akunza 950101, Nasarawa State, Nigeria.
* Corresponding author, email: muskhazli@upm.edu.my

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ABSTRACT

Microbially induced carbonate precipitation by ureolysis is a biomineralization process that has been adapted by various microorganisms in different natural environments. This widespread natural phenomenon can be employed in numerous civil engineering and soil stabilization applications. In the present study, the potential of indigenous soil urease-producing bacteria as potential agents for soil stabilization method was investigated. Assessment of the eight active urease-producing bacterial species isolated from the farm soil samples has demonstrated that all the isolates were Gram-positive rod-shaped bacteria with promising characteristics such as the formation of endospore which is essential for bacterial survival in harsh conditions within the soil environment. The pH profile and growth profile of the isolates were studied and urease activity was measured by phenol hypochlorite assay method. Two isolates designated isolate O6w and isolate O3a were selected based on the highest urease activity recorded at 665 U/mL and 620 U/mL, respectively, and they were able to increase and sustain alkaline culture condition (pH 8.71 ± 0.01 and 8.55 ± 0.01) which was suitable for $CaCO_3$ precipitation. The isolates were identified based on 16S ribosomal RNA sequencing to be *Bacillus cereus* (O6w) and *Bacillus paramycoides* (O3a). This current study suggested that indigenous soil ureolytic bacteria are potential raw material for the biotreatment of soils stability.

INTRODUCTION

Geotechnical engineers termed a particular soil as problematic when it is observed to have inferior engineering characteristics and cannot be effectively utilised for relevant construction purposes, without the application of an improvement procedure (Rabenhorst & Buchanan 2020). Recently, urease-producing bacteria potential in the biotreatment of problematic soils via biocalcification has presented encouraging and impressive results in the literature (San Pabio et al. 2020; Miftah et al. 2020). Biocalcification also referred to as microbially induced calcite precipitation (MICP) is a biomineralization technique involving a biochemical process of precipitating calcium carbonate ($CaCO_3$) crystals induced by active ureolytic bacterial activity due to urea hydrolysis (ureolysis) occurring within the environment (Zamer et al. 2018; Chen et al. 2019; Yang et al. 2020). The success of the MICP process is promoted primarily by ureolytic bacterial species such as *Bacillus sphaericus*, *Pararhodobacter* sp., *Mor-*

ganella morgana, *Bacillus licheniformis* and *Bacillus cereus* which are capable of utilizing urea as a source of nitrogen by passively diffusing or actively transporting the urea into the cytoplasm of the cell and the bacterial cell wall acting as nucleation sites (Dardau et al. 2021). In search for alternative soil improvement technology with minimal environmental consequences, less adverse effect on the ecosystem and maintaining ecological balance (Khaliq & Ehsan 2016), over conventional methods (cement, chemical grouting & deep mixing technique) that varied in terms of environmental impact, cost, penetration depth, energy consumption and treatment uniformity which portrays their merits and demerits (Hiranya et al. 2018; Duo et al. 2018; Bui Truong et al. 2020), and advances in material and geotechnical research, led to the development of an innovative, novel bio-mediated soil improvement technique utilizing urease-producing bacteria as potential agents.

Several genera of ureolytic bacteria have been recognised as potential MICP agents, including *Clostridium*, *Bacillus*, *Desulfotomaculum*, *Sporolactobacillus* and *Sporosarcina* (Ivanov & Chu 2008) with *Sporosarcina pasteurii* widely utilised in most studies on MICP (Wen et al. 2018), due to tolerance to high pH and precipitation of large amounts of calcite due to high urease activity (Minto et al. 2018; Ruan et al. 2019). Noteworthy, ureolytic bacteria species with the potential of forming endospores, have the advantage of enduring harsh environmental conditions such as nutrient deficiencies, extreme temperature, absence of humidity, and exposure to radiation, disinfectants, antibiotics and chemicals (Badiee et al. 2019). Generally, the selection of desired MICP bacterial agent with high potential survival in an alkaline environment and tolerant to extreme conditions, endospore-forming urease-producing bacteria should be the first choice (Li et al. 2019). On the other hand, the ureolytic bacterial success during the MICP process is promoted primarily by *in situ* environmental conditions such as pH, soil particle size and distribution, competition, predation, osmotic pressure, water content and the conditions of treatment like cementation solution, concentrations of bacteria, availability of suitable nutrients, and temperature (Burbank et al. 2011; Dadda et al. 2018). For example, the rate of ureolysis is higher at 30°C while extreme temperatures may affect the microbial urease activity, nucleation rate and solubility. Further, microbial urease enzyme may be denatured irreversibly at a pH value lower than 5.0 (Ng et al. 2012). In addition, urea concentrations higher than 0.75mol/L may inhibit bacterial ureolytic activity due to too high transportation of urea molecule into the cell membrane which could inhibits other cellular processes (Wu et al. 2019).

