UNIVERSITI PUTRA MALAYSIA

MICROBIALLY-INDUCED CALCITE PRECIPITATION BY UREOLYTIC BACTERIA AS AGENT FOR SOIL BIO-STABILIZATION METHOD

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FS 2022 9

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By

DARDAU ABDULAZIZ ALIYU

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

December 2021

DEDICATION

This thesis is dedicated to ALLAH (SWA) and to my beloved parents Alhaji Dardau Aliyu Mailafiya (Falakin Lafiya) and Hajiya Salaha Dardau Aliyu, for their words of motivation, moral supports, guidance, patient, and dedication towards my upbringing.
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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Chairman : Muskhazli Mustafa, PhD
Faculty : Science

Microbially-induced calcite precipitation (MICP) refers to the biochemical process of precipitating calcium carbonate ($CaCO_3$) induced by bacterial urease activity with a complex microbial biochemical reaction occurring within the environment for the purpose of stabilizing loose soils. A loose soil poses great concern worldwide leading to severe environmental hazards such as building collapse, destruction of roads and railways, landslides, loss of lives and properties, with an estimated US$6 billion spent annually to finance over 40,000 soil improvement projects worldwide. This study is limited to calcite precipitation and bio-cementing effect of indigenous soil urease producing bacteria. The aim of this study is to explore the potential of indigenous urease producing bacteria towards soil stabilization. Isolation using $CaCO_3$ precipitation media within 7 d to target highly active urease producing bacteria has successful isolated eight isolates (O6w, O42, O5w, O3a, O6a, O41, S73 and S70) from farmland soil samples at Ladang 15, Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia. Farmland soils of Ladang 15 are known to be urea rich soil due to utilization of synthetic urea and organic manure as fertilizer for crop cultivation. Thus, favours distribution and diversity of urease producing bacteria. Phenotypic analysis indicates all isolates are Gram-positive, rod-shaped and produced circular colonies. The pH profile and growth profile of the isolates were studied and urease activity was measured by phenol hypochlorite assay method (O.D 626 nm) at 24 h interval for 120 h. The experimental results showed that all the isolates were able to sustain a steady growth up to 96 h, which later had produced significant precipitation of $CaCO_3$. Among the eight isolates evaluated, isolate O6w and isolate O3a were selected based on the highest urease activity at 665 U/mL and 620 U/mL, respectively and able to increase and sustain alkaline culture condition (pH 8.71 ± 0.01 and 8.55 ± 0.01) which is suitable for $CaCO_3$ precipitation. The isolates were identified based on 16S ribosomal RNA sequencing to be *Bacillus cereus* (O6w) and *Bacillus paramycoides* (O3a). An amount of 943 ± 57 mg/L and 793 ± 51 mg/L $CaCO_3$ had been precipitated by *B. cereus* and *B. paramycoides,* respectively after 96 h of incubation. Studies on characterization of the precipitated $CaCO_3$ crystals by scanning electron microscope (SEM) microanalysis have shown $CaCO_3$ crystals of various sizes (2.0 µm - 23.0 µm) with different morphologies such as agglomerated

rhomboids, cubic, flower-like and irregular shaped crystals. Confirmed by XRD indicated that precipitated $CaCO_3$ is mostly calcite and a few aragonites. SEM micrographs on microstructural analysis of organic and sandy clay soils treated by both *B. cereus* and *B. paramycoides* have shown the formation of bio-precipitated $CaCO_3$ deposited on soil particles (bio-cementing soil grains). Overall, observed experimental results attributed $CaCO_3$ formation as a bacterial-associated process. Hence, the dynamic process of MICP leading to precipitation of $CaCO_3$ is not chemically induced, but a microbially induced biochemical process directly linked with urea hydrolysis via urease activity. This study suggests that indigenous soil ureolytic bacteria with high urease activity are potentially useful as agent for soil bio-stabilization.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMENDAKAN KALSIT TERARUH MIKROB OLEH BAKTERIA UREOLITIK SEBAGAI AGEN BAGI KAEDAH BIO-PENSTABILAN TANAH

