QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

ISOLATION, IDENTIFICATION, DIFFERENTIATION AND SCREENING OF LOCAL AEROBIC UREOLYTIC BACTERIA INVOLVED IN MICROBIALLY INDUCED CALCIUM CARBONATE PRECIPITATION IN QATARI SOIL.

BY

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ABSTRACT

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Title: Isolation, Identification, Differentiation and Screening of Local Aerobic Ureolytic Bacteria Involved in Microbially Induced Calcium Carbonate Precipitation in Qatari Soil.

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Biomineralization plays an important role in stabilizing the soil by modifying its geological and physical properties, hence, acting as a stabilizer against wind erosion specially in regions characterized by harsh soil (calcareous and dry) and weather such as the Arabian Gulf. Among the micro-organisms existing in soil, ureolytic bacteria can modify the characteristics of soil, thereby inducing biotic mineralization. One of the aim of this study was to explore the occurrence and diversity of ureolytic bacteria in soils of Qatar, for further application in soil stabilization, among other applications. The focus was to study the potential of bacteria adapted to the harsh environmental conditions to exhibit urease activity. Indigenous bacteria were isolated from six locations across Qatar. The most common genus found to exhibit ureolytic activity was *Bacillus* while the most common species was *Bacillus cereus.* 18 isolated ureolytic bacteria were identified and differentiated by MALDI-TOF MS while for 6 isolates, molecular 16S rRNA technique was used. All isolates were screened for the urease activity based on a modified method optimized in this work. Arbitrary urease activity (AUA) and specific production (AUA/cfu) were considered as main screening parameters. The isolates with high specific production of ureolytic activity are considered as inducer of calcium carbonate formation. The analysis of precipitates obtained in cultures using SEM-EDS analysis showed the

precipitates of calcium carbonates while XRD analysis confirmed the presence of calcium carbonate in two mineral phases: aragonite and calcite. The quantification of $CaCO_3$ precipitated was done using back-titration and by analysis of calcium ions using atomic absorption spectrometer. Under lab conditions, the process of Microbial induced calcite precipitation (MICP) increased the calcium carbonate content to 142.96 mg/g of soil (acid treated) in 20 mL liquid cultures quantified by titration, however, bacteria were able to induce 29 mg of $CaCO_3$ per gram of natural soil. The application of MICP on large scale was done in Qatar University Al-Khor farm where an increase in the $CaCO_3$ content was quantified to be of 16.2 %. This research illustrated high occurrence and distribution of indigenous *Bacillus sp.* in soil of Qatar that are capable of biomineralization and hence, can be proved helpful if properly utilized in enhancement of soil properties.

DEDICATION

This work is dedicated to my beloved parents and my lovely family.

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
AH	Abu Hamur
AA	Airport Area
AW	Al-Wakrah
AUA	Arbitrary Urease Activity
DL	Duhail
DN	Dukhan
EPS	Extracellular polymeric substance
M	Mesaieed
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MICP	Microbially Induced Calcite Precipitation
OD	Optical Density
PCR	Polymerase Chain Reaction
PM	Particulate Matter
QNRS	Qatar National Research Strategy
QNV	Qatar National Vision
rRNA	Ribosomal Ribonucleic Acid
SEM-EDS	Scanning Electron Microscopy/Energy Dispersive X-Ray Spectroscopy
XRD	X-Ray diffraction

Chapter 1: INTRODUCTION

Microbial induced calcite precipitation (MICP) is one of the most important research field linking many aspects of science; biology, geology and engineering (Krajewska, 2017). Micro-organisms change the geological and physical properties of the soil by induction of mineral precipitation. This induction happens due to the interaction of micro-organisms or their metabolites with their environment and hence, can precipitate minerals. These interactions are exploited for different engineered technologies (Sessitsch *et al*., 2001).

The involvement of micro-organisms to stabilize soil make them of special interest particularly in regions where windy and desert-like conditions are prevalent. Windy conditions tend to increase soil erosion which increases the level of particulate matter in the air. The presence of high calcium content in calcareous soils, and high temperature in the region helps in the biomineralizing activity through ureolytic bacteria. The ability of bacteria to produce urease either constitutive or inducible is an important factor for calcification. In Qatar, *Sporosarcina koreensis* is the only isolated bacterium from Qatari sand dunes that is identified as potential producer of urease activity, based presence of the corresponding gene on its genome sequence (Majid *et al*., 2016).

The State of Qatar is located in the middle of the Western coast of the Arabian Gulf between 24° 27´-26° 10´ N and 50° 45´-51° 40´ E with an area of 11, 600 Km^2 (Blanchard, 2014).

Arabian Gulf is warm, arid or semi-arid region of the world where the average temperature in the month of January is 17 °C while the average temperature in the

months of July and August is 35 °C (Al-Thani & Yasseen, 2013). Whereas for winter season, the maximum rainfall rate could reach 152 mm per year. Overall, Gulf region have high rate of evaporation that results in high salinity in soil and water (Bari *et al*., 2007; Shomar, 2015). Qatar is a peninsula land known for its aridity and desert-like conditions. The prolonged arid climate has provided support for topographic features. Qatari climate is known for its high temperature, high relative humidity and low precipitation rates (Sofotasiou *et al.*, 2015). However, weather phenomena such as fogs can occur during winter and contribute as a water source for flora species (Norton *et al*., 2009). Some reports have recorded that rainfall in Qatar is unpredictable and irregular in time and space. Based on a study, the average rainfall from years 1962 to 2011 in Doha is approximately 76.6 mm (Mamoon *et al*., 2014).

Qatar has been enjoying a period of unprecedented prosperity with remarkable development and economic progress. Qatar National Vision 2030 published in 2008 specifically highlight four major pillars to achieve the proposed vision. These pillars are human development, social development, economic developments, and environmental development.

The environmental development pillar in the QNV 2030 specifically highlights the investment of the state in techniques that will help to improve and protect the environment. The vision also encourages people to use environmental friendly technologies as well as adopt the sustainable policies and implantation plans to develop a sustainable state. Therefore, Qatar Foundation in 2011 included a goal of targeting the health impacts of particulate molecules and methods of reducing their levels in the air

within Qatar National Research Strategy (QNRS, 2014).

The proposed research is in-line with the Vision of 2030 and is timely given the expected significant infrastructure development in preparation for FIFA World Cup 2022 that will be held in Qatar. The proposed research represents an investigation on the use of bio-modification of soil not only to resist wind loading but also minimize the particulate matter in soil and the environment. The aim of this research is to investigate processes to stabilize soil biologically and sustainably to prevent the erosion of soil from turbulent wind and to focus on soil and environmental conditions for MICP in Qatar. The research combines micro-scale aspects of bio-mediated soils behavior. Furthermore, this study will focus on the research gaps that are encountered when small-scale response of biologically modified soils is studied.

RESEARCH OBJECTIVES:

1. Isolation, molecular identification and differentiation by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) of endogenous soil bacterial species.
2. Investigation of potentialities of isolated bacteria for MICP.
3. Mode of MICP for selected bacteria based on SEM/EDX and XRD analysis.
4. Application of MICP on Lab and Field scale to investigate its use in soil stability.

SIGNIFICANCE/NOVELTY OF THE RESEARCH:

The novelty of our research resides in the characterization of the ureolytic bacterial populations in Qatari soil and the study of the biodiversity and distribution of such populations in harsh environmental conditions that prevails, and in calcareous soil. Also, the native ureolytic bacteria isolated from Qatari soil will be used to bio-modify the soil. Furthermore, the significance of the research lies in the establishment of screening method to investigate the potentialities of ureolytic bacteria for their urease activity as well as to study the response of soil subjected to biomodification on small laboratory and field scales.

Chapter 2: LITERATURE REVIEW

PARTICULATE MATTER IN QATARI AIR

Particulate matter concentrations in air is an important indicator in terms of pollution and its implications on the health status of well-living and well-being. According to Ambient Air Pollution database (2014), Doha ranked 12th among 20 most polluted cities based on the yearly mean levels of particulate matters. The annual average concentrations of particulate matter set by World Health Organization is 10 µg/m^3 for $PM_{2.5}$ and 20 µg/m^3 for PM_{10} respectively (IAMAT, 2016). The national air quality standards for $PM_{2.5}$ and PM_{10} are very different than those set by WHO. There is no standard limit set for $PM_{2.5}$, however, the 24 hours average concentration for PM_{10} is 150 µg/m^3 while annual average concentration is set to be 20 µg/m^3 (Environmental Protection Law 30, 2002). The data obtained from the monitoring station at Qatar University in 2012 measured the annual average concentration of $PM_{2.5}$ to be 119.19 µg/m^3 and for PM_{10} to be 219.57 µg/m^3 (Environment Statistics Annual Report, 2013).

Another report states that the airborne particulate matter levels are in the range of 105 to 185 µg/m^3, which is more than 2 to 3 times the Qatari standard for particulate matter (Qatar Today, 2013). Air quality measurements indicate that the particulate matter in Qatar is among the highest in the region. According to Qatar Today (2013), the data of the Desert Research Institute of the US Department of Defense at US military bases in the Middle East shows that the particulate molecules of size 10 µm or smaller (PM_{10}) in Qatar (166 µg/m^3) ranks second after Kuwait. In addition, the particulate molecules of size 2.5 µm or smaller ($PM_{2.5}$) in Qatar (67 µg/m^3) ranks third following Kuwait City and

Baghdad. The values of PM_{10} and $PM_{2.5}$ in Qatar are more than 4 to 5 times than those recorded in the south east urban areas in the USA. It is important to note that the high particulate matters resulting from sandstorms effects on human health include asthma, coughing, and chronic respiratory problems. In addition, sandstorms affect economical activities such as halting oil exports and affecting airports operation.

BIODIVERSITY OF SOIL BACTERIA IN QATAR

Bacteria are a large group of unicellular organisms that are found everywhere in the world. A study was conducted by Umlai *et al* (2016) in Al Rayyan, Doha-Qatar to investigate the diversity of bacteria in Qatar. The results indicated that the microbes found mainly in sand were *Bacillus thuringensis* (50%) followed by *Bacillus cereus* (40%), *Methylobacterium* (5%), *Enterobacteriophages* (3%) and other forms of *Bacillus* species (2%) along with minor traces of *Streptococcus pneumonia*. *Enterobacteriophages* were also found but in least abundance. In another study, different strains of bacteria involved in degrading hydrocarbons were identified which include *Pseudomonas aeruginosa, Klebsiella pneumoniae, Arthrobacter sp., Citrobacter sp., Citrobacter amalonaticus, Enterobacter helveticus, Cronobacter muytjensii, Pseudomonas aeruginosa* and *Bacillus cereus* (Al-Disi *et al*., 2016). Furthermore, the microbial community associated with Barchan sand dunes of Qatar have also been investigated. The reported microbes collected from 18 Barchan sand dunes belonged to *Actinobacteria, Firmicutes,* and *Proteobacteria* with percentages of 58%, 27% and 15% respectively (Majid *et al*., 2016). In another report, the prevalence and existence of bacterial strains in Qatar were studied. Four kinds of bacteria were reported that include; *Escherichia coli,*

Salmonella, *Campylobacter* and *Listeria monocytogenes* (Cornell University, 2014).

SOIL STABILIZATION AND MICP

Calcite is about 4% by weight of surface area of the earth and is the most abundant mineral. The precipitation of the calcite is very well-known phenomenon that is important part in the formation of natural rocks and is present in different aquatic habitat like, marine and fresh water. It is also present in soils. Organic mineralization also known as organo-mineralization is the precipitation of fine calcium carbonate crystals by interaction with organic matter or macromolecules (Riding, 2000). This process of mineralization occurs in nature without the involvement of microbes (Riding, 2000). This indicates that the presence of organic macromolecules would interfere with the biomineralization processes and could yield wrong observations by abiotically precipitating calcium carbonates.

There are many techniques known to stabilize soil such as using bituminous substances, thereby using a lot of energy or hazardous material (GRT, 2017). As an alternative, bio-stabilization of soil using ureolytic bacteria is more environment friendly technique as compared to the conventional technologies such as use of cement, fly ash, lime and cement (GRT, 2017). The processes involved in biomineralization provide a precise control from nano to macro-level over the composition, structure, morphology and size of biominerals by developing materials with extraordinary complexity, distinct structures and properties. The chemical synthesis of these minerals requires high temperatures and strong chemical solutions and also produces toxic by-products as compared to the process of biomineralization that occurs under mild conditions

and at ambient temperatures (Krajewska, 2017). So, biomineralization is an efficient, eco-friendly and energy saving process that can be applied in different fields and can also be considered for carrying out *in situ* applications (Krajewska, 2017).

Microbially induced calcite precipitation (MICP) is a technique used to biologically induce the precipitation of calcium carbonate. This technique utilizes the pathway of ureolytic bacteria to precipitate calcium carbonate, thereby, stabilizing and strengthening the soil (Cheng & Shahin, 2016). MICP using ureolytic bacteria is a cheaper technique for soil stability. Urea, being one of the nitrogen fertilizer is easily available in the Arabian Gulf countries as one of the petroleum products. Urea has highest content of nitrogen and its cost of transportation per unit of nitrogen is very low (GRC, 2008; Hvidt, 2013). Hence, the provision of such nitrogen rich urea source to the soil will improve soil stability in the presence of ureolytic bacteria. Every process has certain disadvantages. The process of MICP results in production of ammonium ions that is hazardous in nature and can cause problems. Also, the use of microbes to carry out the process makes it a bit slower as compared to other soil stabilizing techniques (Anbu *et al*., 2016). However, there could be two ways to deal with excess ammonia produced. The effluent of the process could be treated for ammonia before discharging it or the ammonia could be used as a fertilizer for the surrounding plants (Mujah *et al*., 2016).

Amelioration of soil, in general, is carried out either by bioaugmentation or biostimulation. In the process of bioaugmentation, the exogenous ureolytic bacteria are added to the soil, with urea and calcium sources. However, in the process of biostimulation, indigenous bacteria are utilized, and all the necessary nutrients and

conditions are fulfilled. Both methods have their own benefit and drawback. In bioaugmentation, usually monoclonal cultures of bacteria are provided due to which the survival rate is low and hence, MICP is lower. On the other hand, biostimulation, even though, allows indigenous bacteria to carry out MICP, the concentration of native ureolytic bacteria will act as a limiting factor (Gat *et al*., 2014).

Local or native ureolytic bacteria (indigenous bacteria) has been used to perform MICP in different studies. For precipitation, it is important for the bacteria to posses' urease enzyme. This could either be constitutive or induced by the provision of urea (Burbank *et al*., 2011).

APPLICATIONS OF MICP

MICP is considered as an environmental and economical friendly technique to stabilize soil, buildings, cracks, concrete and others. Many known ureolytic bacteria are used in different applications. *Bacillus cereus, Bacillus pasteurii, Shewanella, Sporosarcina pasteurii, Bacillus sphaericus, Bacillus pseudofirmus,* and *Bacillus cohnii* are known microorganisms used in applications for fixing cracks, remediation and self-healing of buildings (Bang *et al*., 2001; Ramachandran *et al*., 2001; Ghosh *et al*., 2005; Jonkers *et al*., 2007; Ramakrishnan, 2007; De Belie *et al*., 2008; De Muyunck *et al*., 2008a; De Muyunck *et al*., 2008b; Achal *et al*., 2011a; Achal *et al*., 2011b). Furthermore, the ureolytic bacterial strain *Sporosarcina pasteurii* NCIM 2477 is also used to manipulate the properties of brick by improving its quality through precipitation of calcite that deposits in the void spaces which causes reduction in the absorption of moisture (Sarda *et al*., 2009; Wong, 2015). Reduced permeability is another outcome of

biologically modified materials such as concrete or soil. Calcium carbonate that precipitates due to biological activity deposits over the concrete resulting in reduced permeability, thereby, increasing its life span (Nolan *et al*., 1995). Calcium carbonate precipitation by MICP can act as an additive in decreasing the overall porosity of building materials, thereby improving the durability, strength and quality of the material. This can help to prevent the use of conventional chemicals that can be dangerous or hazardous to improve the construction materials (Annamalai *et al*., 2012).