Previous studies have documented the potential MICP technological application of urease-producing bacteria towards the improvement of soil (Ming-juan et al. 2017; Junjie et al. 2020), biotreatment of calcareous beach sand (Miftah et al. 2020), strengthening compressed interlocking earth blocks (Zamer et al. 2018), microbial restoration of degraded marble structures (Minto et al. 2018), biohealing of cracks in concrete (Ruan et al. 2019) and wind erosion control (Zomorodian et al. 2019) as an effective, economically engineered natural occurring green biotechnological process. Conversely, despite the numerous advances in MICP, most urease-producing bacteria utilised for various MICP applications are commercially procured from culture collection centres, which contribute to cost (Zomorodian et al. 2019). According to the present global market price, it cost approximately US$402.0 to procure the original patent of *S. pasteurii* ATCC 11859, which suggests the low-cost advantage of utilizing indigenous ureolytic bacteria for various MICP applications (Ezzat & Ewida 2021). Further, the procured microorganisms are often associated

with drawbacks regarding reduction in the population of the introduced bacteria into the soil due to competition, mechanical stress and predation arising from the non-adaptability of the organisms to the local environment (Burbank et al. 2011). In addition, the introduced bacteria can negatively influence the soil microbial communities by affecting the ubiquitous interactions among the soil microorganisms and altering the traits expressed by these microbial communities (Badiee et al. 2019).

Meanwhile, species of ureolytic bacteria documented in literature as promising MICP agents for various civil engineering applications include; *Micrococcus* sp., *Virgibacillus* sp., and *Pseudoalteromonas* sp. applied for coastal erosion protection (Al imran et al. 2019), *Bacillus sphaericus* employed to stabilize dispersive soils (Moravej et al. 2018) while *Pararhodobacter sp.* was utilized for coral sand solidification (Khan et al. 2016). A similar study on concrete healing with *Bacillus cereus* by Wu et al. (2019) reported a crack healing of 100 – 800 µm after 28 days of treatment with a decrease in rate of water permeability by about two orders of magnitude. On the other hand, the findings from the study of Zamani & Montoya (2019) on improvement in the cyclic strength of silty sand utilizing *Sporosarcina pasteurii* as MICP agent have shown a decrease in rate of excess pore water generation with a significant increase in cyclic resistance in comparison to their untreated state. Hence, increases the number of cycles essential to reach liquefaction at a constant cycle stress ratio value. A study by Tiwari et al. (2021), observed 205% increase in calcite content of bio-stimulated MICP treatment of expansive soil with indigenous urease-producing bacteria. An increase in split tensile strength and unconfined compressive strength as well as a decrease in swell strain and swelling pressure were also reported.

Noteworthy, indigenous microorganisms distributed within the soil environment can be enriched *in situ* (bio-stimulation) by modifying local environmental conditions which favour the diversity and distribution of existing bacterial community with required urease capabilities for various MICP applications (Gowthaman et al. 2019; Graddy et al. 2021). Thus, it confirms the promising nature of utilizing indigenous urease-producing bacteria as agents for MICP applications. Hence, research on the utilization of indigenous soil ureolytic bacteria with high urease activity as an alternative towards biotreatment of problematic soil becomes paramount and still a budding line of research. This study aimed to assess the potential of indigenous ureolytic bacteria towards biotreatment of problematic soils. Therefore, the objectives were to isolate and screen for *in situ* soil ureolytic bacteria for their urease activity and their potential for calcite production.

MATERIALS AND METHODS

Soil sampling

A total of ten soil samples were collected from the topsoil layer of Ladang 15, Faculty of Agriculture (2°36'05"N 102°42'11"E), Universiti Putra Malaysia in Selangor, Malaysia based on methods adapted from Kang et al. (2015). The soil samples type and texture were determined as described by Towner (1974) and Ritchey et al. (2015). Meanwhile, pH of soil samples was measured using a standard pH meter as described by Kalra (1995).

Isolation and preservation of ureolytic bacteria

The method described by Navneet et al. (2011) and Wei et al. (2015) was adapted for ureolytic bacteria isolation. Soil samples were suspended in a sterile physiological solution (8.5g/L of NaCl in distilled water). A serial

dilution of the soil sample suspensions (10-fold) was prepared and each serial dilution was plated on Calcium Carbonate Precipitation (CCP) agar (3g/L nutrient agar, 20g/L urea, 10g/L NH_4Cl, 20g/L agar, 2.12g/L $NaHCO_3$ and 25g/L $CaCl_2.2H_20$). Cultures were incubated at 28°C ± 0.5° C and assessed on daily basis within 7 days. The appearing colonies were selected and streaked on CCP Minimal Medium agar (3g/L nutrient agar, 20g/L urea, 10g/L NH_4Cl, 20g/L agar, 2.12g/L $NaHCO_3$) and used for urease screening.

Qualitative Screening for Urease Activity

All the pure isolates were qualitatively screened for urease activity on urea agar base (1.0gm/L peptone, 1.0gm/L glucose, 5.0gm/L sodium chloride, 1.2gm/L disodium phosphate, 0.8gm/L potassium dihydrogen phosphate, 0.012gm/L phenol red, 15.0gm/L agar). The pure isolates were streaked and incubated at 28°C ± 0.5°C for 120 hours and observed every 6 hours. A change in medium colouration from orange to pink was interpreted as positive urease production (Akyol et al. 2017).

Basic characterization of ureolytic bacteria as MICP

Colony morphology of the urease-producing bacteria was observed as a preliminary step towards their identification based on Algaifi et al. (2020). Gram's staining was performed based on the method by Smith & Hussey (2005). Meanwhile, Eosin Methylene Blue agar plates were conducted as a confirmatory test for Gram staining results as described by Leininger et al. (2001). The endospore staining technique was carried out as previously described by Kim et al. (2018).

