QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

INVESTIGATION OF THE ROLE OF CARBONIC ANHYDRASE IN MINERAL FORMATION AND MAGNESIUM INCORPORATION BY BACTERIA FROM QATARI SABKHAS AND MARINE SEDIMENTS

BY

RIM ABDEL SAMAD

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COMMITTEE PAGE

The members of the Committee approve the Thesis of
Rim Abdel Samad defended on [Defense Date].

Nabil Zouari
Thesis/Dissertation Supervisor

Samir Jaoua
Committee Member

Roda Fahad Al-Thani
Committee Member

Note: the empty committee member names should be removed.

Approved:

Ibrahim AlKaabi, Dean, College of Arts and Sciences

ABSTRACT

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Title: Investigation of the Role of Carbonic Anhydrase in Mineral Formation and Magnesium Incorporation by Bacteria from Qatari Sabkhas and marine Sediments

Supervisor of Thesis: Nabil Salem Zouari.

Mineral-forming bacteria are of great interest in arid environments specifically. These environments are favorable for enhancing the microbial carbonate deposition that is thought to largely influence the sedimentary structure years ago and continue to do so. Qatar's ecosystem serves as an attractive area for such investigation. In this work, microbial biodiversity in living mats of Dohat Faishakh sabkha was studied using MALDI-TOF mass spectrometry and categorized by identifying characteristic mass peaks and principle component analysis (PCA). Coral sedimentary marine sites were also investigated and carbonate precipitating strains were identified; *Vibrio alginolyticus* and *Psychrobacter sp.* The precipitated crystals were examined using SEM/EDS and XRD analysis. This study also investigated 2 enzymatic activities of Carbonic Anhydrase (CA) claimed to be responsible for catalyzing the reversible reaction converting CO_2 to HCO_3^-; esterase and hydration. Both enzymatic assays showed high activity in mineral-forming strains and almost no activity in non-mineral forming bacteria. In this work, it was demonstrated that the presence of an active CA enzyme is strongly correlated to the ability of mineral-forming bacteria to deposit carbonates and incorporate Mg.

DEDICATION

To my dear family for believing I could do this long before I did.

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So much of the effort put into this research work was a group effort, I would therefore like to express my appreciation to my mentor Prof. Nabil who knew exactly what can be achieved and how, my committee members; Prof Samir Jaoua and Dr. Roda Al-Thani for their continuing support; Dr. Zulfa Al-Disi for always being there for guidance; the Departement of Biological and Environmental Sciences for all the experiences and lessons; Dr. Yousaf Ashfaq for being extremely patient; Mr. Essam from the Central Lab Unit; and everyone who has made this journey possible.

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CHAPTER 1: INTRODUCTION

Since the very beginning of life on Earth, bacteria have contributed to the formation of minerals and rocks as well as having essentially played a role in the biogeochemical cycles of multiple important elements such as Ca, Mn, Fe, S and P, etc. (Gonzalez-Munoz et al, 2010). Our planetary environments through these cycles have been continuously altered over billions of years by microorganisms capable of extracellularly (inorganic crystals) and intracellularly precipitating pivotal minerals. Do they not only act as key factors for researching the creation of ancient topography and environments but also possess numerous potential commercial usages (Lin et al, 2014). However, the relationship between biomineralizing bacteria and their surrounding environment can be better described as double-sided, i.e. as much as the environment is influenced by the minerals formed by bacteria, the precipitation itself is also strongly affected by the conditions in which the cells are growing (Zhu & Dittrich, 2016).

It is of no surprise that the precipitation of calcium carbonates is one of the most studied carbonate deposition; particularly the formation of calcite ($CaCO_3$). This very abundant carbonate mineral has been extensively studied in the past few years and three mechanisms are postulated to try to explain its formation (Zhu & Dittrich, 2016): (a) biologically controlled mineralization, which is directly a consequence of cellular activities. (b) Biologically influenced precipitation in which minerals form in a passive manner because of extracellular polymeric substances (EPS) and other cell surface organic matter. (c) Biologically induced mineralization in which bacteria play a role in the kinetics and function as a catalyst; therefore, stimulating the mineralization or formation of precipitates of a mineral in a solution that is supersaturated with the mineral (Anbu et al, 2016).

Calcium carbonate being one of the minerals that is largely available among all those

formed from geological processes, is a primary source of the carbon reservoir (Romanov et al., 2015). Bacteria and plenty of other organisms are said to be responsible of the biotic precipitation of calcium carbonates, and the process has been extensively studied and mentioned in previous publications (Dhami et al., 2013; Anbu et al., 2016; Zhu and Dittrich, 2016). Studies have been performed on microbially induced calcite precipitation, referred to MICP. Although the mechanism is known to the scientific community, a lot of evidences have led the way to confirm that the availability of bacterial cells and certain enzymatic activities in a supersaturated medium does in fact induce the mineralization of calcium carbonate (Bose & Satyanarayana, 2017).