The effect or impact of MICP on soil properties could be at the macro-scale or micro-scale. The macro-scale behavior of soil is affected by the content of calcium carbonate precipitated, including relative density, confining stress and gradation of soil. On the other hand, micro-scale behavior of biologically treated soil will allow to study the precipitation of calcium carbonate with respect to bacterial cells as well as the inter-particle bonds formed by the precipitation of calcium carbonates between the soil grains (Lin *et al*., 2016).

POLYMORPHS OF CALCIUM CARBONATE PRECIPITATES DURING MICP

Different polymorphs of calcium carbonate are reported to be formed due to the phenomenon of biomineralization. The polymorphs could either be anhydrous or hydrous. Calcite, vaterite and aragonite are the type of anhydrous polymorphs while the hydrated polymorphs include monohydrocalcite, ikaite or hexahydrocalcite and amorphous calcium carbonate (Anbu *et al*., 2016). The most stable known form of calcium carbonate crystals is rhombohedral.

Calcium Carbonate precipitates exist in two forms; amorphous and crystalline. The crystalline phase is further divided in to two groups; crystalline and hydrated crystalline. The crystalline structures include calcite, vaterite and aragonite while the hydrated structures include monohydrocalcite and ikaite (Phillips *et al*., 2013; Dhami, Reddy & Mukherjee., 2013). Among these crystalline structures, vaterite is considered as a transition phase towards the formation of calcite. Micro-organisms are known to precipitate different crystalline forms of calcium carbonate such as; calcite, calcite-magnesium carbonate trihydrate, As(III)-calcite, aragonite, vaterite, calcite-strontianite, halite, monohydrocalcite, struvite, anhydrite, rhombohedral calcite and hexagonal vaterite (Hammes *et al*., 2003; Achal *et al*., 2012; Cheng, Hin & Cord-Ruwisch., 2014; Kang, Kwon & So., 2016; Arias, Cisternas, & Rivas., 2017).

According to Al-Thawadi and his colleague (2012), *Bacillus sp.* were able to induce the precipitation of calcium carbonate as; clusters, spherical (vaterite) and non-spherical aggregates and rhombohedral (calcite) crystals. The responsible bacterial species with most crystal precipitation was reported to have urease activity of 1.6 mM/minute (Al-Thawadi & Cord-Ruwisch, 2012). Calcite and aragonite in trace amounts are commonly reported in research studies. Ronholm *et al*., (2014) reported heterotrophic bacterial isolates to precipitate calcite and aragonite when grown in B4 media provided with calcium citrate or calcium acetate. Calcite crystals are homogenously deposited on the surface of the soil; however, amorphous calcium carbonate is also reported to be precipitated by *B. sphaericus* (Dick *et al*., 2006). The three-dimensional structures of calcite, aragonite and vaterite are shown in Figure 1.

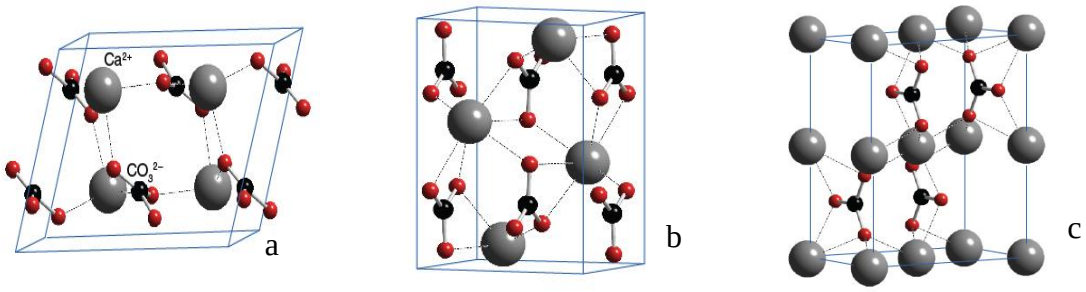

Figure 1. 3 Dimensional structures: (a) Calcite, (b) Aragonite and (c) Vaterite

Source: http://www.chemtube3d.com/solidstate/SS-CaCO3.htm

SOIL UREOLYTIC MICROORGANISMS FOR BIOMINERALIZATION

Photosynthetic organisms (cyanobacteria & algae), sulfur reducing bacteria, ureolytic bacteria under aerobic and anaerobic conditions, and bacteria that utilizes organic acids are all known to increase pH of the environment and precipitate carbonates (Castanier *et al*., 1999; Braissant *et al*., 2002; Hammes & Verstraete, 2002; Ariyanti *et al*., 2012).

The focus of this research is to use ureolytic bacteria to carry out MICP. Ureolytic bacteria are commonly found in the sub-surface soil. To stimulate the activity of ureolytic bacteria, usually nutrients are provided from outside source. Providing nutrients will enhance or stimulate the ureolytic activity of the local bacteria, preventing the need of

bio-augmentation (Mortensen *et al*., 2011). About 17-30% of the aerobic, micro-aerophilic and anaerobic bacteria can perform ureolysis and are found in the soil. These soil microbes could be utilized to the best of their ability by inducing the urease enzyme by providing urea as sole nitrogen source (Burbank, 2012). Urease enzyme belong to a group of enzymes that is found in invertebrates, algae, fungi, bacteria and plants. Bacteria harboring gene for the expression of urease enzyme is widespread and hence, its use for the calcium carbonate precipitation is highly studied (Hammad, 2013).

The role of ureolytic bacteria is important in biomineralization. A variety of bacteria are known to induce the precipitation of calcium carbonate. Bai and his team (2017) reported 200 types of bacteria involved in MICP including both Gram-positive and Gram-negative bacteria. Gram positive bacteria contains homopolymeric urease while Gram negative bacteria contains three distinct subunits (α, β, γ) of urease (Mobley & Hausinger, 1989; Konieczna *et al*., 2012).

The reference micro-organism known to have highest urease activity and higher rate of calcium carbonate precipitation is *Sporosarcina pasteurii* (Paul, 2014). The most commonly known ureolytic bacteria is from the genus *Bacillus.* Many species are known to have ureolytic activity such as *B. sphaericus* (Cheng & Cord-Ruwisch, 2012) *B. lentus* (Kim & Youn, 2016) *B. licheniformis* (Helmi *et al*., 2016) and hence, can induce biomineralization.

MECHANISMS INVOLVED IN BIOMINERALIZATION

There are three different mechanisms known through which bacteria induces the

precipitation of minerals. (i) Precipitation of minerals by bacteria under controlled conditions. This type of mechanism is assisted by the activities of the cells. The nucleation site and minerals growth, both are controlled by the organisms only under certain specific conditions (Anbu *et al*., 2016; Benzerara *et al*., 2011; Phillips *et al*., 2013). (ii) Biologically influenced precipitation of minerals where precipitation occurs due to the excretion of extracellular polymeric substances (EPS). The components of EPS play a role in trapping ions hence, assisting biomineralization (Benzerara *et al*., 2011; Phillips *et al*., 2013, Sarayu *et al*., 2014). (iii) Biologically induced precipitation of minerals where the precipitation occurs due to a chemical change within the environment. The chemical change occurs due to biological activities. Overall, the saturation index reaches, and the precipitation of minerals occurs (Anbu *et al*., 2016; Phillips *et al*., 2013).

UREOLYTIC BACTERIAL METABOLISM AND ITS ROLE IN BIOMINERALIZATION

Precipitation of calcium carbonate occurs from a supersaturated solution based on the chemical changes within the environment through enzymatic activities of ureolytic bacteria. Certain enzymes including ureases are present in micro-organisms, which can be induced (Chahal *et al.*, 2011). This enzyme can hydrolyze urea and produce ammonia and carbon dioxide. Production of ammonia results in an increase in the alkalinity of the environment due to which the minerals are precipitated as the saturation index is reached. Hence, ureolytic bacteria can play a role in biomineralization. In MICP, ureolytic bacteria hydrolyze 1 mole of urea into 1 mole of carbamic acid and 1 mole of ammonia (eq 1). The carbamic acid hydrolyses further and produce another 1 mole of ammonia and 1

mole of carbonic acid. The carbamate further is hydrolyzed to form ammonia and carbonic acid (eq 2). Carbonic acid further undergoes in a reversible reaction with water to produce bicarbonate (eq 3). Similarly, the ammonia reacts with water to form ammonium ions and hydroxide ions resulting in an increase in the basicity of the environment (eq 4). Increased alkalinity of the environment shifts the equilibrium of bicarbonate, hence, produces carbonate ions (eq 5). Increased pH and presence of carbonate ions would precipitate calcium carbonate in the presence of free calcium ions (eq 6) (Okyay & Rodrigues, 2014).

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad \text{(eq 1)}$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad \text{(eq 2)}$$

$$H_2CO_3 \leftrightarrow HCO^-_3 + H^+ \quad \text{(eq 3)}$$

$$2\,NH_3 + 2H_2O \leftrightarrow 2NH^+_4 + 2OH^- \quad \text{(eq 4)}$$

$$HCO^-_3 + H^+ + 2OH^- \leftrightarrow CO_3^{-2} + 2H_2O \quad \text{(eq 5)}$$

$$CO_3^{-2} + Ca^{2+} \leftrightarrow CaCO_3\,(K_{sp} = 3.8 \times 10^{-9}) \quad \text{(eq 6)}$$

The rate of hydrolysis determines the form of calcium carbonate crystals that will form. If the rate of urea hydrolysis is higher, vaterite and amorphous calcium carbonate crystals are observed, however, if the rate of hydrolysis of urea is low, rhomboidal calcite formation is observed (Lin *et al*., 2016). The process of nucleation and formation of calcium carbonate crystals are both affected by the bacterial species. This process is considered as one of the most common activity carried out by bacteria at different environmental conditions.

The biomineralization under natural conditions does occur but the process is slow.

If any ureolytic micro-organism is present in the environment, biomineralization is assisted as they act as a catalyst in the process. Again, the whole process could occur through three mechanisms; controlled precipitation, microbially influenced precipitation through EPS secretion, and microbially induced precipitation where micro-organisms alter the environmental conditions to allow precipitation (Dhami, Reddy & Mukherjee., 2013).

FACTORS AFFECTING UREOLYTIC ACTIVITY AND BIOMINERALIZATION

There are many factors affecting the morphology and composition of the precipitated crystals such as the type of bacteria and its concentration used, pH, temperature, and composition of the growth media or culture (Anbu *et al.*, 2016), the strain specificity regarding urease activity as well as precipitation of minerals. The selection of proper growth media or culture also plays an important role. It not only affects the rate of calcium carbonate precipitation but also affect the morphology of the precipitates. Similarly, use of inappropriate growth media or culture can result in abiotic precipitation. On the other hand, the process of biomineralization in the environment not only depends on the metabolic processes of the micro-organisms but also on the amount of active microbial activities (Versteegen, 2010).

BACTERIAL CULTIVATION AND GROWTH MEDIA FOR UREOLYTIC ACTIVITY

To ensure efficient urease activity, better growth of ureolytic bacteria is required. The measurement of optical density (OD) at 600 nm is used to confirm efficient growth of bacteria. An optical density in range of 0.8-1.2 (10^7 cells/mL) is reported for bacteria to have high urease activity (Al-Qabany, 2012). Kim & Youn (2016) reported inoculum to have concentration of 10^8 to 10^9 cells per mL. Optical density of 600 nm of ureolytic bacteria is also reported between 2 to 2.5 for soil stabilization using bioslurry technique (Cheng & Shahin, 2016). There are many growth media reported in the literature and the composition vary from one medium to another. Urease enzyme is activated in the cells by growing them in appropriate growth media. A similar growth media was reported in another research where nutrient broth and 2% urea was used to grow the bacteria (Hammad, Talkhan, & Zoheir, 2013). Other study conducted in 2018 by Phang and colleagues reported the use of nutrient broth (3 g/L), sodium bicarbonate (2.12 g/L) and urea (20 g/L). Research conducted by Burbank and colleagues (2012) grew ureolytic bacteria in nutrient broth with nitrogen source; 100 mM urea or 100 mM ammonium sulphate to obtain urease enzyme. Also, urea medium (5 g/L NaCl, 2 g/L KH_2PO_4, 1 g/L glucose, 0.2 g/L peptone, 20 g/L urea) was used to grow bacteria for measurement of urease activity (Burbank et al., 2012). Following a similar recipe with additions, Canakci and colleagues (2015) used urea media (5 g/L peptone, 5 g/L NaCl, 2 g/L yeast extract, 1 g/L Beef extract and 20 g/L urea) to grow the bacteria for experimental analysis.

TEMPERATURE

Temperature is another important factor that should be considered while studying urease activity, as it can be different for different strains. Such as ureolytic bacterium: *Sporosarcina pasteurii* is known to exhibit urease activity between the temperature range of 25 °C to 45 °C, however, the specific urease activity was observed to be highest at temperatures of 25 °C and 30 °C respectively (Omoregie *et al*., 2017). Other bacteria such as *A. sulfureus* and *B. atrophaeus* showed efficient urease activity between temperature range of 25 °C to 30 °C. Highest urease and biomineralization activity was observed at 37 °C for *B. muralis.* (Otlewska & Gutarowska, 2016). Other bacteria; *B. thuringiensis* and *B. subtilis* were found to have biomineralization activity at temperature of 25 °C (Baskar *et al*., 2006). According to Nemati and colleagues (2005), impact of temperature on the biomineralizing activity of ureolytic bacteria is not significant, however, at lower temperature of 20 °C, biomineralization occurred at slow rate as compared to the biomineralization rate achieved at temperature of 35 °C. This difference could be due to the optimal temperature required by the urease enzyme to perform ureolytic activity. Based on the review it can be said that calcium carbonate can be precipitated at different temperature ranges; however, the rate of precipitation and polymorph will be different.

PH

Ideal pH for urease enzyme activity is in range 5 – 8 (Fisher *et al*., 2017). The activity of urease and biomineralization are linked to each other. Higher the urease activity, higher will be the biomineralization rate. At pH range of 8 to 9, high rate of

biomineralization is reported while at pH 6, no biomineralization is known to occur (Otlewska & Gutarowska, 2016). Gorospe and colleagues (2013) has similar findings; *Sporosarcina pasteurii* KCTC 3558 and ATCC 6453 performs efficient biomineralization activity at pH of 8. For efficient activity of urease enzyme, an alkaline pH is recommended (Anne et al., 2010). The pH for urease activity could be more alkaline depending on the type of media used; B4 media is reported to produce acetic acid due to which a pH of 10 is required for the efficient activity of urease enzyme (Prah *et al*., 2011).

UREA CONCENTRATION

One of the most significant factor in ureolysis and biomineralization activities is the concentration of urea. In most of the researches 20 g/L of filter sterilized urea is used (Burbank *et al*., 2012; Hammad, Zoheir, & Talkhan, 2013; Gat *et al*., 2014; Wei *et al*., 2015; Xu *et al.*, 2017). Xu and colleagues (2017) established a relationship between the concentration of urea and the growth of bacteria. A positive correlation was reported for a range of urea concentration (20 g/L – 60 g/L) whereas, higher concentrations (80 g/L & 100 g/L) are reported to inhibit the growth of bacteria, therefore, no biomineralization is assisted too (Xu *et al*., 2017). Another research reports that for certain bacteria, urea (20 g/L & 30 g/L) with calcium chloride (30 g/L & 75 g/L) at certain conditions inhibits the growth and activity of urease enzyme (Nemati, Greene, & Voordouw, 2005).