Molecular identification

For the identification of the unknown ureolytic bacteria culture, a single pure colony on CCP agar was incubated at 28°C ± 0.5°C overnight and molecular identification base on 16S ribosomal RNA sequencing (Zhang et al. 2019). The 16S rDNA, full length 1.5 kb, were amplified using the universal primers 27F (5' - AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5' - GGTTACCTTGTTACGACTT- 3') (Wu et al. 2014). The bi -directional sequencing of purified PCR products was done with universal sequencing primers 785F (5' -GGATTAGATACCCTGGTA- 3') (Manoharan et al. 2020) and 907R (5' -CCGTCAATTCCTTTRAGRTT - 3') (Reysenbach et al. 2000) using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Massachusetts, U.S.A). The forward and reverse sequencing results are edited and assembled into one full-length sequence. The genes were compared with the generated sequence of National Centre for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST), (https://www.ncbi.nlm.nih.gov/nuccore/NR_115714.1); (https://www.ncbi.nlm.nih.gov/nuccore/NR_157734.1).

Growth and pH profile

The CCP broth medium was used for the measurement of bacteria growth and pH profiles as adapted from Navneet et al. (2011). Meanwhile, the pH profile was studied by measuring the pH of the culture medium using pH meter every 24 hours throughout the incubation period under study (120 hours).

Quantification of urease activity

The quantification of bacterial urease activity was done in accordance with the method described by Navneet et al. (2011) based on the phenol

hypochlorite assay method. Approximately 2.5mL of 0.1M urea was mixed with 0.1M potassium phosphate buffer (pH 8.0) followed by 250 µl of the culture filtrates. The mixture was incubated at 37˚C ± 0.5˚C for 5 minutes. Then 1mL of alkaline hypochlorite (56g/L phenol, 0.25g/L sodium nitroprusside, 0.02g/L EDTA) and phenol nitroprusside (2.1g Sodium hypochlorite in 25g/L NaOH), respectively and further incubated for another 25 minutes at 37˚C ±.0.5˚C. Optical density was measured at 626 nm and one unit of urease is defined as the amount of enzyme hydrolysing 1µmol urea per minute. Ammonium chloride (50 to 100 µM) was used as standard.

Calcium carbonates precipitation

Bacterial isolates were grown individually in 100 mL of CCP broth and incubated for 48 h at 28˚C ± 0.5˚C, respectively and an uninoculated CCP medium was used as a control. Later, 1 mg/mL of lysozyme was added to the whole suspension and incubated further for another 60 min at 37˚C ± 0.5˚C. The cell debris was removed by centrifuging at 4,000 g for 4 min and the precipitates were thoroughly washed with distilled water (pH 8.5) (Wei et al. 2015) before the washed precipitates were viewed under the light microscope. The confirmatory test for the precipitated calcium carbonate was carried out using a quick acid test (Richardson et al. 2014). The precipitated calcium carbonate produced was poured into dried test tubes and a few drops of 10% (v/v) of hydrogen chloride was dropped into the test tube and rapid effervescence with bubble formation was interpreted as a positive response.

Statistical analysis

The statistical analysis for the results from this study was carried out using Microsoft Excel 2016. One-way Analysis of variance (ANOVA) was used to study the ureolytic bacterial isolates growth and urease enzyme production variations at 95% confidence level using Graph Pad Prism version 9.1.2.

RESULTS AND DISCUSSION

Isolation and screening for indigenous soil ureolytic bacteria

All soil samples designated as A – E were identified as brown sandy clay soil with a fine texture and a pH value ranging from 3.93 to 4.12 while soil samples designated as F – J were identified as black organic soil with a silky texture and a pH value ranging from 4.11 to 4.51. Soils from farms are known to be rich in urea due to frequent use of organic manure and synthetic urea fertilizers which improve microbial activity through stimulating *in situ* urease-producing bacteria already distributed within the soil pore spaces (Al-Thawadi & Cord-Ruwisch 2012). It is normal that organic soils and urea-rich soils favours the distribution and diversity of ureolytic bacteria which utilizes urea as a sole source of nitrogen and energy (Zhu & Dittrich 2016). Thus, enhancing the rapid growth of ureolytic bacteria within the soil environment (San Pabio et al. 2020; Svane et al. 2020). Previous literatures successfully reported the potential isolation of ureolytic bacteria from urea-rich soils (Phang et al. 2018; Noor et al. 2021).

The fact that ureolytic bacteria are common natural inhabitants of urea-rich soils, the CCP media was supplemented with urea (20g/L) as suggested by Wei et al. (2015) to selectively target active ureolytic bacteria that are tolerant to higher concentrations of ammonia and urea. This was observed by a pungent smell indicating the release of ammonia gas from the culture plates due to the degradation of urea by the bacterial

isolates. Thus, it is an indication of the bacterial isolates' suitability for calcite precipitation towards the soil stabilization process (Zomorodian et al. 2019). In the current study, a total of 16 pure cultures of bacterial isolates with distinct morphological colonies were isolated. Noteworthy, all the bacterial isolates were isolated from soil samples with pH values ranging from 3.73 to 4.51 indicating an acidic environment. In a similar study, Phang et al. (2018) isolated five ureolytic bacterial strains of the genus *Bacillus* from an acidic tropical peat soil of pH value 3.8 to 4.9 in Miri, Sarawak, Malaysia. Most studies on MICP applications reported the isolation of ureolytic bacteria from slightly neutral to alkaline soils (Gat et al. 2014; Dhami et al. 2017) because generally harsh acidic conditions might result in a total loss of bacterial urease activity. However, current findings suggest the adaptation of urease-producing bacteria to an acidic environment where for such bacteria to adapt to the acidic environment, a large amount of urease enzyme is secreted within the microenvironment. Hence, urea hydrolysis will neutralize the acidic condition which favours bacterial survival within the environment and thus favouring the MICP process (Gowthaman et al. 2019). The findings from these different studies implies that isolated strains of ureolytic bacteria vary between alkaline and acidic soil environment.