Oleh

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Pemendakan kalsit teraruh oleh mikrob merujuk kepada proses biokimia pemendakan kalsium karbonat ($CaCO_3$) yang diaruhkan menerusi aktiviti bakteria urease menerusi tindakbalas biokimia mikrob yang kompleks bagi tujuan penstabilan tanah longgar. Tanah longgar yang menimbulkan kebimbangan yang besar seperti runtuhan bangunan, kerosakan jalan raya dan jalan keretapi, runtuhan tanah, kehilangan nyawa dan harta benda dengan dianggarkan bernilai USD 6 bilion dibelanjakan setahun untuk membiayai lebih 40,000 projek penambahbaikan tanah di seluruh dunia. Tujuan kajian ini ialah untuk menerokai potensi bakteria tempatan yang menghasilkan urease bagi tujuan penstabilan tanah. Pemencilan menggunakan media pemendakan $CaCO_3$ dalam tempoh 7 hari yang menyasarkan bakteria penghasil urease yang aktif telah berjaya memencilkan lapan isolat (O6w, O42, O5w, O3a, O6a, O41, S73 dan S70) dari sampel tanah Ladang 15, Fakulti Pertanian, Universiti Putra Malaysia, Selangor, Malaysia. Tanah di Ladang 15 dikenali sebagai tanah yang kaya urea di sebabkan oleh penggunaan urea sintetik dan baja organik sebagai baja penanaman tanaman. Oleh itu, ianya menyumbang kepada taburan dan kepelbagaian bakteria penghasil urease. Analisis finotipik menunjukkan semua isolat adalah Gram-positif, berbentuk rod dan menghasilkan spora bulat. Profil pH dan pertumbuhan isolat telah dikaji dan aktiviti urease telah diukur menggunakan kaedah biocerakin fenol hipoklorit (OD 626 nm) pada sela masa 24 jam selama 120 jam. Keputusan kajian menunjukkan bahawa semua isolat berupaya mengekalkan pertumbuhan yang malar sehingga 96 jam, yang mana kemudiannya telah dapat menghasilkan pemendakan $CaCO_3$ yang ketara. Daripada lapan isolat yang dinilai, isolat O6w dan isolat O3a telah dipilih berdasarkan penghasilan aktiviti urease yang tertinggi dengan masing-masing pada 665 U/mL dan 620 U/mL, serta berupaya meningkatkan dan mengekalkan keadaan kultur beralkali (pH 8.71 ± 0.01 dan 8.55 ± 0.01) yang mana ianya sesuai untuk pemendakan $CaCO_3$. Isolat telah dikenal pasti berdasarkan penjujukan 16S ribosomal RNA sebagai *Bacillus cereus* (O6w) dan *B. paramycoides* (O3a). Sebanyak 943 ± 57mg/L dan 793 ± 51mg/L $CaCO_3$ masing-masing telah dimendakkan oleh *B. cereus* (O6w) dan *B. paramycoides* (O3a) selepas 96 jam pengeraman. Kajian pencirian terhadap endapan kristal $CaCO_3$ menggunakan analisis imbasan mikroskop elektron (SEM) menunjukkan berbagai saiz kristal $CaCO_3$ (2.0 µm

- 23.0 μm) dengan morfologi yang berlainan seperti rhomboid bergumpal, kubik, berbentuk bunga dan kristal berbentuk tidak seragam. Pengesahan menggunakan XRD menunjukkan bahawa kebanyakkan endapan $CaCO_3$ adalah kalsit dan sedikit aragonit. Mikrograf SEM pada analisis mikro-struktur tanah organik dan tanah liat berpasir yang telah dirawat oleh kedua-dua *B. cereus* dan *B. paramycoides* memaparkan pembentukan bio-endapan $CaCO_3$ pada zarah tanah (bio-simen butiran tanah). Secara keseluruhan, hasil kajian yang diperhatikan telah dapat mengaitkan pembentukan $CaCO_3$ sebagai proses yang berkait dengan bakteria. Oleh itu, proses dinamik tindakbalas biokimia mikrob yang kompleks ke arah pemendakan kasium karbonat bukanlah diaruh secara kimia, tetapi oleh proses biokimia bakteria teraruh yang berkait rapat dengan hidrolisis urea menerusi aktiviti urease. Kajian ini mencadangkan bahawa bakteria ureolitik tanah tempatan dengan aktiviti urease yang tinggi adalah berpotensi sebagai agen penstabil bio tanah yang berguna.