CEMENTATION SOLUTION CONCENTRATION

The precipitation of calcium carbonate requires both urea and calcium source. On hydrolysis, urea produces carbamate which on further reaction produces carbonate. The

carbonates react with calcium ions if available and produce calcium carbonate. The calcium carbonate precipitates in an alkaline environment. Different sources of calcium are used to precipitate calcium carbonate polymorphs in the presence of urea such as calcium acetate, calcium chloride, calcium nitrate and calcium oxide (Lee, 2003; Burbank *et al*., 2013; Achal & Pan, 2013; Otlewska & Gutarowska, 2016). For precipitation of calcite, medium with 49 g/L was used along with 20 g/L urea (Kumar, Prabhakara & Pushpa, 2013). The precipitation of calcium carbonate is reported to not increase any further if the concentrations of calcium ions is increased beyond 90 g/L (Okwadha & Li, 2010). Furthermore, a mixture solution of 13 g/L nutrient broth, 111 g/L calcium chloride, 0.17 M sodium acetate and 0.0125 M ammonium chloride is also used as cementation solution for bio-cementation activity (Omoregie *et al*., 2017). The concentration of cementation solution is important because it affects the homogeneity of the precipitated calcium carbonate. A highly concentrated cementation solution (111 g/L $CaCl_2$) tends to reduce the homogeneity of precipitate over the soil as compared to less concentrated cementation solution (55.5 g/L $CaCl_2$) (Zheng & Kadhim, 2017).

INSTRUMENTS USED FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF $CACO_3$

SEM-EDX analysis, XRD analysis and AAS were used for analyzing the precipitates of $CaCO_3$.

Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy (SEM-EDX) Analysis

Scanning electron microscopy (SEM) is an instrument that uses beam of electrons to study the surface of the samples. It uses electron beams that has high energy. Signals are produced when the atoms in the samples interact with the beam of electrons (Newbury & Ritchie, 2013). It provides morphological and chemical information about the specimen under observation. Moreover, it also helps to analyze the orientation of the material.

X-Ray Diffraction (XRD) Analysis

XRD is an analytical technique used to characterize materials. XRD provides a 2-D diffraction pattern of the samples by providing concentric rings or the peaks. These peaks correspond to the d spacings within the lattice of the crystals. The peak intensities and positions are used to identify the mineral phase of the samples (Pandian, 2014).

Atomic Absorption Spectrometry (AAS)

The measurement of Ca^{2+} ions using atomic absorption spectrometry is a very developed and advanced technology which uses a flame and electro-thermal automizers (Ferreira *et al*., 2018). Atomic absorption spectroscopy (AAS) is considered as one of the three most important instruments that are used to analyze the elements within the sample hence, enabling us to investigate the soil minerals and help in their quantitative analysis (Singh & Agrawal, 2012). AAS is used to investigate about the chemical composition of the sample. The technique is one of the sensitive analytical instrument measuring very minute details about the elemental composition of the samples (Alhawdar, 2014).

CHAPTER 3: MATERIALS AND METHODS

COLLECTION OF SOIL SAMPLES

Soil sampling was done from six sites across Qatar; Airport area (AA) [25.2647º N, 51.5596º E], Duhail (DL) [25.3491º N, 51.4677º E], Al-Wakrah (AW) [25.1659º N, 51.5976º E], Mesaieed (M) [24.9909º N, 51.5493º E], Dukhan (DN) [25.4280º N, 50.7833º E] and Abu-Hamur (AH) [25.2388º N, 51.4914º E]. The soil samples were collected from the surface and to a depth of 3-4 cm and were transferred to the lab in ice box.

MEDIA AND CHEMICALS

Enrichment Media for isolation of Ureolytic bacteria

The enrichment medium used to isolate ureolytic bacteria from soil cultures comprises of the following ingredients (per liter): glucose 0.25 g, sodium acetate 13.8 g, Bacto yeast extract 0.5 g and filter sterilized urea 20 g.

Phosphate-Buffered Saline Solution (PBS)

PBS was used in enrichment procedure with composition per liter; NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g.

Urea Media

Urea Media was used to grow ureolytic bacteria and to measure the urease activity. The composition of media per liter (autoclave); NaCl 5 g, KH_2PO_4 2 g, glucose 1g, phenol red indicator 0.012 g, peptone 0.2 g, urea 20 g (filter sterilized).

Modified Urea Agar

Modified urea agar was used to screen the bacteria for their ureolytic activity. The

composition per liter (autoclave); NaCl 5 g, KH_2PO_4 2 g, glucose 1g, phenol red indicator 0.012 g, peptone 0.2 g, agar 15 g, urea 20 g (filter sterilized).

Luria Broth (LB) – Liquid and Solid

Luria Broth media (liquid/solid) was used to prepare pure cultures for experiments.

Urease Buffer

Urease buffer was used for measurement of urease activity. It is composed of EDTA 1 mM, HEPES 50 mM, Urea 20 g/L (filter sterilized)

Phenol-nitroprusside solution

Composition of phenol-nitroprusside solution per liter: phenol 70 g, nitroprusside 0.34 g. Store in dark bottle at 4 °C.

Sodium Hypochlorite Solution

Composition per liter: NaOH 17.5 g, Na_2HPO_4 59.45 g.

The solution shall be freshly prepared and stored in dark bottle at 4 °C.

Cementation solution

A solution used as source of calcium ions (Ca^{2+}). It was composed of calcium chloride ($CaCl_2$) used as a supplement with urea media to help ureolytic bacteria to induce calcium carbonate precipitation. Two concentrations of calcium chloride were used; 0.03 M (3.5 g/L) and 0.2 M (22.2 g/L)

Chemicals

70 % Formic Acid, 100 % Acetonitrile and Matrix solution HCCA (α-cyanohydroxycinnamic acid) were used for protein extraction in MALDI-TOF MS for

identification and differentiation of bacteria.

For molecular identification by 16s rRNA, PCR mix was used. It included; 2.5 µL of $DNTP_{mix}$, 2.5 µL RibS73, 2.5 µL RibS74, 0.5 µL Taq polymerase, 11.5 µL distilled water, 1 µL of $MgCl_2$, 2.5 µL of buffer, 2 µL sample. Universal primers; RibS74 and RibS73 were used for pcr amplification.

For titration; 0.33 M HCl and 0.16 M NaOH were used.

ENRICHMENT AND ISOLATION OF SOIL UREOLYTIC BACTERIA

Enrichment of bacteria involved in ureolysis

Soil from each location was added to sterile syringe barrels up to 30 mL mark. 20 mL of enrichment solution for each sample was added to the syringe barrels. This method used was the modification of technique performed by Burbank *et al*., (2012). To prevent any loss of solution from the syringes, a tube closed by a clamp was connected to the end of each syringe. The syringes were incubated for 3 days. After completion of incubation time, the solution was drained from the syringe and was re-treated for next 48 hrs. 1 mL of the effluent from the syringe was collected into sterile centrifuge tubes and were washed with PBS cold solution three times followed by centrifugation. The pellets obtained after centrifugation were suspended in 5 mL cold PBS solution.

Isolation of Ureolytic bacteria

Isolation of ureolytic bacteria was done by performing serial dilutions of enrichment suspension obtained in step (3.3.1). The plating was done on urea agar which was the modified form of Burbank *et al* (2012) method. The plates were incubated at 37 °C. Any color alteration from yellow to pink indicated the ureolytic activity by bacteria.

The cells that showed a color change were transferred to sterile centrifuge tubes and were further enriched with enrichment solution. The selected colonies were incubated in the rotatory shaker at 37 °C. After growth was observed in the form of turbidity in the tubes, the cultures were centrifuged to obtain pellets. Sterile cold PBS was used to wash the pellets. The pellets were re-suspended in PBS and serial dilutions were carried out to obtain pure cultures of the isolates. The cells were then purified and preserved in 30 % glycerol at -80 °C.

ISOLATES IDENTIFICATION AND DIFFERENTIATION BY MALDI-TOF-MS

Preparation of samples

Protein extraction was done using ethanol/formic acid as described by Wang *et al*., (2012). Isolates that were to be identified were grown overnight. A loop full of cells were suspended in 300 µL water to which 900 µL ethanol was added. The tubes were centrifuged for 2 min at 13,000 rpm. The supernatant obtained after centrifugation was discarded and re-centrifuged for 2 more minutes. The residual ethanol was then discarded. To the pellet obtained, 50 µL of 70% formic acid and 100% acetonitrile was added. The solution was mixed by vortex and then centrifuged at 13,000 rpm for 2 min. 1 µL of supernatant obtained after centrifugation was transferred onto a MALDI Biotarget (48 or 96 sample spots) plate. 2 sample spots were used for each sample for Identification and Typing. The sample after drying was overlaid with 1uL of HCCA matrix solution. The sample spot was then air dried before loading it into the machine. The biotarget plate with samples overlaid with matrix solution was loaded in the

machine.

Result analysis

Bruker Biotyper software was used to analyze the spectra obtained from the identification. The spectra of each isolate were compared with the database within the system (Nacef *et al*., 2017). A log scale from 0 – 3 range is used to interpret the results for similarities and differences among the bacterial protein profiles.

IDENTIFICATION OF BACTERIAL ISOLATES BY MOLECULAR 16S RRNA TECHNIQUE

Preparation of samples

The isolates to be identified by 16s rRNA technique were grown overnight on LB agar. DNA of isolates was extracted by thermal lysis. In this method, 200 µL of sterile water was added into sterile eppendorf tube. A colony of bacterial strain that needed identification was suspended in the same tube with sterile water. To obtain suspension, the mixture was vortex. After vortex, the tube was incubated at -80°C for 20 min. After the incubation period was completed, the tubes were transferred immediately to boiling water (100°C) for exactly 10 min. After 10 min, the tubes were transferred to ice for cooling them. After approximately 2-3 min, they were centrifuged for 10 min at 13000 rpm (Al-Disi *et al*., 2017). The supernatant obtained after centrifugation was amplified using Polymerase Chain Reaction (PCR).

PCR Amplification

To amplify the isolates by PCR, PCR mixture was made. The primers used to amplify the DNA were RibS73 and RibS74 (Al-Disi *et al*., 2017). The amplicons

obtained were purified and sequenced using Applied Biosystems 3500 Series Genetic Analyzer System. The sequences were compared to the data base by BLAST to find similar and identical sequences in the Gene Bank. National Center for Biotechnology Information (NCBI) is the provider of Gene Bank database.

Gel Preparation and Electrophoresis

To prepare gel for running the PCR products, 1 g of agarose was weighed and added to 100 mL of 0.5x TAE buffer. The solution mixture was heated for approximately 2-3 min in oven. 5 µL of ethidium bromide was added to the heated solution and the solution was mixed properly. The solution was poured in trays with comb and was left for solidification.

The PCR products obtained after PCR amplification were loaded in gel to confirm whether the amplification was successful or not. In the gel, the ladder, water as a control and the pcr products were loaded. The total volume loaded in the gel was 12 µL. For ladder sample preparation, 8 µL of water, 2 µL of dye and 2 µL of ladder were added in a sterile tube. For control, 5 µL of water, 5 µL of PCR product (water) and 2 µL of the loading dye was added. For our samples, 5 µL of water, 5 µL amplified DNA and 2 µL of loading dye was added. The gel was run for 45 min. This procedure was modified with in the lab.

Purification of PCR products

A purification kit was used to purify the PCR products to identify the strains. The protocol given with in the kit was followed to purify the pcr products. To begin purification, 15 µL of PCR product was added to a sterile eppendorf tube and 60 µL of

B2 solution from the kit was added in the middle of the tube. The tubes were centrifuged at 14.6 rpm for 1 min. After completion of centrifugation, 650 µL of ethanol was added to the tubes and then centrifuged again for 1 min at 14.6 rpm. The solution obtained in the lower compartment of the tube was discarded and re-centrifuged for 3 min at 14.6 rpm. Any solution obtained in the lower compartment was discarded. The tube below the main tube was replaced with a new sterile eppendorf tube. After washing, 50 µL of elution buffer was added and then the tubes were incubated at room temperature for 1 min. After incubation, the tubes were centrifuged for 2 min at 14.6 rpm. The supernatant obtained after centrifugation should contain the purified DNA. Nanodrop was used to quantify the DNA purified.

MEASUREMENT OF UREASE ACTIVITY

Preparation of samples

Bacteria suspension with OD_{600} of 0.1 was used to inoculate urea media of 20 mL volume. The cultures were incubated in rotatory shaker at 37 °C at rpm 150. Urea concentrations varying from 5 to 50 g/L were used to investigate the optimal urea concentration at which the bacteria can have highest urease activity (Bibi *et al*., 2018).

Analysis of results

To analyze the results, samples from each culture were collected in sterile tubes and were centrifuged at 13000 rpm for 10 min. The supernatant was used to measure the urease activity.

Urease activity by Modified Phenol-hypochlorite assay

Measurement of urease activity was done following the procedure by Burbank *et*

al., (2012) with modifications. The supernatant obtained in step 3.6.2 were used to measure the activity by measuring the ammonia produced. 200 µL from the supernatant was added to 1800 µL of urease buffer. The mixture was incubated for 10 min at 30 °C. For control tubes, the solutions were incubated in ice bath for 30 min to prevent any enzyme activity. After addition of sample, the solution was incubated for 10 min at -20 °C. After completion of incubation period for both control and treatment tubes, 200 µL of phenol nitroprusside was added to them followed by 200 µL of fresh sodium hypochlorite solution. The treatment tubes were incubated for 20 min at 30 °C. after incubation, the absorbance was measured at wavelength of 640 nm. Triplicates were done for each sample. The activity of enzyme was inhibited by the cold temperature in the negative control. Any possible activity if measured would be due to the already present ammonia in the supernatant (Bibi *et al*., 2018). The blank for the experiment included all the three components of the solution; urease buffer, phenol nitroprusside and sodium hypochlorite, whereas the volume of supernatant was replaced by urease buffer. Urease activity is defined as the arbitrary urease activity (AUA) which is the quantity of urease responsible for producing 1 µmole per minute of ammonium ions (NH^{+}_{4}) under experimental conditions (incubation at 30 °C for 10 min). A standard curve of ammonia was prepared by serially diluting stock solution (5×10^{-4} M) of ammonium chloride.

Scanning Electron Microscopy and X-Ray Diffraction (SEM-EDS)

SEM-EDS was used to investigate about the composition and structure of the precipitated calcium carbonate. To obtain precipitates for analysis, urea media cultures were inoculated with ureolytic bacteria with an initial OD_{600} of 0.1 and were incubated at

37 °C at rpm 150 for 30 days. For culture preparation, 0.4 g of soil from Abu Hamur was weighed and added to 20 mL urea medium and calcium chloride. The culture was then inoculated with ureolytic bacteria. Cultures without soil were also inoculated with AA3 and incubated. Three control cultures for the experiment were conducted. First culture composition includes; urea medium and calcium chloride, second culture composition includes; 0.4 g soil, urea medium and calcium chloride and the third culture contained; 0.4 g soil and water (Bibi *et al*., 2018). After incubation period, any precipitate obtained were collected with a spatula in centrifuge tubes, washed three times with water by centrifugation. The pellet was oven dried for 72 hrs at 40 °C. Modified urea agar plates streaked by DL2, M5, M3 and DL4 were analyzed for there biomineralizing activity.

XRD analysis

The mineralogical composition of the samples were investigated by XRD analysis. Peaks obtained were analyzed using OriginPro 8 software to find out the mineral form of precipitated calcium carbonate.

APPLICATIONS OF MICP FOR SOIL STABILITY

The process of MICP was applied on lab scale and in field using indigenous bacteria. Following are the experiments done to investigate if the process is applicable in Qatari soil.

Application of MICP in syringe columns

50 mL syringes were filled with sterile soil from Abu-hamur up to 30 mL mark. The syringes were closed with sterile Whatman filter paper from the bottom to avoid the soil passage from the syringe. 3 controls were carried out for the experiment; Urea

medium, cementation solution (0.2 M $CaCl_2$) and urea medium with cementation solution. The treatments were inoculated with *Bacillus cereus* (AA3) grown overnight on LB plate and then suspensions were made in each respective solution and were added to the syringes. 10 mL of each respective solution was added to the syringes followed by suspension of AA3 and then incubated at 35 ^{O}C overnight. For the syringe with both urea medium and cementation solution, first 10 mL of urea medium was added followed by AA3 and then cementation solution directly. After completion of incubation period, the solutions were drained, and the soil was washed by passing distilled water through it. After washing, the addition of solutions was continued and re-incubated. The experiment was ran for two weeks and was stopped on observing any differences between control and treatment syringe soil columns. This procedure for the application of MICP was modified in the lab.