All 16 bacteria isolates were qualitatively screened for urease activity on Urea Agar Base media containing a pH indicator, phenol red. The change in colouration of the medium was caused by an increase in pH due to the generation of hydroxyl ions from ammonium ions production as a result of urea degradation which is detected by the phenol red (DeJong et al. 2010). In addition, the glucose and peptone present in the medium enhance the rapid growth of diverse species of ureolytic bacteria (Dortey et al. 2020). A change in medium colouration from orange to pink was interpreted as positive urease production and all the isolates showed those responses at different time intervals (Table 1).

Table 1. Bacteria isolates' test for urease activity on urea agar base (UAB).

No	Isolates	(hours)
1	O6w	18
2	O5w, O3a	24
3	O6a,	30
4	O42, S73	36
5	O41	42
6	S70	48
7	O32, O31	78
8	S75, S74	84
9	S72, S76, S77, S71	90

Hence, indicates the different rates of urease enzyme production by the bacterial isolates. Subsequently, in order to target potential high urease producers, only isolates that result in colour change within 48 hours were selected, as isolates that results to change in urea agar base colouration within 48 to 72 hours are potential bacteria with urease activity (Akyol et al. 2017). Thus, justify the selection of 8 isolates (O6w, O42, O5w, O3a, O6a, O41, S73 and S70) utilized in subsequent analysis.

Characterization of ureolytic bacteria as MICP

The colony appearance of 8 selected urease-producing bacteria was studied through visual observation and recorded under standard protocols. There were notable morphological differences across the isolated ureolytic bacteria. Although all the isolates form circular colonies, the close morphological difference noticed might be due to enrichment methods fa-

vouring dominant species during isolation and cultivation periods (Stocks-Fischer et al. 1999). All isolates were Gram-positive, and having a thick layer of peptidoglycan gives an advantage for these isolated Gram-positive bacteria, as the peptidoglycan layer contains 60% teichoic acids which are negatively charged due to high phosphate groups in it (Swoboda et al. 2010; Rauch & Leigh 2015). Thus, it creates a dense network of negative charges on Gram-positive cell wall surfaces to attract positively charged ions such as calcium and this forms the fundamental principle behind the MICP potential of ureolytic bacteria (Wong 2015). Sharma et al. (2021) demonstrated soil biotreatment via MICP in Narmada sand, India using the following Gram-positive bacteria; *Sporosarcina pasteurii*, *B. subtilis* and *B. sphaericus*. In a similar study, Ali et al. (2020) reported all six urease-producing bacteria isolated from urea-rich soils with potential for calcification were Gram-positive. A similar tendency was reported by Gowthaman et al. (2019).

However, the Gram staining technique is subject to inherent limitations leading to technical variation, arising probably due to under decolourization, over decolourization and misinterpretation (Thairu et al. 2014). Thus, further confirmation was carried out by cultivation of the ureolytic bacterial isolates on EMB agar, as the EMB agar contained methylene blue and eosin Y that inhibits the growth of most Gram-positive bacteria (Leininger et al. 2001). None of the ureolytic bacterial isolates grew on the agar medium. Hence, confirming a Gram-positive result across all isolates.

The results from the Endospore staining procedure indicate all isolates under study are spore-forming ureolytic bacteria. Bacterial endospores are unique dormant structures formed especially within the cell, essential for bacterial survival in harsh environmental conditions such as extreme soil temperatures and chemical exposure (Algaifi et al. 2020). A significant characteristic feature preventing ureolytic bacterial death at extreme conditions such as mechanical stress and variations in temperature during the MICP application processes. This makes ureolytic bacterial isolates suitable for a wide variety of MICP applications, particularly stabilization of problematic soils and biocementation in concrete (Khadhim et al. 2019). To emphasize, it has been documented that dormant spore-forming ureolytic bacteria have potential survival at extreme pH (above pH 12) and remain viable in Portland cement-based concrete for a very long period of up to 200 years (Gavimath et al. 2012). It is significant to note that endospores contribute to the success of the MICP process with their capacity to survive desiccation, crosslinking, mixing of concrete and have experimentally been proven to have a positive effect on calcium carbonate precipitating potential on treated concretes (Nielsen et al. 2020). Generally, endospores prolong the survival of bacteria within concrete and soil environment by encasing the vegetative bacterial cells with a multi-layered protein complex structure referred to as coat, which provides two main functions toward the success of the MICP process; (i) it protects against bactericidal chemicals and enzymes such as chloroform and lysozyme, hence enhance spore's resistance viability and properties and (ii) it contributes to endospore's ability to monitor its microenvironment and response within minutes to germinate when exposed to appropriate nutrients (De Muynck et al. 2010; Erşan et al. 2015; Grabiec et al. 2017). Thus, contributing to a greater extent the endospore's calcium carbonate precipitation capacity for applications in MICP (Basha et al. 2018). Several other studies have reported endospore-forming ability of numerous ureolytic bacteria, mostly from the genus Bacillus and Sporosarcina (Harikrishnan et al. 2015; Kim & Youn 2016).