ACKNOWLEDGEMENTS

I express my thanks to Allah Almighty (S.W.A) the giver of time through grace for his blessings of wisdom and life to witness the completion of this program despite the encountered hurdles and challenges. I remain grateful to Allah for making Associate Professor Dr. Muskhazli Mustafa as my supervisor, advisor and mentor who contributed greatly towards my research achievements and scientific reasoning. His support means to me more than words can ever adequately express. May Allah reward him abundantly. My great and sincere special thanks go to my wonderful supervisory committee member Associate Professor Dr. Nor Azwady Abd Aziz for his support, positive critics, intense supervision and encouragement throughout the period of my studies. May Allah (S.W.A) continue to guide and protect him.

I owe my endless gratitude to my beloved parents, Alhaji Dardau Aliyu Mailafiya (Falakin Lafia) and Mrs. Salaha Dardau Aliyu for their relentless lifetime supports, love and prayers throughout the period of my studies. May the blessings of Allah (S.W.A) be upon them for inspiring me to go for higher degrees since from childhood while they persistently remain optimistic and affirmative. These made me to work day and night so that I would graduate on time to come back home. I appreciate the persistent support and prayers from precious siblings and friends.

To all my research fellows, too numerous to mention, I sincerely appreciate your friendship. I would like to acknowledge the supports of Maryam Muhammed Mailafia and my senior colleagues Dr. Hamza Abdulqadir, Dr. Sulaiman Bala and Dr. Ibrahim Musa Adamu who groomed my research and writing skills. I am grateful for their contributions to the design and presentation of my work. Their efforts shall remain good memories because they sacrificed their time and energy to make me progress by motivating me and providing all the necessary assistance I needed.

Worthy to mention is the relentless support of FULAFIA scholars in UPM, and Nigerian community in UPM for the joyful living environment during the study period. I would like to acknowledge the supports of Research Assistants, Laboratory Science Officers of Department of Biology and Laboratory Science Officers of Faculty of Science, Universiti Putra Malaysia.

Finally, I appreciate Universiti Putra Malaysia for offering me Putra Research Grant (Grant No: GP-IPS/2020/9691000) which enabled me to start my research on time. I am most grateful to Federal University of Lafia and Tetfund for giving me the golden opportunity of furthering my studies. Indeed, their financial support, enormous contributions and endless support towards the success of this study cannot be over emphasize. My appreciation also goes to members of staff, Department of Microbiology, Federal University of Lafia for their support and understanding even in my absence.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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%	Percentage
ACC	Amorphous calcium carbonate
ANOVA	One-way analysis of variance
As	Arsenic
ATP	Adenosine triphosphate
BCM	Biologically controlled mineralization
BIM	Biologically induced mineralization
BIM	Biologically influenced mineralization
BLAST	Basic Local Alignment Search Tool
CCB	Calcium carbonate broth
CCP	Calcium carbonate precipitation
cm	Centimetre
cm^2	Centimetre square
Co	Cobalt
CO_2	Carbondioxide
$CaCO_3$	Calcium carbonate
$CaCO_3.H_2O$	Monohydrate calcium carbonate
$CaCO_3.6H_2O$	Hexahydrate calcium carbonate
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	Calcium chloride dihydrate
Ca	Calcium
Ca^{2+}	Calcium ion
Cells/ml	Cells per millilitre
CO_3^{2-}	Carbonate ion

$CO(NH_2)_2$	Urea
^oC	Degree Celsius
Cd	Cadmium
CdS	Cadmium sulphide
Cr	Chromium
Cu	Copper
deg/min	Degree per minute
DNA	Deoxyribonucleic acid
“E	East
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin methylene blue
Fe	Iron
g	gram
g/L	gram per litre
h	Hour
ha	Hectare
Hg	Mercury
HCO_3^-	Carbonate
kpa	Unconfined compressive strength
Ksp	Solubility product constant
K	potassium
Kg	Kilogramme
Log	Logarithm
M	Mole
Mg	Magnesium
MICP	Microbially induced calcite precipitation