Application of MICP in liquid cultures with soil and quantification of calcium carbonates by Back-titration

To quantify the calcium carbonates, the technique of back-titration was modified and experimented in the lab. The soil sample from Abu Hamur were spread out, and dried at 105 ^{O}C temperature overnight. Organic matter from the soil sample were removed manually and the soil was grounded to pass through a 2 mm sieve. The soil is then sterilized at high temperature of 150 ^{O}C for 2-3 hrs. Following sterilization, cultures of 20 mL with 0.4 g of soil were prepared. Ureolytic *B. cereus* (AA2) was grown overnight on LB plate to obtain pure culture. Bacterial suspension was made with OD_{600} of 0.1. Control for the experiment included; dry soil, soil with water, soil with cementation

solution, soil with urea medium, soil with urea medium and cementation solution. Other control culture was performed at the same conditions but without using soil. For precipitation, the cultures were inoculated with AA2. The cultures were incubated at 35 ^{O}C in rotatory shaker at 170 rpm for 1 week. Back-titration was carried out in triplicates for each day to quantify the calcium carbonate precipitates. Since, carbonate in the soil is quantified by dissolving it in the acid. After dissolution, the volumetric analysis of carbon dioxide produced is done. Generally, HCl drops are added to the mineral or rock and the change on the surface is measured. If bubbles were seen, the presence of carbonate was indicated. However, this technique does not quantify calcium carbonate.

The following chemical reaction occurs when carbonates are measured (Blinkova & Eliseev, 2005):

$$CaCO_3 + 2H^+ \rightarrow Ca^{2+} + CO_2\uparrow + H_2O$$

The release of carbon dioxide occurs in the form of bubbles; weak or strong depending on the size and quantity of the soil under examination. The calcareous soils are rich in calcium carbonate and the pH ranges from 7.0 to 8.3.

The quantification of calcium carbonates precipitated biologically in the cultures was achieved by back-titration. To dissolve any precipitate in the liquid cultures, 0.33 M HCl was used. The culture after addition of HCl was heated for 10 min to dissolve calcium carbonate in the sample. The sample was then cooled in an ice bath for 10 min and then brought to room temperature by incubating for 10 min at room temperature. After completion of incubation period, 3 drops of phenolphthalein indicator was added to indicate a change in pH from acidic to basic conditions. Standardized NaOH (0.16 M)

was used to titrate the sample until a permanent faint pink color was observed (pH ~ 8.6). The buret readings were noted down. Based on a calibration curve between NaOH used and concentration of calcium carbonate, the concentration of calcium carbonates with in the cultures was calculated.

Acid Treatment of Soil

This process was done to achieve soil with the minimum amount of minerals to better quantify the precipitates formed by the treatment. 2 L container was used and soil from Abu-hamor was filled up to 500 mL mark in it. Approximately 900 mL of acetic acid was added to it and allowed to shake constantly overnight. After overnight shaking, the shaking was stopped to allow the solution to settle. The process was repeated and the next day all the acid was replaced with distilled water. The soil was washed with distilled water 3-4 times to ensure removal of any remaining acid. The obtained soil was oven-dried at 105 ^{O}C. The process was modified within the lab.

RESPONSE OF BIOLOGICALLY MODIFIED SOIL SURFACES ON LAB-SCALE

200 g of acid-treated soil from Abu-Hamor was taken in 12 x 8 cm tray. Holes for drainage were made in the bottom. Figure 2 shows the set up for the experiment. For controls; soil with distilled water, and soil with urea medium and cementation solution were prepared. For treatment, ureolytic bacterium AA2 was added. 50 mL of each respective solution was sprayed on each tray in intervals of 1.5 hrs thrice. After incubation period completed, the soil was dried at 105 ^{O}C and then further analysis was completed using atomic absorption spectrometer.

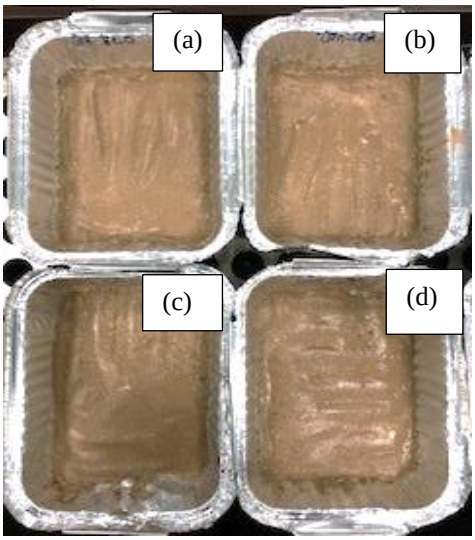

Figure 2. Application of solutions for bio-modification of soil, a) Soil only b) Soil + Water c) Soil + Urea Medium + Cement Solution d) AA2 + Soil + Urea Medium + Cement Solution

INVESTIGATION OF FIELD RESPONSE OF BIO-MODIFIED SURFACE SOILS SUBJECTED TO REAL WORLD, LONG-TERM WIND CONDITIONS AND TREATMENT

Based on the analysis of results obtained from the modified experiments carried out in the lab, experiment in field was carried out for soil stability using microbially induced calcite precipitation (MICP):

Study site: Qatar University Farm is located in Al Khor, Qatar

(25°48'42.1"N 51°20'43.2"E).

Many types of soils may be observed in the Farm. The most representative area is composed of calcareous sandy clay, with abundance of rocks at high size distribution. This area is managed for agricultural activity under Qatar University authority.

Site preparation: A representative area was selected, and rocks were removed from the 10-20 cm upper layer to provide a sandy surface, appropriate for accurate and reproducible results.

Sampling: All soil samples were collected from the 1-2 cm surface under the corresponding experimental conditions. Soils were air-dried at 105 ^{O}C, ground to pass a 2 mm sieve and analyzed (three replicates from each sample were used).

EXPERIMENTAL SET-UP

Bacteria cultivation

B. cereus (AA2) was grown in urea media for 48 hours at 35 ^{O}C until an optical density at wavelength of 600 nm of 0.8-1.2 was achieved.

Field work

Soil surface was divided into 8 quadrants for control (water, cementation solution (CS), urea media (UM), and (CS+UM) and treatment (water + AA2, CS + AA2, UM + bacteria, CS + UM + AA2). Each quadrant was treated with 400 mL of the respective solution and then incubated for 1.5 hrs. This was done 3 times daily for 3 days. By the end of each day, upper layer (1-2 cm) and lower layer (10 cm below surface) of soil were collected from each treatment. Figure 3 shows the steps a till d.

a) Four quadrants were set on the

b) Quadrant perimeter was drawn

c) Quadrants were used again for the next set of treatment

d) Stones and grass were removed to make a clear surface

Figure 3 (a-d). Summary of field work

MEASUREMENT OF $CACO_3$ BY ANALYZING CA^{2+} CALCIUM IONS IN THE SAMPLES USING ATOMIC ABSORPTION SPECTROMETER (AAS)

The quantification of calcium carbonates was done by back-titration and by using

AAS. The calcium carbonate percentage is estimated by measuring the Ca^{2+} ions in the sample using AAS model AA-6800. A calibration curve ranging from 0 to 350 ppm of Ca^{2+} ions was established for measuring the Ca^{2+} ions concentration. The sample preparation for analysis in atomic absorption spectrum was done by weighing 15 g of each soil sample in a glass tube. 15 mL of 5 M HCl was added to the glass tube and mixed to dissolve any precipitated carbonates. After dissolution, 0.5 mL of extract was diluted 500 times and then measured by the instrument.

The following formula was used to measure the calcium carbonate content in the samples;

$CaCO_3$ content (%) = $[W_{CaCO3} / W_{tube + sand} - W_{tube} - W_{CaCO3}] * 100\%$

Where $W_{CaCO3} = C_{ca} * 500/40$ g/mol * 100 g/mol * 15 mL

W_{CaCO3} = Weight of $CaCO_3$ in sample

$W_{tube + sand}$ = Weight of tube and sand

W_{tube} = Weight of tube

C_{ca} = Concentration of Ca^{2+} ions in diluted sample of extract.

CHAPTER 4: RESULTS AND DISCUSSION

ISOLATION OF UREOLYTIC BACTERIA FROM SOIL SAMPLES

The purpose of this study was to isolate local, indigenous ureolytic bacteria from Qatari soil and to estimate their growth and ureolytic activity. In Qatar, the sampling was performed from six locations. Isolation of bacteria was performed by enrichment cultures (Section 3). The ureolytic bacteria were grown in urea media and the activity was estimated by plating on modified urea agar. Table 1 shows the isolated bacteria from different locations and the ureolytic ones. Out of the 30 isolates, 18 were shown positive for ureolytic activity at the experimental conditions.

Table 1. *Number of total isolates from six locations in Qatar and number of isolates tested positive for Ureolytic activity.*

Location	**Airport Area (AA)**	**Duhail (DL)**	**Al-Wakrah (AW)**	**Mesaieed (M)**	**Dukhan (DN)**	**Abu Hamur (AH)**
Total Isolates	6	8	4	7	4	1
Positive for Ureolytic Activity	4	6	2	5	1	0

Figure 4 shows the plates of modified urea agar inoculated with bacteria to screen them for ureolytic activity. Fig. 4 (a, b, c) shows the change of color from yellow to pink in the plates indicating the ureolytic activity of isolated bacteria. Fig. 4 (d) shows partial

color change which means that the isolate is hydrolyzing urea at a slow rate, hence, the pH increase is slower and the color change from yellow to pink is not complete. Fig. 4 (e, f) shows no color change indicating that no ammonia was accumulated in the plate due to which the pH did not increase. Similar screening technique or method was used by Chahal *et al*., (2011), Burbank *et al*., (2012) and Phang *et al*., (2018) to screen the bacteria with ureolytic activity. The authors also reported that, certain bacteria can test false positive due to the presence of chemicals such as peptone, contaminating the commercial medium which might undergo hydrolysis and produce acid residues. Urea agar is also used in slants to test the bacterial urease activity as reported by Hammad *et al*. (2013).

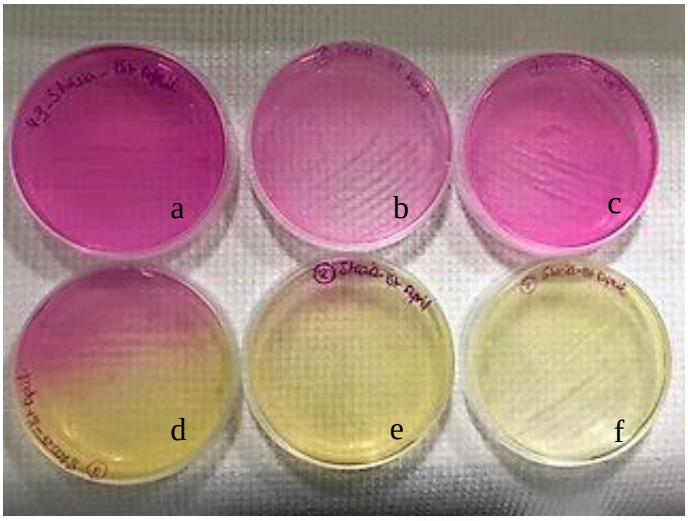

Figure 4. Ureolytic activity test on Modified Urea Agar

IDENTIFICATION OF THE ISOLATED UREOLYTIC BACTERIA BY MALDI-TOF-MS

MALDI TOF-MS was used to identify and differentiate bacterial isolates to investigate the occurrence and diversity of bacteria with urease activity in Qatari soils. The comparison of the protein peaks obtained during identification were matched with the data base. The score ranges between 0 to 3, according to which the isolates were identified. The comparisons were done based on the instructions and information provided by the manufacturers. The identifications were divided into three categories based on similarity scores; highly identical at species level (2.3-3), identical at genus and probable species level (2-2.299) and genus level identification (1.7-1.999) (Bibi *et al*., 2018). The Bruker Daltonics manufacturers provided standard bacterial tests (Item #255343) for calibration. Table 2 summarizes the liability score and the identified bacteria by MALDI-TOF-MS.

Table 2. *Isolate name, liability score and Identified bacteria*

Isolate Name	Liability Score	Identified Bacteria
AA1	1.7	*Bacillus subtilis*
AA2	2.27	*Bacillus cereus*
AA3	2.2	*Bacillus cereus*
AA4	2.17	*Bacillus cereus*
DL1	2.01	*Bacillus cereus*
DL2	2	*Bacillus licheniformis*
DL3	2.15	*Bacillus cereus*
DL4	2.21	*Bacillus cereus*
DL5	2.10	*Bacillus cereus*
DL6	2.07	*Bacillus cereus*
AW1	2.10	*Bacillus cereus*
AW2	2.19	*Bacillus cereus*
M1	1.88	*Bacillus licheniformis*
M2	2.10	*Bacillus cereus*
M3	2.19	*Bacillus cereus*
M4	2.18	*Bacillus cereus*
M5	2.14	*Bacillus cereus*
DN1	2.16	*Bacillus cereus*

As shown in Table 2, the most identified bacteria among the isolates from different regions of Qatar is *B. cereus. B. subtilis* and *B. licheniformis* were also among the isolated ureolytic bacteria. *B. subtilis* identified with a score of 1.7 on the identification reliability scoring should be *Bacillus* at the genus level identification only, but it was also considered as *subtilis* as species in the data base. On the other hand, *B. licheniformis* (codes; DL2 and M1) with scores higher than 2 and 1.88 respectively were also identified at the species and genus levels. All the other identified species were scored above 2 indicating probable species level identification.

DIFFERENTIATION OF THE ISOLATED UREOLYTIC BACTERIA BY COMPARING PROTEIN PROFILES USING MALDI-TOF-MS

Most of the isolated bacteria were identified as *B.* cereus, thereby, it is important to differentiate them from each other to know whether they are the same bacterial isolates. The differentiation of bacteria was performed by comparison of protein profiling and peak intensities using MALDI TOF-MS. Signals of ribosomal proteins ranging from 2000 to 20, 000 Da size were obtained and were used to draw protein profiles that could be found in all the three levels; genus, species and sub-species level. Species with similar peaks were categorized together in 1 group. Total of 5 groups were formed. Four groups (A, B, C, D) contain species with similar protein profiles while the last group (E) contains species that belongs to none of the group and are different from all the four categorized groups. Figure. 5 shows the protein profiles of AA2, AA4 and DL1 in group A.