Ureolytic bacterial pH profile

Six out of eight isolates (O6w, O3a, O42, S73, O5w and O6a) sustain a steady rise in pH up to 96 hours (Figure 1). This steady rise in pH changes the microenvironmental conditions which inhibit all other competitive processes within the system. Thus, enhancing bacterial urea hydrolysis which favours permanent precipitation of more $CaCO_3$ crystals between soil grains (DeJong et al. 2010; Jiang et al. 2020). Urea decomposition by ureolytic bacteria generates ammonium ions which increase the pH of the culture medium (Al-Thawadi 2011). Several literatures stated the range of pH values of between 8.3 and 9.3 to be ideal for bacterial calcium carbonate precipitation for biotreatment of problematic soils (DeJong et al. 2010; Sidik et al. 2014). Therefore, the ability of the bacterial isolates to survive at the aforementioned alkaline pH demonstrated their potential to be utilized as agents for MICP towards soil stabilization. According to Hammes & Verstraete (2002) and Krajewska (2018), an increase in pH within the microenvironment is crucial in creating a physiological condition favourable for bacterial cell walls acting as the nucleation site for mass $CaCO_3$ precipitation. In addition, a rise in pH value played a significant role in bacterial adhesion and transportation on and in-between soil grains, to achieve improved homogeneous distribution of precipitated $CaCO_3$ across treated problematic soils (Al Imran et al. 2019).

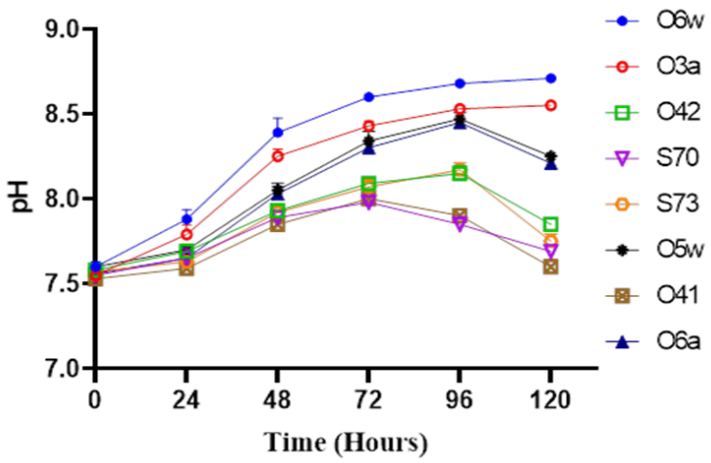


Figure 1. pH profile of selected bacterial isolates that result in colour change of urea agar base medium from orange to pink within 48 hours. Error bars represent standard deviation of the mean.

However, in the present study, the steady rise in pH was followed by a continuous decline after 96h. This might be due to exhaustion of urea within the culture medium due to hydrolysis by ureolytic bacteria leading to accumulation of ammonia as by-products (Whiffin & Paassen 2007). This by-product is usually accumulated either as ammonium salt (NH_3) or ionized (NH_4^+), while the former contributed mainly to the toxicity. Noteworthy, most of the ammonium produced during ureolysis are converted to ammonium salt when the medium pH exceeds 9.5 while bacterial denitrification converts the remaining fraction to nitrate (NO_3^-) (Soon et al. 2014). The ammonia gas is highly detrimental to human health when inhaled, particularly at high concentrations (Omoregie et al. 2016) and this is the major shortcoming of MICP.

Ureolytic bacterial growth profile

All isolates show a similar growth pattern with corresponding progressive cell growth in response to time (Figure 2). The logarithmic phase of growth was observed within the first 24 hours after incubation, which

was sustained up to 72 hours. This growth phase is characterised by an exponential increase in cell growth which favours precipitation of calcium carbonates due to two factors; (i) alteration of the environmental pH through the production of more urease enzyme and (ii) the new available bacterial cells provide surfaces that act as a heterogeneous nucleation site for $CaCO_3$ precipitation by attracting calcium ions rapidly on to its negatively charged cell wall (Phang et al. 2018). Isolate O5w recorded the highest bacterial growth with an optical density (OD) of 1.05 with other isolates maximum growth varied between 0.82 to 1.0 OD. Thus, demonstrating diversity in their metabolic requirements. The maximum optical density recorded can be influenced by the bacterial strain of choice, environmental and growth conditions (Richardson et al. 2014). However, after 96h, the stationary growth phase had started to show a negative rate of growth leading to the death phase, indicating continuous cell death due to limited transfer of nutrients and extremely high alkaline environment inhibiting cell growth (Li et al. 2019). Nevertheless, the growth profile demonstrated the potential of the isolates as promising agents for sustaining steady growth up to 96 hours, which is sufficient to favour mass precipitation of $CaCO_3$ (Kim et al. 2018).

Quantification of urease activity

All isolates recorded a steady rise in urease activity with an increase in incubation time up to 72 hours (Figure 3). The higher urease activity observed with time implies an increase in bacterial growth and an increase in bacterial production of urease enzyme due to the availability of urea as the sole nitrogen source within the medium (Omoregie et al. 2019a; Omoregie et al. 2019b). However, a continuous decrease in urease activity after 120 hours was observed due to exhaustion of nutrients, metabolism inhibition, cell death and enzyme degradation with time leading to an irreversible loss of urease activity (Jiang et al. 2016). Isolate O6w and isolate O3a recorded the highest urease activity of 665 U/mL and 620 U/mL respectively. Thus, an indication of their suitability for application as potential agents towards biotreatment of problematic soils via MICP. One-way ANOVA analysis with a 95% confidence level shows a significant difference in urease activity of all isolates across all the time intervals, which implies the different rate of urease enzyme produced by the individual bacterial isolates. Several studies have reported native ureolytic bacterial strains with different urease activity (Dhami et al. 2017; Jain & Arnepalli 2019; Ma et al. 2020).