mg/L	Milligram per litre
mol/L	Mole per litre
mL	millilitre
mm	millimetre
mM	millimole
N	Nitrogen
“N	North
No	Number
nm	nanometer
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NH_4^+	Ammonium ion
NH_4Cl	Ammonium chloride
NH_3	Ammonia
NH_2COOH	Carbamate
OD	Optical density
OH^-	Hydroxyl ion
P	Phosphorus
pH	Potential of hydrogen
Pb	Lead
PCBs	Polychlorinated biphenyls
Ra	Radium
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

rpm	Revolutions per minute
Sb	Antimony
SD	Standard deviation
SEM	Scanning electron microscope
Spp	Species
Sn	Tin
Sr	Strontium
Th	Thorium
U	Uranium
U/ml	Urease per millilitre
UAB	Urea agar base
UBB	Urea broth base
µm	micrometer
µmol	micromole
UK	United Kingdom
USA	United State of America
US	United State
UCS	Unconfined compressive strength
(v/v)	Volume/Volume
(w/v)	Weight/Volume
XRD	X-Ray diffraction machine
Zn	Zinc

CHAPTER 1

INTRODUCTION

1.1 Research background

There is rapid population growth in both developing and developed nations across the globe and it is expected that the world human population would continue to record a rapid increase at an alarming rate (Sinha & Chattopadhyay, 2016). On the contrary, landmass available for various construction purposes continue to become relatively scarce (Bernardi *et al.,* 2014). This fast growth enhances the high demand for land for civil infrastructure, particularly in urban areas. In response to meeting various human basic needs, necessitate infrastructural development on problematic soils (Chang *et al.,* 2016; Hiranya *et al.,* 2018). These problematic and weak soils affect the safety and stability of structures constructed on them, due to over shear stress or limitation of shear strength applied during loading onto the soil, which consequently results in failure of the built structures. It is almost impossible or expensive to replace problematic soils such as soft marine clay commonly found at river banks and coastal areas, as they can extend to a great depth (Saad *et al.,* 2019). This makes the improvement of soil mechanical properties crucial. An investigation carried out in 2008 reported that over USD 6 billion was spent annually to finance over 40,000 soil improvement projects worldwide (Kalantary & Kahani, 2015). In the Nilgiri district of India, an estimated 1150 landslides of various sizes destroy roads, houses, railway lines and claim the lives of about 80 people within five days in November, 2009 (Suresh *et al*., 2019). Further, in the United Kingdom, erosion threatens 30,000 people with over 90,000 ha of agricultural lands within the Humber estuary flood plain due to destabilized sandy soil foreshores (Salifu *et al*., 2016). Meanwhile, for the last two decades, within the high areas of Malaysia, more than 400 landslides comprising of over 30 major landslides, involving both natural and cut slopes were reported to have destroyed properties worth of billions of ringgits and claim over 200 lives, due to high compressibility and low shear strength of Malaysian tropical peatland soils (Makinda *et al.,* 2018).

Although, for the past few years, several methods and materials were developed to improve the engineering characteristics of soils. However, they varied in terms of environmental impact, cost, penetration depth and treatment uniformity which portrays their merits and demerits (Wang *et al.,* 2011; Khan *et al.,* 2016; Duo *et al.,* 2018). The application of chemical grouting for the purpose of soil improvement is limited to a short injection distance of usually 0.3 to 1.0 m and is rather expensive. Further, the chemicals used such as polyurethane, lignosulfonates and acrylamides are toxic and may have adverse impacts on the environment (Paassen *et al.,* 2010) and has been prohibited by several countries due to their dangerous nature to humanity (Khaleghi & Rowshanzamir, 2019).

The use of mineral additives such as cement plays an important role for the construction industry. Concrete is produced from cement with an annual global demand of over 10 billion tons and experts predicted this demand to increase to about 16 billion tons by

2050 (Castro-alonso & Montañez-hernandez, 2019). Although cement is required in high demand, but its industrial manufacturing process have economic and environmental concerns which include, high energy consumption and emission of huge amount of CO_2 that is as high as 7% of the world anthropogenic emissions (Hiranya *et al.,* 2018; Nething *et al.,* 2020). An alarming situation which results to climate change (Wong, 2015). In addition, cement treatment of fine soils is highly ineffective due to its high viscosity in suspension (Stabnikov *et al.,* 2013). This draws the interest of researchers towards the idea of simulating nature for the improvement of loose soils through a microbial enzyme technology known as Microbially Induced Calcite Precipitation (MICP).