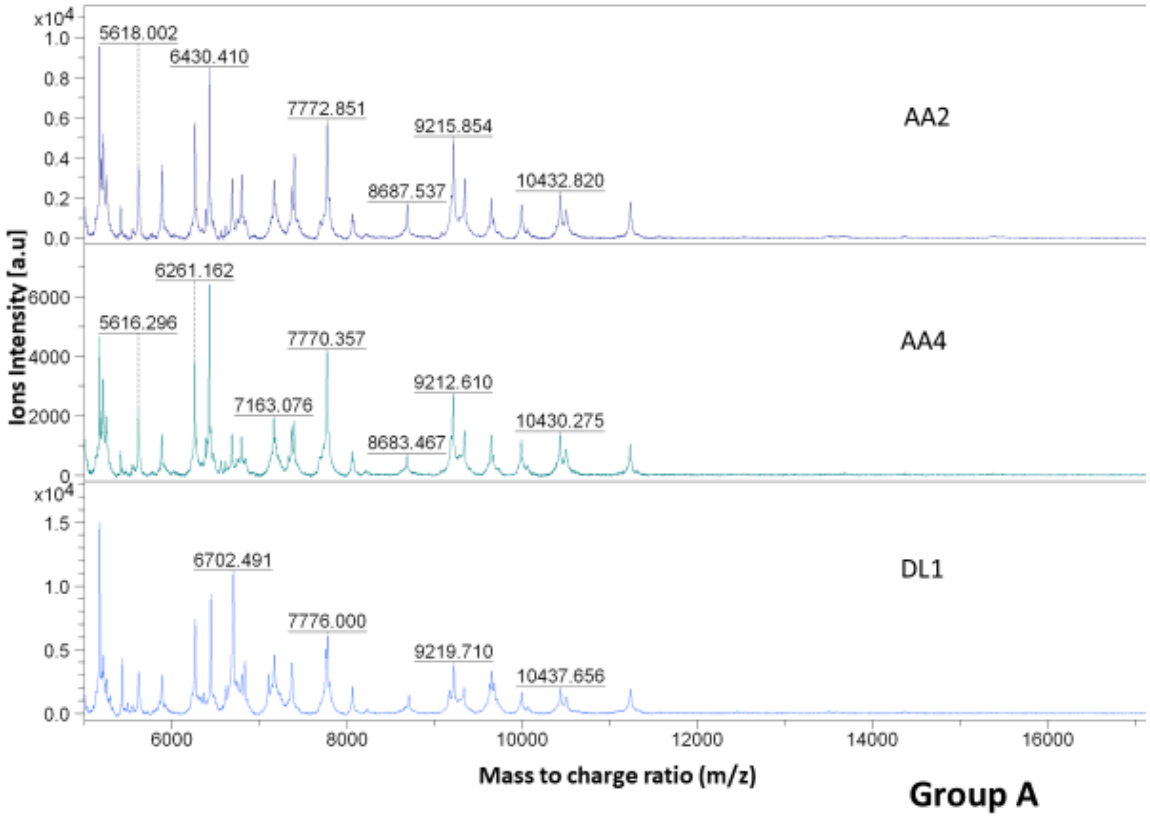

Figure 5. Grouping of Isolates AA2, AA4 and DL1 in Group A.

It can be clearly observed that the isolates AA2, AA4 and DL1 identified as *B. cereus* are highly similar and belong to the same Group A exhibiting highly similar proteins profiles. The most important peaks are common and positioned at m/z ratio of 5616.296, 6261.162, 7770.357, 9212.610 and 10430.275. AA2 and AA4 were isolated from the Airport sample, therefore, they can be the same bacterial strain. DL1 was from

Duhail area. Furthermore, Figure 6 shows the protein profiles of M2, AW1 and M3 respectively.

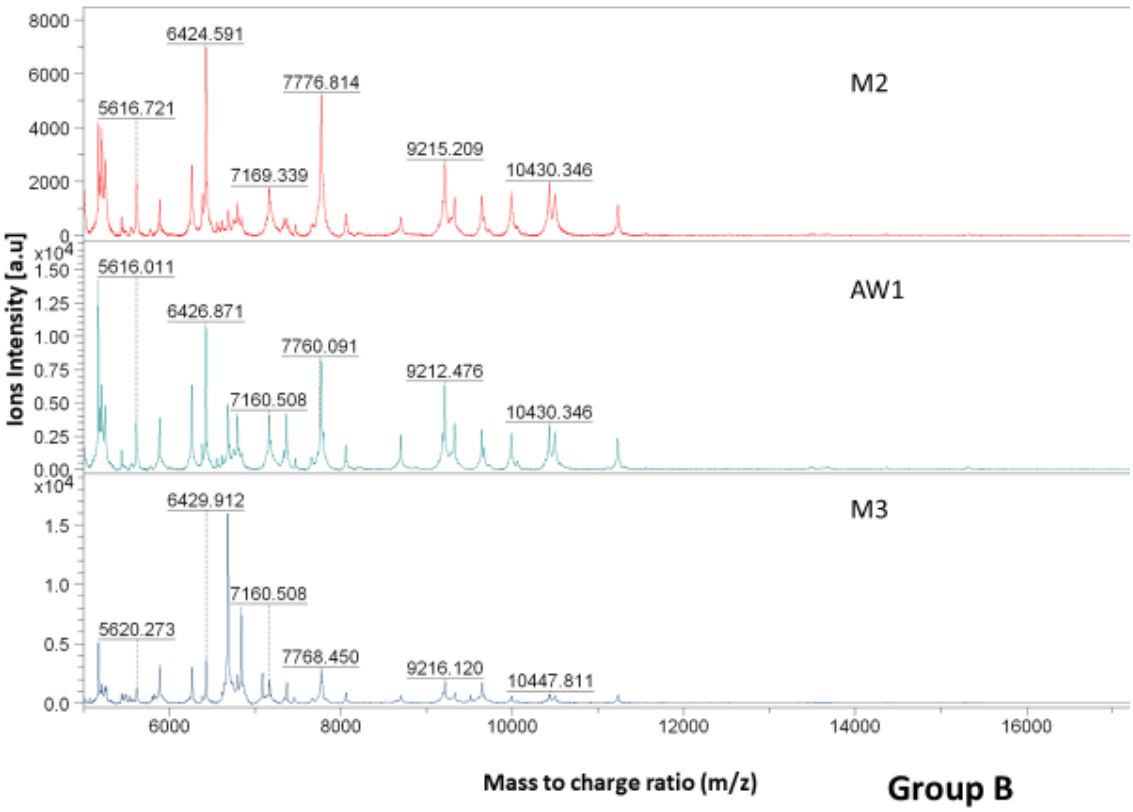

Figure 6. Grouping of Isolates AW1, M2, and M3 in Group B.

In group B, species; M2, AW1 and M3 share similar protein profiles based on their corresponding MS peaks. The common peaks at m/z ratio includes; 5616.011, 6426.871,

7160.508, 7760.091, 9212.476 and 10430.346. M2 and M3 were isolated from Mesaieed soil samples; they can be the same bacterial strain. AW1 was isolated from Al-Wakrah area. Figure 7 shows the protein profiles of AA3, DL3, DL4 and DL5.

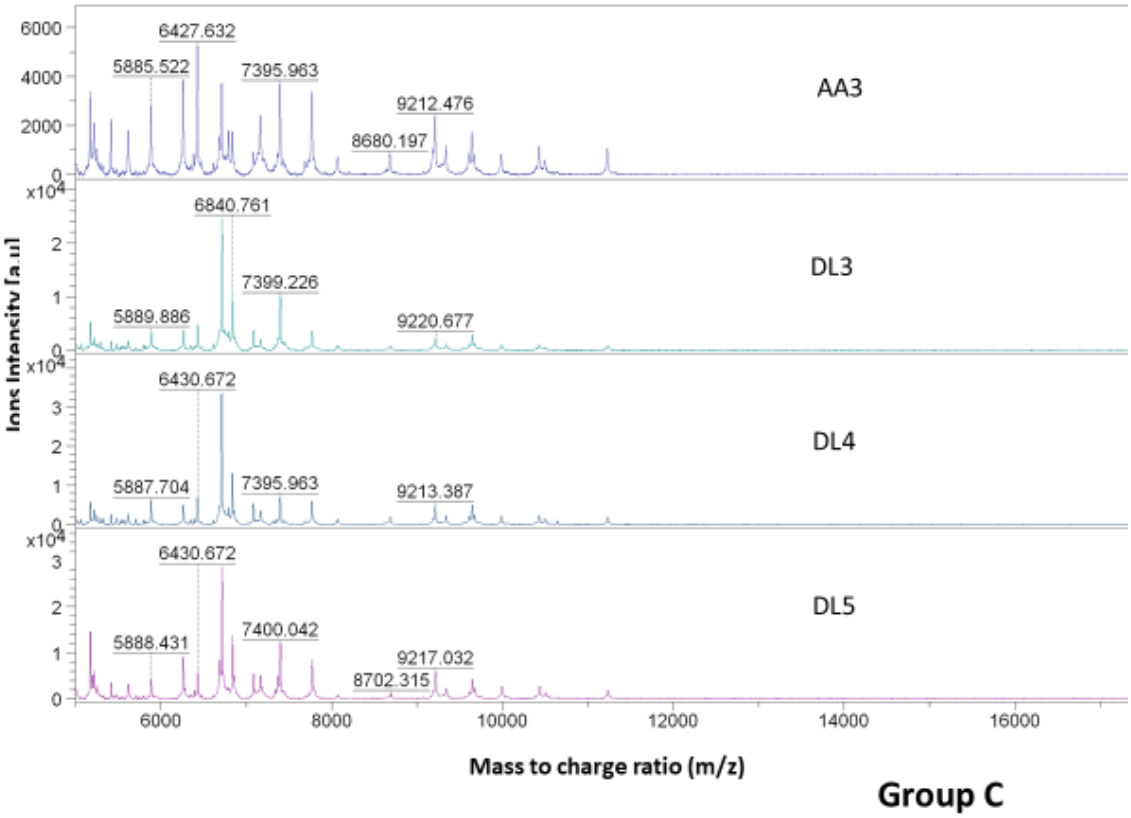

Figure 7. Grouping of Isolates AA3, DL3, DL4, and DL5 in group C.

In Group C; species AA3, DL3, DL4 and DL5 share similar protein peaks at m/z of 5889.886, 6430.672, 6840.761, 73699.226, 8702.315 and 9220.677. AA3 was originated from the airport area, while DL3, DL4 and DL5, which may be the same bacterial isolate, were isolated from Duhail soil sample. Group D has protein profiles of AW2, M4 and DN1 shown in Figure 8.

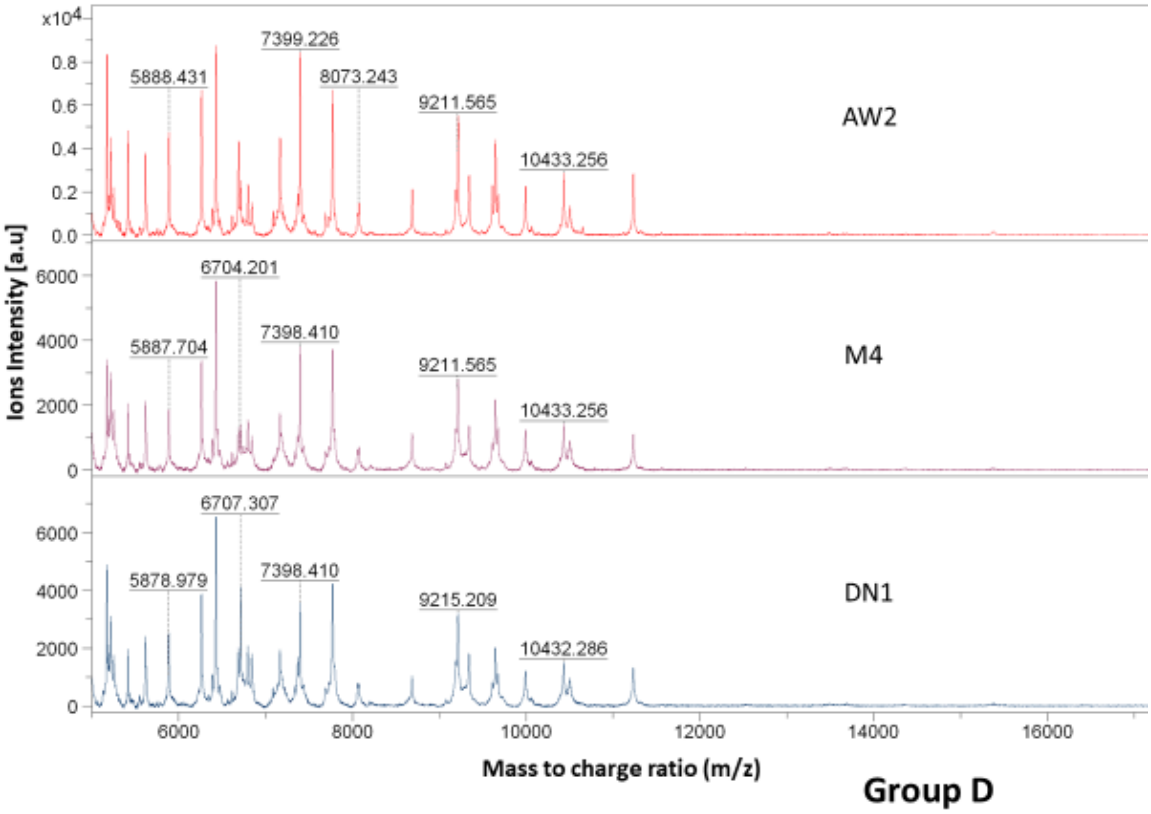

Figure 8. Grouping of AW2, M4, and DN1 in group D.

Species AW2, M4 and DN1 were grouped in category D with similarity in peaks at m/z of 5887.704, 6704.201, 7398.410, 9211.565 and 10433.256. Each of these group members were isolated from a different area.

The grouping of species among the categories is obvious based on the peaks. But, the last category E (Figure 9) comprises of the isolates AA1, M5, M1, DL2, and DL6 that belonged to none of the previous groups. Any similarity may be found in their protein peaks. AA1 was isolated from the airport area, DL2 and DL6 from Duhail, and M1 and M5 from Mesaieed. Relying on protein peaks and differentiation, it was possible to correlate the occurrence, and distribution of ureolytic bacteria in the selected sites in Qatar. The isolated ureolytic bacteria from Airport Area (AA) and Duhail (DL) are distributed in group A, C and E. Isolates from Al-Wakrah are distributed in group B and group D. Isolates from Mesaieed (M) are distributed in group B, D and E. Isolate of Dukhan (DN) was grouped in category D based on the similarities in the protein profiles.

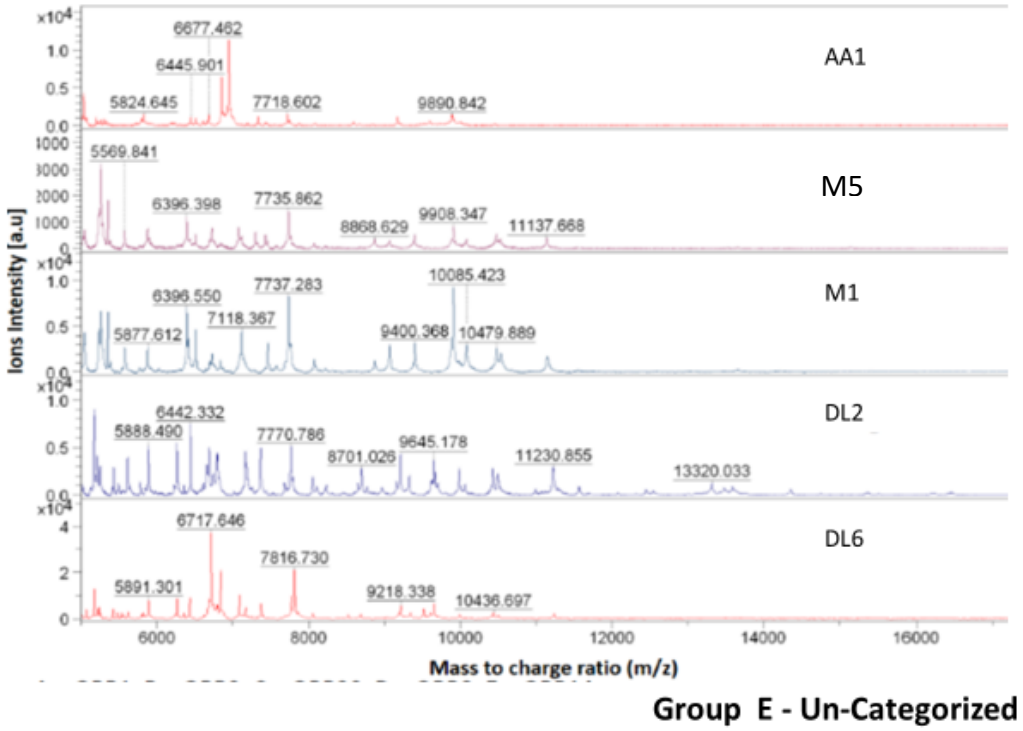

Figure 9. Grouping of AA1, M5, DL2, M1 and DL6 in Group E.