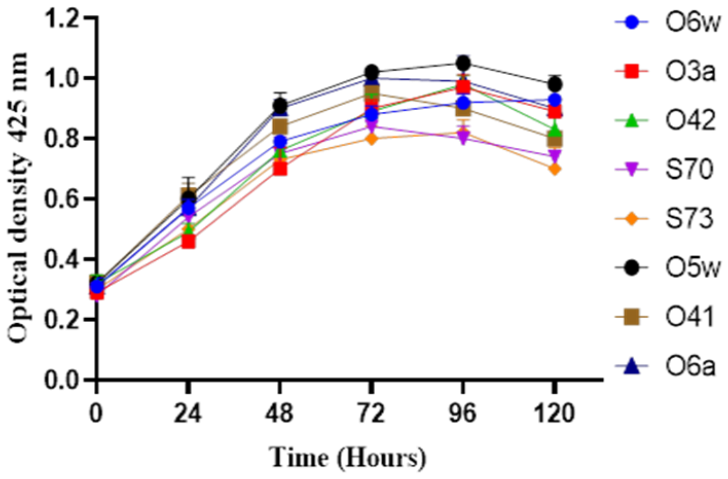


Figure 2. Growth profile (optical density 425 nm) of selected bacterial isolates that result in colour change of urea agar base medium from orange to pink within 48 hours. Error bars represent standard deviation of the mean.

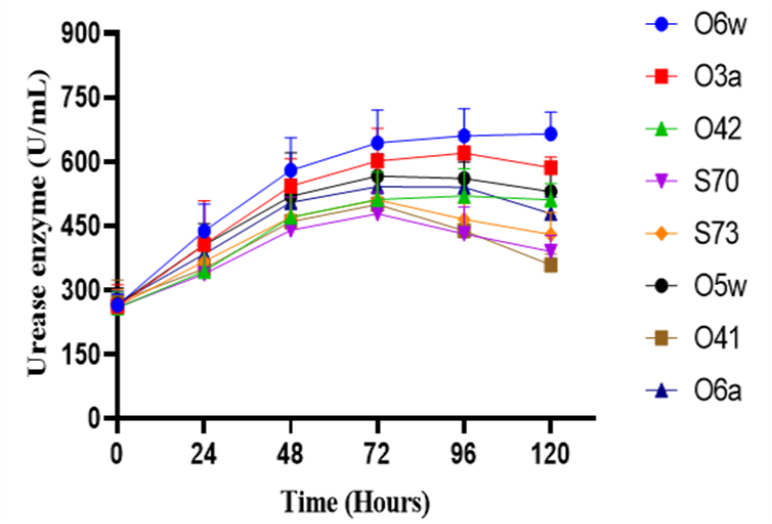


Figure 3. Urease activity (optical density 626 nm) of selected bacterial isolates that result in colour change of urea agar base medium from orange to pink within 48 hours. Error bars represent standard deviation of the mean.

Noteworthy, higher urease activity within the microenvironment, favours the production of more dissolved CO_2 in the form of HCO_3^- or CO_3^{2-} and ammonium ions are generated leading to an increase in pH (Zaghloul et al. 2021). The dissolved CO_2 does not only form part of the precipitated $CaCO_3$ but also act as a buffer within the system (Wu et al. 2017), while the ammonia produced is also advantageous to the ureolytic bacteria towards the generation of energy in the form of Adenosine triphosphate (ATP) (Cheng & Cord-Ruwisch 2013). However, higher concentrations of ammonia are detrimental to ureolytic bacterial growth and affect urease activity via biochemical reactions (Tang et al. 2020). Further, an ideal urea concentration is crucial for the survival and metabolic requirements of the ureolytic bacterial growth within the medium (Ng et al. 2012). Hence, in the current study, a urea concentration of 0.33 mol/L was utilized for bacterial growth as suggested by several earlier studies (Burbank et al. 2012; Wei et al. 2015; Akyol et al. 2017). Based on the experimental conditions on the study conducted by Okwadha & Li (2010) using *S. pasteurii* reported urea concentrations of 0.66 mol/L to be optimum for the bacterial growth and MICP processes. Higher urea concentrations can inhibit the ureolytic activity of even the bacteria with high urease activity due to too high transportation of urea molecule into the cell through the cell membrane which inhibits other cellular processes. Hence, urea concentrations in excess of 0.75mol/L are not recommended for applications in MICP (Wu et al. 2019).

Noteworthy, Bibi et al. (2018) reported that higher bacterial cell growth may not correspond to higher urease activity which does not necessarily translate to higher $CaCO_3$ yield. Rather, higher urease activity and the isolate potential to sustain and survive at a higher alkaline pH, other than bacterial growth are the basic parameters favouring the fundamental success of the MICP application process towards soil biostabilization (Wath & Pusadkar 2016; Bibi et al. 2018). Based on the aforementioned basic factors (urease activity and pH), isolate O6w and O3a were favoured as the bacterial candidates that sustained the optimum conditions correct for calcium carbonate precipitation activity towards biotreatment of problematic soils.