MICP is a biomineralization technique involving a biochemical process of precipitating calcium carbonate ($CaCO_3$) crystals induced by active bacterial activity such as *Sporosarcina pasteurii* due to chemical reactions occurring within the environment (Yang *et al.,* 2020). There are various metabolic pathways leading to the biosynthesis of microbial precipitation of $CaCO_3$ such as denitrification, photosynthesis, methane reduction, ammonification, sulphate reduction and ureolysis (Suresh *et al.,* 2019). Among all, ureolysis has been reported to be more favourably utilized for various technical applications due to energy efficiency and capability of inducing high amount of $CaCO_3$ in a short duration of time (Krajewska, 2018; Mukherjee *et al.,* 2019).

Ureolysis as a recent technology, utilizes eco-friendly features of urease producing bacteria in providing favourable conditions of hydrolysing urea in a series of complex biochemical reactions to generate ammonium and carbonate ions. The ammonium ions produced favours precipitation conditions by increasing the pH of the microenvironment (Terzis & Laloui, 2019; Filet *et al.,* 2020). The bacteria cell surface has a net negative charge as negative zeta potential (Renner & Weibel, 2011), thus under sufficient super saturation conditions provide a binding site for the bonding of carbonate ions with available divalent calcium ions within the micro-environment, hence precipitate cementitious calcite crystals on the cell surface. These crystals further cement soil grains together, filling inter-particle voids and in turn improving soil strength and stiffness (Torres-aravena *et al.,* 2018; Yan *et al.,* 2019; Cui *et al.,* 2020). The soil properties improve with high amount of calcite precipitation (Lutfian *et al.,* 2020). Further, MICP technique is possible in different natural water conditions ranging from freshwater to 100% seawater at various degrees of saturations, however a low degree of saturation gives better result (Oliveira *et al.,* 2017). Noteworthy, the technique can also be applied to a variety of soil types ranging from coarse and well graded sands to finer soils. However, MICP technique is more effective when applied on coarse and well graded sands in comparison to finer soils (Mortensen *et al.,* 2011).

Research on MICP as an emerging discipline has been a continuous process with a diverse documented successful story of soil stabilization and measured as one of the most effective soil improvement technology (Ghosh *et al.,* 2019; Ivanov *et al.,* 2020). Previous literature presented encouraging and impressive results (Hoang *et al.,* 2019; San Pabio *et al.,* 2020; Miftah *et al.,* 2020), thus proven its potential for addressing a wide range of geoenvironmental and geotechnical projects, including controlling erosion in rivers and coastal areas, enhancing the stability of non-piled and piled foundations, treating pavement surface, decreasing dust levels on exposed surfaces by binding together the dust particles, reinforcing soil for the enhancement of underground constructions (Wath

& Pusadkar, 2016). Bacteria suitable for MICP are alkali tolerant ureolytic bacteria, hence model strains are from genera *Clostridium*, *Bacillus*, *Desulfotomaculum*, *Sporolactobacillus* and *Sporosarcina* (Ivanov & Chu, 2008). However, *S. pasteurii* is widely utilised in most studies on MICP (Wen *et al.,* 2018), due to its high urease activity, tolerance to high pH and precipitation of large amounts of calcite (Rowshanbakht *et al.,* 2016; Minto *et al.,* 2018; Ruan *et al.,* 2019). An experiment carried out by Salifu *et al.,* (2016) demonstrated the efficiency of MICP using *S. pasteurii* as a model strain for protection of foreshore slope sites against erosion. These bacteria cemented the sandy soil by precipitating up to 120 kg of calcite/m^3 of the soil, filling 9.9% of inter-particle voids and in turn cemented the sandy soil which withstood unconfined compressive stress of 470 kpa. This amount of precipitated calcite satisfies the range for several soil improvement projects.