MALDI-TOF MS has been used in researches to differentiate among species that are closely related. *Acidocorax oryzae* and *Acidovoran citrulli* are two species differentiated based on their specific protein peaks (Wang *et al*., 2012). Furthermore, the sub-species level identification is not fully studied so far (Sandrin, Goldstein & Schumaker, 2012). Using MALDI-TOF MS, bacteria like *Yersinia enterocolitica* (Rizzardi, Wahab & Jernberg, 2013), *Staphylococcus aureus* (Wolters *et al*., 2011), *Staphylococcus enterica* (Dieckmann & Malorny, 2011) *and Streptococcus agalactiae* (Lartigue *et al*., 2009) were successfully differentiated.

IDENTIFICATION OF ISOLATES BY MOLECULAR 16S RRNA TECHNIQUE

The identification of six isolates; AA2, AA3, AW1, DN1, DL3 and DL4 were done by molecular technique to confirm the identification done through MALDI-TOF MS. Table 3 shows the code, identity, accession number and similarity percentages of the isolates. Based on the results obtained, there was no inconsistency among the results of MALDI-TOF MS and 16s rRNA technique.

Table 3. *Identified bacteria with codes and GenBank number.*

Code	Identified Bacteria	Accession Number	Similarity Percentage	GenBank number
AA2	*B. cereus*	KP743133.1	99 %	MG745362
AA3	*B. cereus*	KB743133.1	100 %	MG745365
DL3	*B. cereus*	KY316447.1	96 %	MG745366
DL4	*B. cereus*	CP011151.1	99 %	MG745367
AW1	*B. cereus*	LC260003.1	100 %	MG745332
DN1	*B. cereus*	CP011151.1	96 %	MG751339

OPTIMIZATION OF CONCENTRATION OF UREA FOR BACTERIAL GROWTH AND KINETICS OF UREASE ACTIVITY PRODUCTION

To evaluate the activity and growth of each of the ureolytic bacterial strain, they were grown at different urea concentrations and the cells count and their urease activity was determined daily. All the isolates showed a similar growth pattern; highest urease activity at a urea concentration of 20 g/L on day 3. After 3 days of incubation, the cell count showed a decline for each isolate despite the concentration of urea provided (Fig 10 a).

The pH of the cultures was also measured to investigate the hydrolysis of urea which ultimately should increase the pH. The pH started to increase from day 2 indicating urease activity of the cells. On day 3, the pH of the cultures reached 8.5 and then remained constant (Fig 10 b). The growth inhibition after 3 days could be because of two reasons; an increased pH or catabolic repression. The alkaline conditions in the cultures might have inhibited the growth of the cells due to which no further hydrolysis of urea has occurred. On the other hand, the inhibition might have occurred due to catabolic repression by excess availability of substrate urea (Bibi *et al*., 2018).

Based on the kinetics of growth and ureolytic activity of the isolates, 20 g/L was selected as an appropriate concentration of urea for further experimental investigations. It is also reported from literature that urea concentration of 20 g/L was appropriate for evaluation of urease activity and biomineralization applications (Burbank *et al*., 2012; Hammad *et al*., 2013; Wei *et al*., 2015; Lin *et al*., 2016).

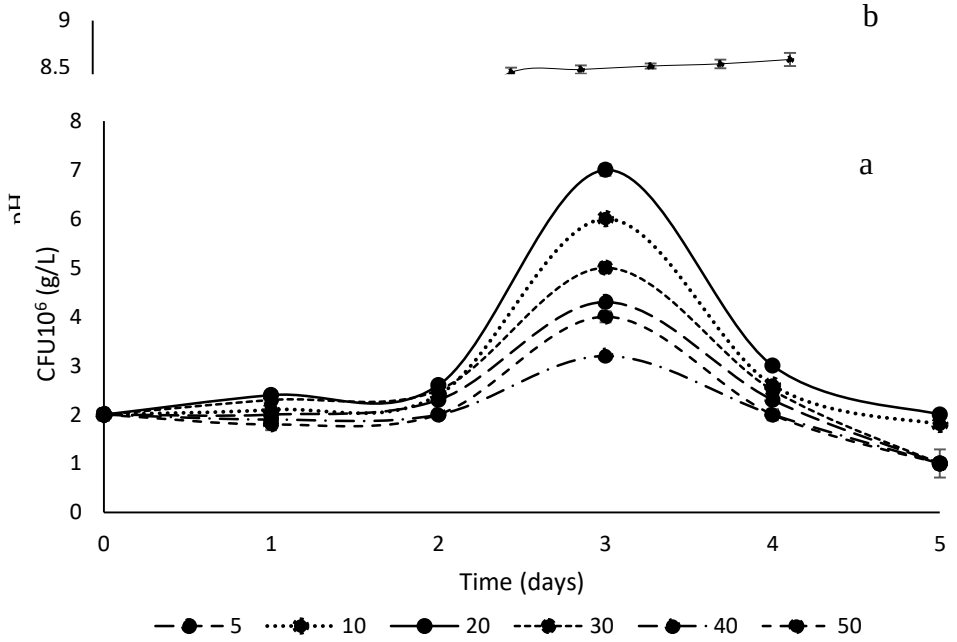

Figure 10. Urea concentration optimization ([5 g/L ( ), 10 g/L ( ), 20 g/L ( ),30 g/L ( ), 40 g/L ( ), 50 g/L ( )]; a) CFU b) pH of cultures

MEASUREMENT OF UREASE ACTIVITY OF THE ISOLATES

The categorization of ureolytic bacteria in to groups based on the protein profiles helped in knowing the occurrence and distribution of these species in Qatar. The ureolytic activity of the isolates can differs because of the genes content, responsible for the expression of urease enzyme. The isolate with higher urease activity is an indicator for higher gene expression as compared to isolates with lower urease activity. The gene expression is affected by different factors including the metabolic pathways or regulations within the cells (Hammes *et al*., 2003). Table 4 shows the location of the

isolates, code, identity, colony forming unit (cfu/mL), arbitrary urease activity (AUA/mL), specific production (AUA/cfu 10^7) and the 95% confidence interval of 15 isolates out of 18.

Table 4. *Arbitrary Urease Activity (AUA/mL) and Specific Production (AUA/cfu) of Isolates from different Locations across Qatar*

Location	Code	Identity	Colony Forming Unit (cfu/mL)	Arbitrary Urease Activity (AUA/mL)	Specific Production (AUA/cfu 10^7)	Confidence Interval 95%
Airport Area	AA1	*B. subtilis*	20 ±1	3.05 ± 0.020	1.523	0.135 ± 0.012
	AA2	*B. cereus*	27 ±1	14.14 ± 0.128	5.238	0.630 ± 0.114
	AA3	*B. cereus*	29 ± 9	14.77 ± 0.126	5.094	0.658 ± 0.204
	AA4	*B. cereus*	34 ±1	8.49 ± 0.086	2.498	0.378 ± 0.125
Duhail	DL1	*B. cereus*	30 ±1	7.55 ± 0.085	2.517	0.336 ± 0.053
	DL2	*B. cereus*	26 ± 5	10.92 ± 0.106	4.198	0.486 ± 0.078
	DL3	*B. cereus*	35 ±1	11.52 ± 0.110	3.291	0.513 ± 0.172
	DL4	*B. licheniformis*	26 ± 2	5.74 ± 0.056	2.206	0.255 ± 0.044
	DL5	*B. cereus*	37 ± 9	8.05 ± 0.086	2.174	0.358 ± 0.156
Al-Wakrah	AW1	*B. cereus*	35 ± 6	8.13 ± 0.093	2.324	0.362 ± 0.009
Mesaieed	M1	*B. licheniformis*	45 ± 3	5.44 ± 0.053	1.210	0.242 ± 0.038
	M2	*B. cereus*	44 ± 3	4.75 ± 0.044	1.079	0.211 ± 0.037
	M3	*B. cereus*	57 ± 3	7.48 ± 0.084	1.313	0.333 ± 0.029
	M4	*B. cereus*	27 ± 5	9.73 ± 0.104	3.602	0.433 ± 0.132
Dukhan	DN1	*B. cereus*	27 ± 6	22.75 ± 0.148	5.056	1.014 ± 0.667

Measurement of arbitrary urease activity and cell count corresponding to the growth of the bacterial isolate by hydrolyzing urea is an efficient way to investigate the ureolytic activity of the bacteria. The highest arbitrary urease activity was measured for DN1 (22.75 ± 0.148) with cfu of 27 ± 6. On the other hand, M3 with higher cell count of

57 ± 3 was measured to have arbitrary urease activity of 7.48 ± 0.084 which is very low compared to the growth of cells. Similarly, for isolate M1, the measured arbitrary urease activity was 5.44 ± 0.053 with cell count of 45 ± 3. It can be said that the specific production per colony forming unit would be an appropriate indicator for the cells' urease activity. Consequently, AA2, AA3 and DN1 have higher specific production of 5.238, 5.094 and 5.056 AUA/10^7 cfu respectively. Following the trend, DL2, DL3 and M4 also have high specific production of 4.198, 3.291 and 3.602 AUA/10^7 cfu respectively. The highest producer among the isolates is AA2 regardless of the low cell count. However, the lowest producer is M2 with relatively higher cell count of 44 ± 3.

Isolates AW2, DL6 and M5 were not efficient growers on both liquid and solid urea media therefore, there activity was not determined and compared with other isolates. The measurement of arbitrary urease activity through determination of specific production per cell count is a new technique used in this research. The ANOVA analysis for the measured urease activity showed no significant difference among the isolates, however, through the specific production (AUA/10^7 cfu) of the isolates, they could be grouped as over-producers or low producers of urease enzyme. There is a possibility that the isolates need more growth time to hydrolyze substrate; urea, due to variation in the conditions (Al-Disi *et al*., 2017).

INVESTIGATION OF THE ROLE OF UREOLYTIC BACTERIA IN MICP USING SEM/EDS AND XRD ANALYSIS

SEM-EDS analysis

Crystals obtained from each isolate on modified urea agar differed in the

morphology as indicated by orange arrows (Fig 11). The most common shape found in the plates produced by DL2, AW1, M3, and DL4 is spherical calcium carbonate as seen in Fig 10. The imprints of bacteria can be clearly seen in Fig 11 (b, c). No crystallization and color change were observed in plates not streaked with bacteria (Not shown).

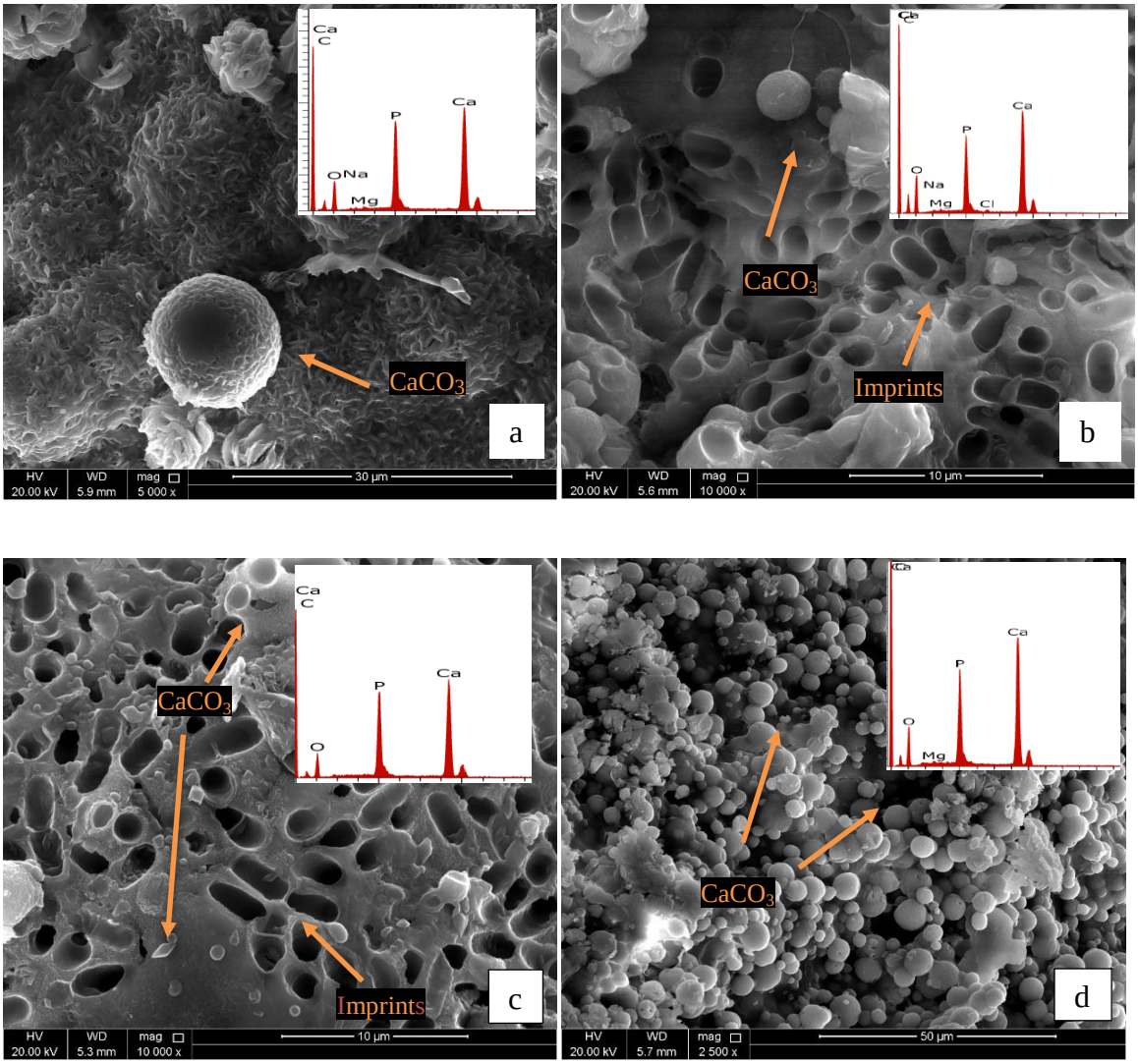

Figure 11. SEM-EDS of crystals obtained from Modified Urea Agar with 0.03M $CaCl_2$, a) DL2, b) AW1, c) M3, d) DL4

Similar crystal morphologies of calcium carbonate have been reported by Versteegen (2011) and Al-Thawadi (2012). The identified isolate *B. licheniformis* (DL2) is reported to be a biomineralizing agent on different media (Chahal *et al*., 2013; Helmi *et al*., 2016). Since, the crystallization of calcium carbonate was found in plates with bacterial isolates (DL2, AW1, M3, DL4), liquid cultures were done with two concentrations of calcium chloride (0.03 M and 0.2 M) at 35 ^{O}C.

Ureolytic bacteria with specific production higher than 2 were analyzed for biomineralization and were able to precipitate calcium carbonate. The precipitate obtained from cultures of AA3 done with urea medium, soil and 0.03M $CaCl_2$ on SEM/EDS analysis concluded the presence of calcium carbonate in both amorphous and crystalline calcite forms (Fig 12).