Identification of potential MICP isolates

BLAST results against NCBI 16S ribosomal RNA sequence using the neighbour joining method were used to construct a phylogenetic tree for

individual isolates as shown in Figure 4. The phylogenetic tree suggests the closest description of isolate O6w to be *Bacillus cereus* while isolate O3a closest description is *Bacillus paramycoides*. This might be due to the dominance of *Bacillus* spp. present within the soil environment and in comparison, to other genus by high degree favoured by their physiological adaptation to harsh environmental conditions (Elmanama & Alhour 2013). In addition, this coincided with previous investigations, which indicate most ureolytic bacteria from soil origin are Bacillus species. Bibi et al. (2018) in their study, isolated eighteen ureolytic bacterial isolates from Qatari soil and found all to be of the genus Bacillus. Another study by Phang et al. (2018) reported the isolation of five Bacillus species with calcifying potential from the tropical peat soil in Sarawak, Malaysia.

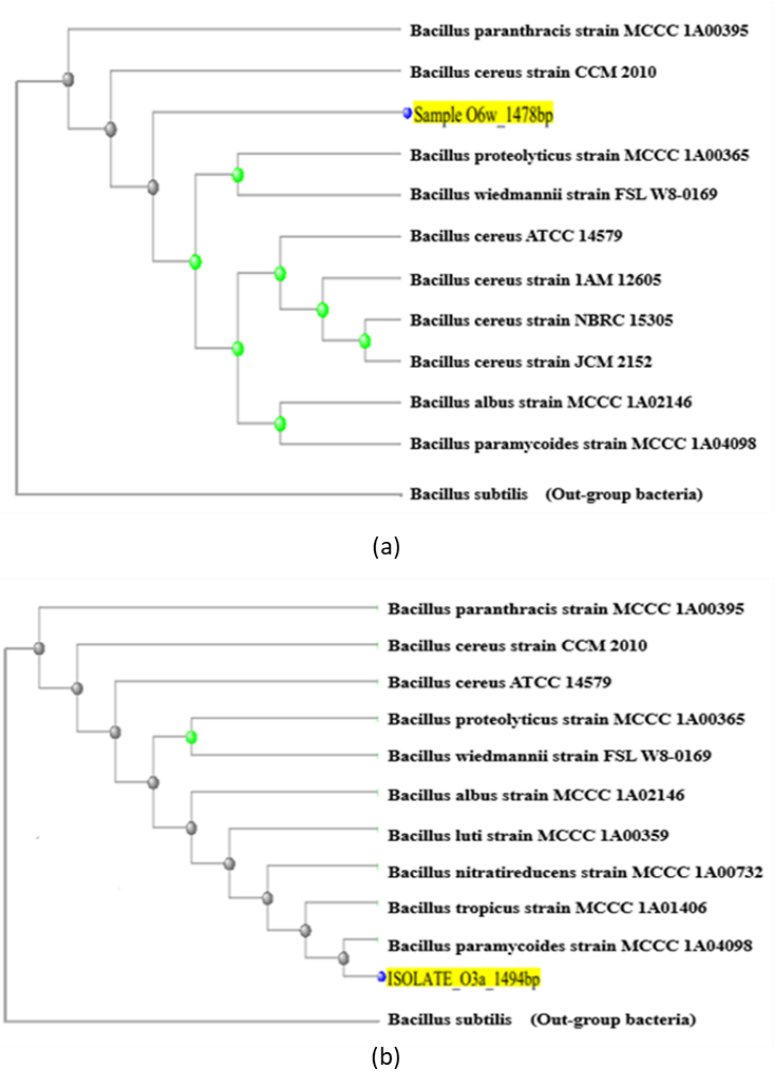


Figure 4. Phylogenetic Tree – Neighbour Joining (Unrooted Tree) by NCBI Blast Tree Method, as compared to known species (a) Isolate O6w and (b) Isolate O3a.

Isolate O3a has 99.93% similarity to *B. paramycoides* (Table 2) which was characterised as non-urease producing bacteria (Liu et al. 2017). However, this current study has shown that isolate O3a recorded high urease activity (Figure 3) thus, contrary to the Liu et al. (2017) claim. A further search had shown that several recent studies have reported bacterial isolate with 98.1% to 98.9% identification similarity with *B. paramycoides* and characterized the isolate as a urease producing bacteria (Mekonnen et al. 2019; Mekonnen et al. 2021; Caglayan 2021), which is consistent to the findings of this study.

Table 2. Molecular identification base on 16S rRNA sequencing data using NCBI nucleotide BLAST database.

No		Isolate O6w	Isolate O3a
1	Closest description	*Bacillus cereus* (16S ribosomal RNA, partial sequence)	*Bacillus paramycoides* (16S ribosomal RNA, partial sequence)
2	Maximum score	2666	2691
3	Total score	2666	2691
4	Query cover	100%	100%
5	Percentage identification	100%	99.93%
6	Accession number	NR115714.1	NR 157734.1
7	Base pair	1478	1494
8	Genus	*Bacillus*	*Bacillus*
9	Species	*Bacillus cereus*	*Bacillus paramycoides*

Ureolytic bacterial calcium carbonates precipitation

Both *B. cereus* and *B. paramycoides* precipitated calcium carbonates after 48 h incubation in CCP medium at 28°C ± 0.5°C, respectively. Precipitated $CaCO_3$ was confirmed by a quick acid test and rapid effervescence of carbon dioxide gas with a continuous formation of bubbles was observed. Hence, confirming the possible presence of an alkaline based material presumptively identified as $CaCO_3$ (Richardson et al. 2014). In the present study, the precipitated $CaCO_3$ was visualized under a light microscope, some of the precipitated crystals formed clusters of two or more, as such visualized as aggregates (Figure 5a), as also been observed in a study by Al-Thawadi and Cord-Ruwisch (2012), while no precipitates were formed in uninoculated medium (Figure 5b).