The success of MICP process is promoted primarily by *in situ* conditions such as particle size and distribution, temperature, water content and the conditions of treatment like cementation solution and concentrations of bacteria (Dadda *et al.,* 2018). Bacteria constitute one of the successful ubiquitous forms of life within the natural environment (Dorost *et al.,* 2018), adapting to varying environmental conditions both physiologically and genetically (Khaleghi & Rowshanzamir, 2019). Despite the numerous advances in MICP, this technique is associated with setback regarding reduction in the population of the introduced bacteria into the soil due to competition, predation and stress arising from abiotic factors like osmotic pressure, pH, availability of suitable nutrients and temperature (Burbank *et al.,* 2011). These limitations may be overcome by the utilization or enrichment of indigenous soil ureolytic bacteria (Burbank *et al.,* 2012). Further, main issue affecting this technique is bioclogging, which occur due to uneven distribution and uniformity of precipitated $CaCO_3$ in treated samples (Rowshanbakht *et al.* 2016; Omoregie *et al.,* 2018). This result in retention of the cementation solution and bacterial culture at the treatment injection point, thus affect the overall sand stiffness.

1.2 Justification of the study

In search for alternative soil improvement technology with minimal environmental consequences over conventional methods, and advances in material and geotechnical research, led to the development of an innovative, novel bio-mediated soil improvement technique termed Microbially Induced Calcite Precipitation (MICP). Previous studies have documented the application of MICP towards the improvement of soil as an effective, economically engineered natural occurring biotechnological process (Martinez *et al.,* 2013; Ming-juan *et al.,* 2016; Ming-juan *et al.,* 2017; Junjie *et al.,* 2020). However, most urease producing bacteria utilised for various MICP applications are commercially procured from culture collection centres, which contribute to cost (Zomorodian *et al.,* 2019), and only a few studies on indigenous ureolytic bacteria have been reported (Bibi *et al.,* 2018). According to the present global market price, it cost approximately US$402.0 to procure the original patent strain of *S. pasteurii* ATCC 11859, which suggest the low-cost advantage of utilizing indigenous ureolytic bacteria for various MICP applications (Ezzat & Ewida, 2021). Further, the procured microorganisms are often associated with drawback, regarding reduction in the population of the introduced microorganism into the soil due to competition, mechanical stress and predation arising from 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 microoorganisms and alter the traits expressed by these microbial communities (Badiee *et al.,* 2019). However, *in situ* ureolytic microorganisms have the least effect on the soil microbial flora (Badiee *et al.,* 2019). Further, it is important to note that ureolytic bacterial strains are not abundant due to complex biochemical reactions and specific environmental conditions (Zhu & Dittrich, 2016a). Thus, research on the utilization of alternative indigenous ureolytic bacteria with high urease activity towards soil improvement become paramount and still a budding line of research.

1.3 Significance of the study

The loss of lives, economic and social infrastructural assets caused by problematic soils and the adverse effect of utilizing cement and chemical grouting in problematic soils treatment could be tackled, by the development and utilization of the MICP biotechnology as a natural self-biotreatment process. In support of earlier successful studies carried out on MICP, this current study may provide additional knowledge on the potential of *in situ* ureolytic bacteria to precipitate $CaCO_3$ as a raw material for soil improvement. This technology converts urea (metabolic waste) to biocement (calcium carbonate crystals). Additionally, the idea of utilizing *in situ* ureolytic bacteria will save cost and enhance the MICP process by eliminating the setback regarding reduction in the population of the introduced bacteria into the soil due to competition and predation because of the adaptation of the *in situ* ureolytic bacteria to the soil environment. The outcome of this study is expected to be seen as a basis for the establishment of reference on an improved, straightforward, environmentally friendly and natural bio-mediated technique of soil improvement method via the precipitation of $CaCO_3$ by *in situ* soil ureolytic bacteria with high urease activity.

1.4 Hypothesis of the study

Null hypothesis: Indigenous ureolytic bacteria cannot hydrolyse urea to induce precipitation of calcium carbonate crystals for soil stabilization method.

Alternate hypothesis: Indigenous ureolytic bacteria can hydrolyse urea to induce precipitation of calcium carbonate crystals for soil stabilization method.

1.5 Objective of the study

Therefore, the objectives were set as follows:

i. To screen for *in situ* soil ureolytic bacteria with active urease activity from farmland soils of Ladang 15, Faculty of Agriculture, Universiti Putra Malaysia.

ii. To characterize the selected ureolytic bacteria with the potential of sustaining the culture conditions optimum for calcite precipitation activity.

iii. To analyze the precipitated calcium carbonate crystals produced in the biocementation of potential ureolytic bacteria using Scanning Electron Microscope (SEM) and X-Ray Diffraction (XRD) technique.

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