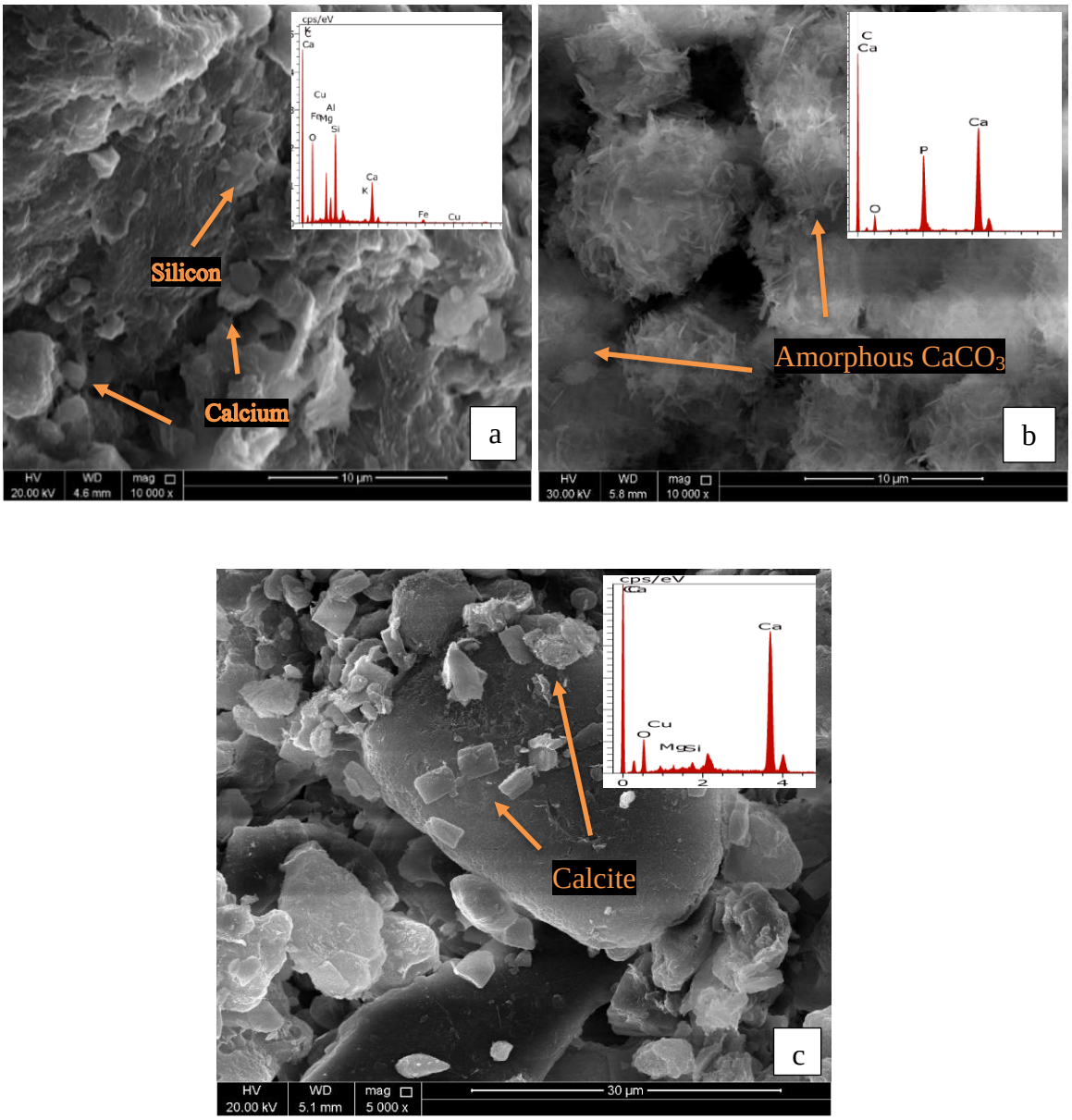

Figure 12. SEM Analysis of cultures with a) Soil + Urea Media + 0.03 M $CaCl_2$ – No AA3 b) Urea Media + 0.03 M $CaCl_2$ + AA3 c) Soil + Urea Media + 0.03 M $CaCl_2$ + AA3

All the strains were cultured to perform biomineralization, however, the incubation time differed from 30 days to 60 days. The pH was checked in intervals until it reached a pH of 8.5. EDS analysis for precipitates obtained from cultures of AA3 (5.094 AUA/10^7 cfu) is mentioned in table 5. Negative controls for the experiment were performed where no abiotic precipitation or mineralization was seen without the presence of soil in the culture. Isolate AA3 was able to induce the precipitation of amorphous $CaCO_3$ (Fig. 11 b) with equal atomic % (16.8 at%) of carbon and calcium while a much higher percentage of oxygen (65.3 at%) was reported. Oxygen is observed to be higher than it should be (50.04 at%) as it should be present in the precipitates 3 times higher than carbon and calcium. In the EDS analysis, phosphorus was also present with at% of 1.8 respectively. The presence of phosphorus is due to the extracellular polymeric substances that are also precipitated along with $CaCO_3$. EPS could be one of the reason that oxygen in the analysis is more than it should be. Fig. 12 (a) shows the SEM image obtained for abiotic culture (not inoculated with AA3) where no precipitation of calcium carbonate is observed, whereas, Fig 12 (b,c) shows the SEM images for cultures with urea media, calcium chloride and inoculum of AA3. The presence of components such as calcium, oxygen, carbon, phosphorus and silicon is normal in all cultures; biotic or abiotic, however, the clear crystal form of calcium carbonate; calcite is only evident in the culture which was inoculated with AA3 (Fig 12 (c)). The EDS analysis of the precipitate shows the at% of calcium, carbon, oxygen and phosphorus to be 23.9%, 24%, 60.5% and 1.1% respectively. Within the same culture, amorphous calcium carbonate was seen precipitated as indicated by the arrow. The EDS analysis shows the presence of carbon, calcium,

oxygen and phosphorus in the region with atomic percentages of 13.9%, 13.4%, 60.8% and 2.6% respectively.

Table 5. *EDS analysis; atomic percentages of Carbon (C), Oxygen (O), Calcium (Ca), and Phosphorus (P) of cultures with urea media, calcium chloride (0.03M & 0.2M) and soil(with/without) and pH.*

Medium	**Isolate AA3**	**Precipitate type**	**Composition in At %**				**pH**
			C	**O**	**Ca**	**P**	
Urea Medium + $CaCl_2$ (No Soil)	No	Not Any	NA	NA	NA	NA	7
Urea Medium + $CaCl_2$ (No Soil)	Yes	Amorphous $CaCO_3$	16.80	65.30	16.80	1.80	8.50
Water (With Soil)	No	No Precipitate	7.80	57.10	2.50	0.60	7.00
Urea Medium + 0.03 M $CaCl_2$ (With Soil)	No	No Precipitate	7.80	57.10	2.50	0.60	7.00
Urea Medium + 0.03 M $CaCl_2$ (With Soil)	Yes	Calcite Amorphous $CaCO_3$	24.00 13.90	60.50 60.80	23.90 13.40	1.10 2.60	8.80 8.80
Urea Medium + 0.2 M $CaCl_2$ (With Soil)	No	No Precipitate	18.07	48.40	6.68	3.76	7.00
Urea Medium + 0.2 M $CaCl_2$ (With Soil)	Yes	Spherical $CaCO_3$	14.46	62.76	13.66	6.65	8.80

With higher calcium chloride concentration (0.2 M), the isolate AA3 was able to induce the precipitation of spherical calcium carbonates as shown in Figure 13 (b). Not only the morphology but also the amount of precipitates was higher when compared to the concentration of 0.03 M $CaCl_2$ keeping urea concentration constant (20 g/L). No

precipitates were observed in abiotic cultures. The EDS analysis of the cultures with 0.2 M $CaCl_2$ shows C, O, Ca and P to be of 14.46 %, 62.76 %, 13.66 % and 6.65 % respectively (Table 5).

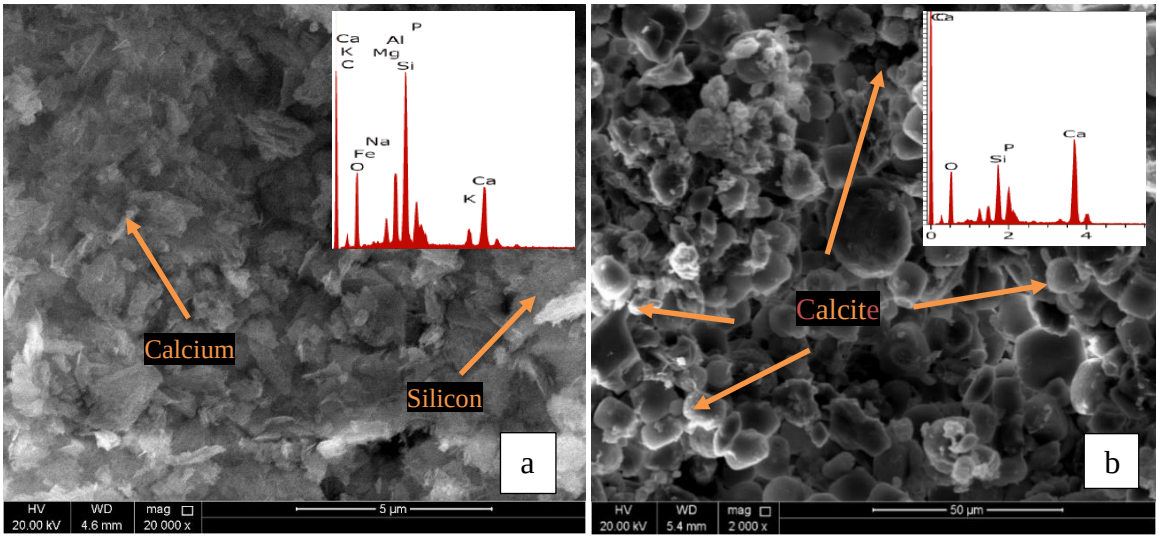

Figure 13. SEM Analysis of cultures with a) Soil + Urea Media + 0.2 M $CaCl_2$ – No AA3 b) Soil + Urea Media + 0.2 M $CaCl_2$ + AA3

XRD analysis of precipitates

Analysis by X-ray diffraction was carried out for the precipitates obtained from the cultures after incubation of 60 days. XRD analysis provides information about the mineral phase in which it exists (Pandian, 2014). Fig. 14 shows the XRD analysis of 7 samples; Control (No Inoculum), soil sample and samples obtained from cultures inoculated with AA2, AA3, AW1, DN1, DL3 and DL4 respectively. In control sample, a

very small peak for aragonite is seen at 26.7 θ, similarly a very small peak for calcite at 29.52 θ is present as well, however, the inoculated cultures with separate isolates showed distinct and high intensity peaks for both aragonite and calcite as can be seen in Fig. 14. This means that the bacterial isolates were able to induce the precipitation of calcium carbonate in two mineral phases: aragonite which has orthorhombic shape while calcite which has rhombohedral shape respectively.

The presence of calcite at 29.52 θ is confirmed by Burbank *et al* (2011). Wei et al. (2015) also reported the dominant form of calcium carbonate induced by *Bacillus sp.* to be calcite. Other researches conducted on micp also showed similar results for XRD analysis indicating the main form of precipitate to be calcite with high peak intensity at 29.5 θ (Li *et al*., 2010; Amidi & Wang, 2015; Balam, Mostofinejad, & Eftekhar, 2017). Not only calcite but aragonite form of calcium carbonate is also reported to be an outcome of biologically assisted mineralization. Liu and his colleagues (2016) mentioned the precipitation of calcium carbonate in its polymorph status; aragonite. This means that aragonite is an expected form of calcium carbonate polymorph precipitated in MICP.

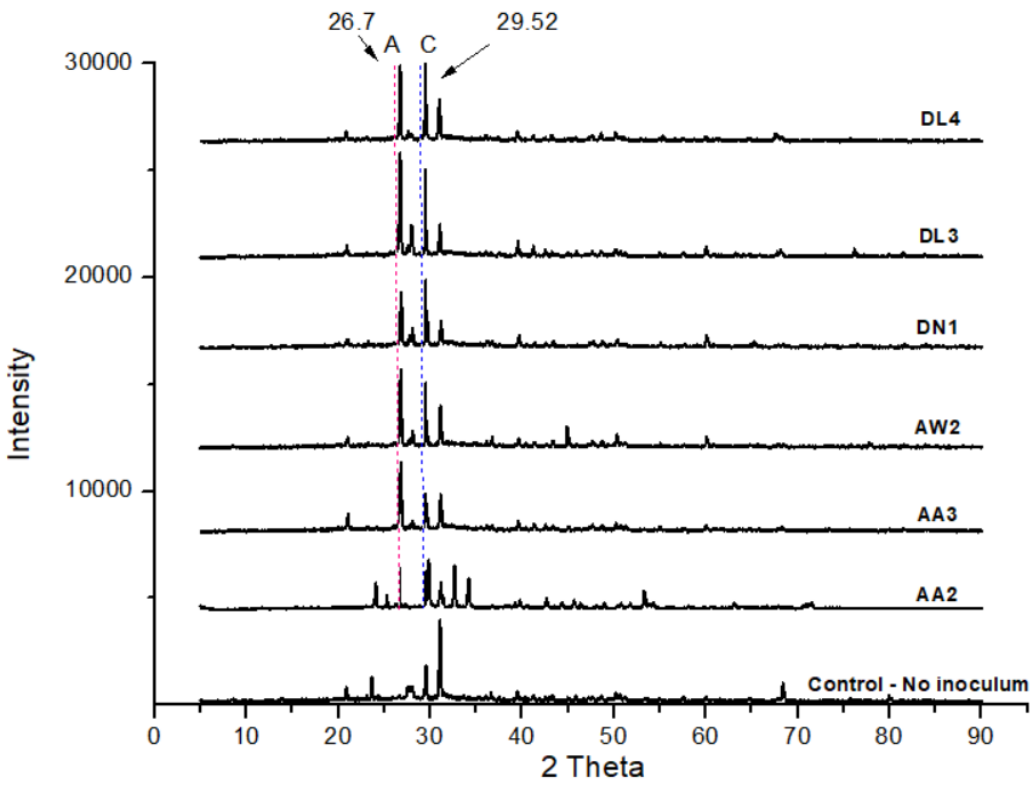

Figure 14. X-ray diffraction patterns of soil samples inoculated with AA2, AA3, AW1, DN1, DL3 and DL4 compared with control sample not inoculated with bacteria.

APPLICATIONS OF MICP ON LAB AND FIELD SCALE

After the cultures were performed with urea media supplemented with calcium chloride and bacterial isolate, and the corresponding analysis confirmed the precipitation of calcium carbonate, the technique was applied in three different ways; 1) Precipitation of calcium carbonates in syringe columns, 2) Precipitation of calcium carbonate in soil trays by applying the required solutions for biomineralization under laboratory conditions, 3) Application of microbially induced calcite precipitation in the Qatar

University Alkhor farm. In all the experiments, 0.2 M of calcium chloride was used.

Application of MICP in syringe columns

Precipitation of calcium carbonate was achieved in syringe columns with both urea medium and 0.2 M calcium chloride. The summary of the results observed from the experiment are mentioned in Table 6.

Table 6. *Summary of solutions with or without inoculation of bacterial isolate (AA3) and the precipitation in the syringes:*

Medium	**Inoculation (AA3)**	**Precipitate type**
Urea medium	No	NA
Urea medium	Yes	NA
Cementation solution (0.2 M $CaCl_2$)	No	NA
Cementation solution (0.2 M $CaCl_2$)	Yes	NA
Urea medium + Cementation solution (0.2 M $CaCl_2$)	No	NA
Urea medium + Cementation solution (0.2 M $CaCl_2$)	Yes	White Precipitate

As shown in Figure 15 (a): control; urea medium and (Fig 15 (b)) urea medium with isolate AA3 had no evident difference and no surface soil precipitation. Similarly, control; cementation solution (Fig 15 (c)) and cementation solution with AA3 (Fig 15 (d)) showed no differences. The only differences observed were between the control (urea medium + calcium chloride) (Fig. 15 (e)) and treatment (urea medium + calcium chloride + AA3) (Fig. 15 (f)). To further confirm the results, XRD analysis of the soil

samples obtained from syringe columns (Fig. 15 (e, f)) was performed. The XRD pattern is shown in Fig. 16.