Further, scanning electron microscopic images (Figure 6) of the $CaCO_3$ precipitated by both *B. cereus* and *B. paramycoides* were morphologically visualized as agglomerated $CaCO_3$ crystals. By comparison, agglomerated $CaCO_3$ crystals precipitated by *B. paramycoides* were similar to the ones produced by *B. cereus* and were found to be in agreement with earlier similar observations (Kakelar et al. 2016). Noteworthy, these precipitated crystals were formed by supersaturation within the medium with availability of bacterial cell wall acting as sites for nucleation (Wang et al. 2017). In addition, the formation of agglomerated $CaCO_3$ crystals occurs due to nucleation and growth of existing $CaCO_3$ crystals, which leads to the precipitation of larger $CaCO_3$ crystals (Al-Thawadi & Cord-Ruwisch 2012; Mujah et al. 2017). Finally, based on survival at alkaline pH and the high urease activity achieved by both *B. cereus* and *B. paramycoides* and their capability to sustain the culture conditions which favours precipitation of calcium carbonate as confirmed by quick acid test and viewed under light microscope and SEM, demonstrated their potential utilization as agents toward biotreatment of problematic soils.

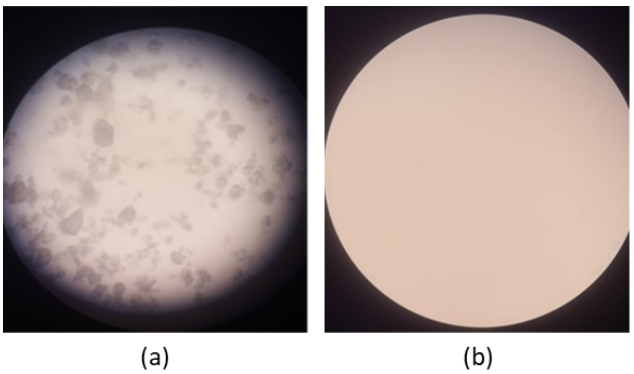
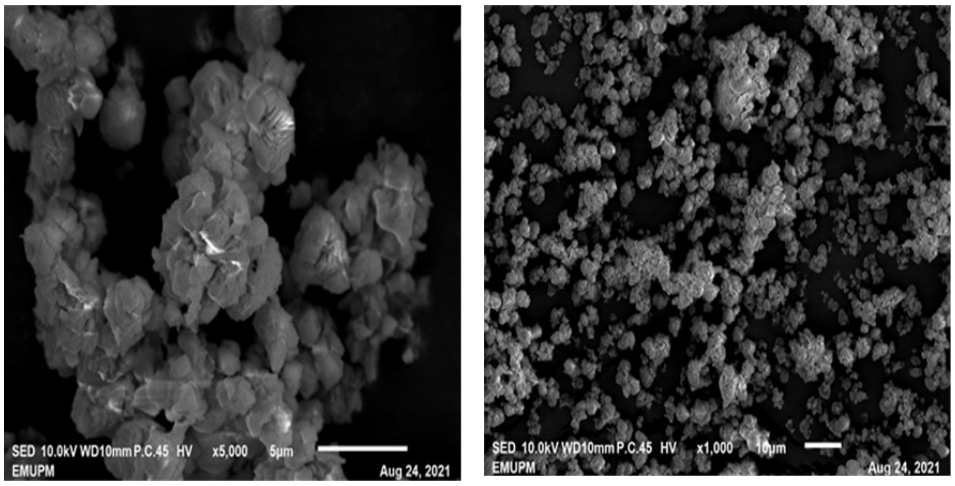


Figure 5. Microscopic images of precipitated calcium carbonates produced by *Bacillus cereus* viewed under light microscope (a) ×40 and (b) Uninoculated calcium carbonate precipitation medium.



(a) (b)

Figure 6. SEM micrographs of precipitated calcium carbonate crystals by (a) *Bacillus cereus* and (b) *Bacillus paramycoides*.

CONCLUSION

This study effectively established the presence of indigenous urease producing bacteria distributed within the farm soil environment. Among the sixteen easily isolated strains of ureolytic bacteria evaluated, *B. cereus* and *B. paramycoides* recorded the highest urease activity, survived a steady growth at alkaline pH and was able to sustain the activity for the precipitation of $CaCO_3$. This study also reported the presence of *B. paramycoides* (known non-urease producing bacteria) within the active indigenous urease $CaCO_3$ precipitating bacteria in the farm soil environment. Both *B. cereus* and *B. paramycoides* had a promising potential application as MICP agents for the soil stabilization method.

AUTHORS CONTRIBUTION

All the co-authors contributed to the research experimental design, data analysis, drafting of manuscript, editing and completing the revisions. All co-authors have agreed and read the final version of the submitted manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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