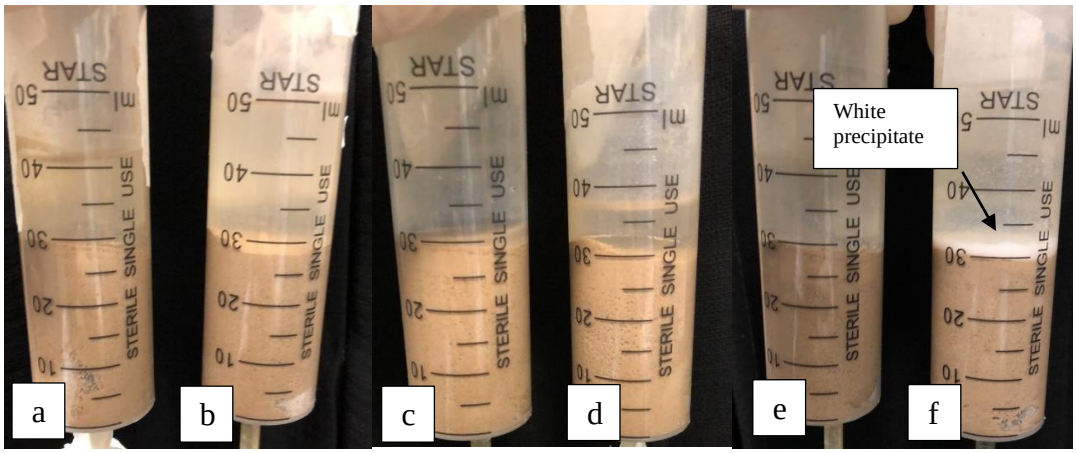

Figure 15. Application of MICP in syringe columns, a) Urea Medium, b) Urea Medium + AA3, c) $CaCl_2$, d) $CaCl_2$ + AA3, e) Urea Medium + $CaCl_2$, f) Urea Medium + $CaCl_2$ + AA3

Distinct and high peak for aragonite is seen at 26.8 θ and 27.60 θ. The peak for aragonite at 26.8 θ is present in the control soil sample as well which indicates the presence of aragonites in the soil, however, in the treated soil, the peak has higher intensity indicating the presence of high concentration of biominerals: aragonite and calcite.

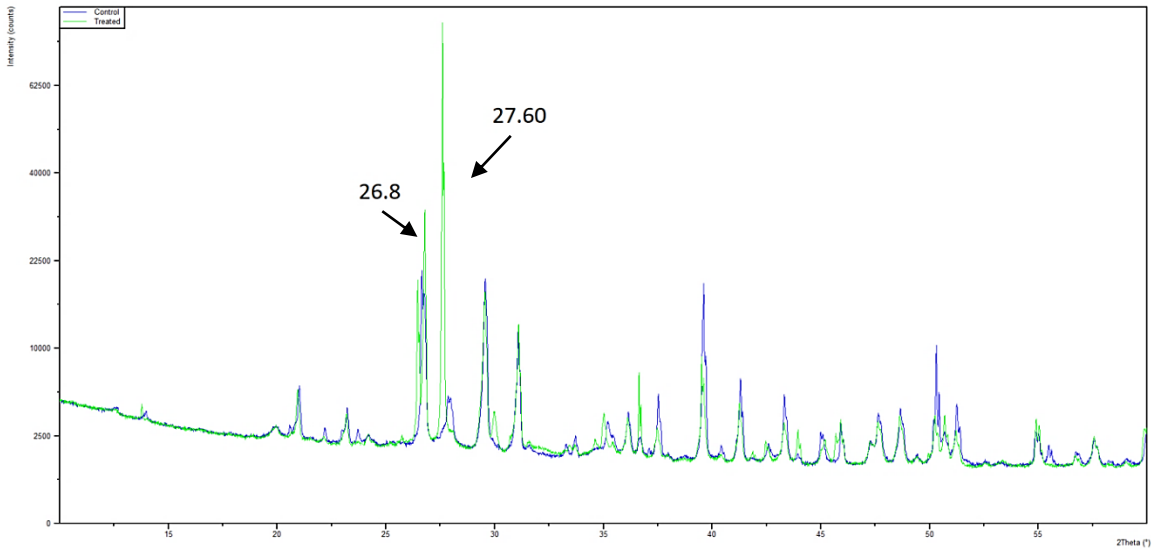

Figure 16. XRD Pattern of soil samples obtained from syringe columns; Control; Urea Medium + 0.2 M $CaCl_2$ (Blue color), Treatment; Urea Medium + 0.2 M $CaCl_2$ + AA3 (Green color).

Syringe columns are commonly used to biologically treat soil samples for stabilization. A greater compaction and strength was achieved when the soil in the soil columns was treated with urea medium supplemented with calcium chloride and bacteria (Dhami *et al*., 2017). Furthermore, Rajasekar *et al* (2017) used the syringe columns to allow soil cementation to occur by both organic and inorganic carbonate precipitation.

Application of MICP in liquid cultures with soil and quantification of carbonates by Back-titration

To quantify the carbonates precipitated in liquid cultures, the technique of back-titration was performed. Based on solubilization and titration of pure calcium carbonate in the urea medium. The technique provided a calibration curve with concentrations of

calcium carbonate per liter based on the consumption of NaOH (0.16 M) in titration. The curve showed a significant negative linear correlation (y = -5.9047x + 35.003, y is carbonate concentration (g/L) determined by volumetric titration method, x is the ratio of volume of NaOH added for titration, R2 = 0.9998). Table 7 shows the results obtained from quantification of calcium carbonate using back-titration. These results are from the soil experiments that were treated with acid. The non-treated soils did not give significant differences in results between control and treatment cultures (inoculum; AA2) on smaller scale. The control sample contained 165 mg $CaCO_3$ per gram of soil, while the culture inoculated with AA2 contained 166 mg of $CaCO_3$ per gram of soil. The reason could be the inactivity of bacterial isolate AA2 either because of higher substrate concentration or certain conditions within the surrounding environment that affected the growth of bacterium and induction of urease enzyme.

Table 7. *Quantification of calcium carbonate precipitated in acid-treated soil using back-titration*

Treatment	**$CaCO_3$ in soil (mg/g)**	**Increase in $CaCO_3$ (mg/g)**	**Improvement of calcium content (%)**
Dry soil	33.54		
Soil + Water	32.10		
Soil + UM	30		
Soil + Cementation Solution (0.2 M $CaCl_2$) + UM	32.53		
Average	32.04		
AA2 + Soil + UM	37	+ 4.96	+ 15.48
AA2 + Soil +Cementation Solution + UM	175	+ 142.96	+ 446.19

From the results, it can be concluded that the soil after treatment with acid contained only 32.04 mg of calcium carbonate per gram of soil. With treatment using AA2 and urea medium only, 4.96 mg of calcium carbonate were produced per g of soil, however, with the application of urea medium followed by AA2 and cementation solution, an increase of calcium carbonate content was determined up to 142.96 mg/g of soil.

The cultures performed without soil as controls showed no precipitation in urea medium inoculated with AA2, however, urea medium supplemented with calcium chloride and AA2 together were able to precipitate 10 g/L of calcium carbonate. The pH of the respective cultures was noted to be around 8.5.

Response of Biologically modified soil surfaces on small scale (Lab scale)

The calcium carbonate precipitated on small scale under lab conditions was analyzed by measuring calcium ions using atomic absorption technique based on which calcium carbonate percentage was calculated. The table 8 concludes the presence of an average of 205 ppm of calcium ions in the control while 215 ppm in the treatment which indicates an increase of 10 ppm of calcium ions in the soil. In this case, AA2 was able to fix 10 ppm of calcium ions. Since, the experiment was performed under lab conditions, the increase in calcium carbonate was not very high. Generally, in biomineralization, the metabolic activities of bacteria as well as environmental conditions play a significant role. These two factors together affect the saturation index of calcium carbonate. On lab-

scale, it is expected to not have enough evaporation as well as optimal temperature for bacterial growth (Al-Qabany *et al*., 2012).

Table 8. *Quantification of calcium carbonate precipitation in acid-treated soil by estimating the concentration of calcium ions using atomic absorption spectrometer.*

Treatment	**Calcium concentration (ppm)**	**Increase in Calcium ions (%)**
Dry soil	203	
Soil + Water	204	
Soil + Cementation Solution + UM	207	
Average	205	
Bacteria + Soil +Cementation Solution + UM	215	+ 10 ppm (+ 4.8%)

For the natural soil from Al-Khor, the quantification of calcium carbonate was performed by titration (Table 9) and atomic absorption technique (Table 10).

Table 9. *Quantification of calcium carbonate precipitated in soil from QU Field using back-titration*

Treatment	**Biomineralization**	**Calcium Carbonate formed (mg/g)**
Soil + Water + AA2	No	ND
Soil + UM+ AA2	No	ND
Soil + Cementation Solution + AA2	Slight	12
Soil + Cementation Solution + UM + AA2	Yes	29

The soil with calcium chloride (cement solution) and AA2 showed precipitation of carbonates without providing urea medium. The reason could be chemical reactions between the solutions provided during the experiment and the soil components leading to precipitation. On the other hand, 29 mg of calcium carbonate per gram of soil was precipitated in the soil**.** It is evident from the analysis that for the precipitation of calcium carbonates, it is important to provide ureolytic bacteria with both urea media and calcium chloride.

Table 10. *Quantification of calcium carbonate precipitation in soil from QU Field by estimating the concentration of calcium ions using atomic absorption spectrometer*

Treatment	**Calcium Concentration (ppm)**	**Increase in Calcium Ions**
Soil + Water	213.9	
Soil + Cementation Solution	214.2	
Soil + UM	210.3	
Soil + Cementation Solution + UM	199.5	
Average	209.5	
AA2 + Soil + Water	216.6	+ 2.7 = + 3.4 %
AA2 + Soil + Cementation Solution	213.0	ND
AA2 + Soil + UM	207.4	ND
AA2 + Soil + Cementation Solution + UM	221.4	+ 21.9 = + 5.7 %

As can be seen from Table 10, the urea medium supplemented with calcium chloride and ureolytic bacterium AA2 was able to fix 5.7 % of calcium in the soil.

Investigation of field response of Bio-modified surface soils subjected to real world, long-term wind conditions and treatment

The soil samples from the experiment carried out in field were analyzed by back-titration and by atomic absorption technique. Figure 17 (b) shows the white precipitates on the surface of soil in blot with urea medium, calcium chloride and ureolytic bacterium AA2 as compared to control blot where no precipitation was observed (Fig 17 (a)).

Figure 17. (a) Control; Urea Medium + $CaCl_2$ (b) Treatment; Urea Medium + $CaCl_2$ + AA2; White precipitates observed on the surface soil in blot with urea medium, calcium chloride and AA2

The quantification of calcium carbonate was performed by back-titration and the results are mentioned in Table 11. The calcium carbonate content is 198 mg per gram of

soil sample in the field soil (Al-Khor). On treatment of soil with urea medium and AA2 alone, 11 % increase in the calcium carbonate content was determined, while treatment of soil with urea medium supplemented with cementation solution and AA2 resulted in an increase of 16.2 %.

Table 11. *Quantification of calcium carbonate precipitated in soil samples after treating for 3 days in field using back-titration*

Treatment	**$CaCO_3$/g soil (mg/g)**	**Increase in $CaCO_3$**
Dry soil	195 ± 4	
Soil + Water	200 ± 5	
Soil + Cementation Solution	200 ± 5	
Soil + UM	195 ± 5	
Soil + Cementation Solution + UM	200 ± 4	
Average	198 ± 5	
AA2 + Soil + water	225 ± 4	+27 mg/g= +13.6%
AA2 + Soil + Cementation Solution	195 ± 4	ND
AA2 + Soil + UM	220 ± 5	+ 22 mg/g = +11%
AA2 + Soil + Cementation Solution + UM	230±5	+32 mg/g = +16.2%

Further analysis of treated field soil with diverse solutions using atomic absorption technique also indicated an increase in the concentration of calcium carbonate in the soil after treatment. In the Table 12, it is seen that the calcium ions in soil samples without treatment with AA2 has an average concentration of 348.81 ppm. On the other

hand, the soil treated with AA2 and urea medium has Ca^{2+} concentration of 389.3216 ppm (+11.6%) while soil treated with AA2, urea medium and cement solution, the Ca^{2+} concentration is 404.815 ppm (+16%). To ensure, if the carbonate precipitated is calcium carbonate or not, the concentration of calcium carbonate was calculated using the values obtained from atomic absorption spectrometry (section 3.9.6). In the soil treated with solutions but without inoculation of ureolytic bacteria (AA2), the concentration of calcium carbonate is 19.4 % while in soil with urea medium and AA2, it is 21.63 % (+11%) and in soil with urea medium, cement solution and AA2, it is 22.49 (+16%). If the results are compared for both Ca^{2+} concentration and percentage $CaCO_3$, the increase in both cases is similar (11% and 16%) indicating that the isolate AA2 successfully fixed the Ca^{2+} ions and precipitated $CaCO_3$.

Table 12. *Quantification of calcium carbonate precipitated in soil after treating for 3 days in field by analysing calcium ions using atomic absorption spectrometer*

Treatment	Calcium Concentration (ppm)	Increase Calcium ions (%)	Calcium Carbonate (%)	Increase in Calcium carbonate (%)
Dry Soil	338.1		18.7	
Soil + Water	319.2		17.7	
Soil + Cement	375.5		20.8	
Soil + UM	353.2		19.6	
Soil + Cement + UM	358.1		19.8	
Average	348.8		19.4	
AA2 + Soil + Water	371.2	+ 22.3 = + 6.4%	20.62	+ 6.4%
AA2 + Soil + Cement	351.7	+ 2.9 = + 0.8%	19.54	+ 0.8%
AA2 + Soil + UM	389.3	+ 40.5 = + 11.6%	21.63	+11%
AA2 + Soil + Cement + UM	404.8	+ 56.0 = + 16%	22.49	+ 16%

Quantification of calcium carbonate in soil is performed either by calorimetric method or by titration. Depending on the soil of the region, a method is selected (Elfaki *et al*., 2015). The use of hydrochloric acid (HCl) to dissolve minerals and then titrating the remaining acid with sodium hydroxide (NaOH) is one of the technique used to quantify the precipitated carbonates (Kassim, 2013). Kumar *et al* (2013) used similar method to quantify the precipitates of calcium carbonate that were induced by *Bacillus sp.; B. flexus*, *B. pasteurii* and *B. sphaericus.* Calcium carbonate up to 6.6 g per litre was quantified using this technique (Kumar *et al*., 2013).

Some drawbacks of this technique of quantification is the presence of clay

minerals, soil organic matter, incomplete dissolution of carbonates and error in pH detection. For this reason, it is suggested to remove the errors of the calculated values (Kassim, 2013).

CHAPTER 5: CONCLUSION

The results of the study confirm the availability of ureolytic bacteria in Qatari soil. The occurrence of such bacteria in the soil proves their adaptability to the harsh environment conditions. MALDI-TOF MS was utilized to differentiate *B. cereus* by obtaining the protein profiles and comparing them. From differentiation, it can be concluded that ureolytic activity can be different at both species and sub-species level based on the expression level of gene responsible for urease enzyme. A new method through this study is obtained to determine and measure urease activity; the method is known as arbitrary urease activity (AUA). The use of ureolytic bacteria will increase the alkalinity in the surrounding environment by hydrolyzing urea. This increase of pH will lead to precipitation of minerals such as $CaCO_3$. For qualitative (morphology, composition) analysis, SEM-EDS and XRD was performed while for quantitative (mg/g of soil) analysis, back-titration and atomic absorption spectrometer was used. Based on the results obtained, it can be concluded that urea media supplemented with calcium chloride and ureolytic bacteria plays a significant role in affecting the composition and morphology of $CaCO_3$. Furthermore, it can be concluded that the local or indigenous ureolytic bacteria isolated from Qatari soil has the potential to exhibit urease activity and induce mineral precipitation. Therefore, the use of MICP to stabilize soil whether for soil stability against wind erosion or for application-based purpose is possible in Qatar provided all the necessary conditions.

Publication; Parts of the results of this research have already been published in RSC Advances journal titled *"Isolation, differentiation and biodiversity of Ureolytic bacteria*

of Qatari soil and their potential in microbially induced calcite precipitation (MICP) for soil stabilization" available at http://pubs.rsc.org/en/content/articlehtml/2018/ra/c7ra12758h

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