Not only do they add to the global primary production and fixation of CO_2, microorganisms also aid in the processes of lithification and precipitation after interacting with the sediments (Prieto-Barajas et al., 2017).

Nowadays, sabkhas are vulnerable areas regarding future climate projections, with little rainfall, restricted freshwater supplies and a rising population (Cusack et al., 2018). Despite the harsh conditions, mangroves, sabkhas and seagrasses along the Arabian Gulf seem to serve as CO_2 sinks (Cusack et al., 2018), impacting the so-called "blue carbon," or the carbon stored in marine systems (Duarte et al., 2013). Until now, carbon storage in blue carbon habitats has been excessively studied in temperate and tropical climates (Cusack et al., 2018). The data for arid regions, including the adjoining Arabian Gulf, are very scarce, and the understanding of organic carbon storage in coastal ecosystems remains restricted (Rui Santo et al., 2019). Naturally, microorganisms enhance their growth and sustain their survival by assembling in different forms of aggregates that can be of one species such as in biofilms, or complex well-established ecosystems in the case of microbial mats (Dupraz, et al., 2008). In

extreme habitats like that of a sabkha, such microbial mats made up of several layers colonize as complete ecosystems enabling them to acquire better defense mechanisms and transfer their resources among the layers (Al-Thani et al, 2014). The adaptation established by the microorganisms to the extreme conditions in a mat of a sabkha is considered as an extra characteristic allowing them to survive in such conditions. However, these microbial mats play a pivotal ecological role particularly in marine intertidal ecosystems. Within a microbial mat, the growth of the species and how they are distributed among the layers is directly affected by the physical and chemical properties of the environment. In Qatar, sabkhas as microbial ecosystems housing biomineralizing bacteria have been carefully studied. The most significantly mineralizing bacterial strains isolated from the hypersaline Dohat Faishakh sabkha which is a coastal sabkha located in the north west of Qatar were *Virgibacillus* (Al Disi et al., 2017) along with other aerobic, halophilic, heterotrophic bacteria. These strains have been identified in Qatar; although also reported in literature by Vreeland et al. (2000) and Satterfeild et al. (2005) in the Permian Salado salts in the Guadalupe Mountains of western Texas, U.S. Thus, the sabkhas of Qatar are today considered a ubiquitous environment for the investigation of the role of aerobic bacteria, specifically *Virgibacillus* in mineral precipitation (Al Disi et al., 2017). It is now well established, that *Virgibacillus* is not only responsible of mineral formation, but also contributes in magnesium incorporation into the carbonates, which leads to formation of protodolomite, which is magnesium calcium carbonate (Arias et al., 2017). This function is unique among microorganisms. However, the mechanism of incorporation of magnesium is still not elucidated.

It has been repeatedly reported in literature that both enzymes urease and carbonic anhydrase are critical for proficient mineralization of calcium carbonate and other

minerals (Achal & Pan, 2011). Carbonic anhydrase, being far less studied, seems a very interesting potential area of investigation to many scientists (Dhami et al., 2014). The Carbonic anhydrase enzyme itself is present in many living organisms (eukaryotes, prokaryotes, archaea) and it works on catalyzing the reversible hydration of carbon dioxide according to the reversible reaction (Equation 1) (Achal & Pan, 2011 and Bose & Satyanarayana, 2017):

$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$

This reaction when taking place in the forward direction will provide the fundamental molecules required for the formation of $CaCO_3$. Nevertheless, the role of this enzyme in $CaCO_3$ precipitation is still ambiguous. Indeed, the marine actinobacterium *Brevibacterium* linens BS258 was responsible of forming precipitates of calcium carbonate precipitates using the urease activity (Zhu et al. 2017). The same authors also showed that this strain dissolves the formed precipitates when the calcium concentration was increased. They finally attributed this role to carbonic anhydrases activities as they were up-regulated at high calcium concentration (Zhu et al., 2017). In fact, microenvironment around the cell becomes alkaline due to hydrolysis of urea by urease in urease-based MICP, resulting precipitation of carbonates (Zhu et al., 2017).

In an attempt to draw a correlation between calcium carbonate precipitation and carbonic anhydrase activity in biomineralizing bacterial strains, this research intends to:

1. Investigate the activities of carbonic anhydrase in confirmed mineral forming *Virgibacillus spp.* strains and non-mineral forming strains, all isolates from Dohat Faishakh Sabka in Qatar.
2. Isolate, identify and characterize, using MALDI-TOF MS, the mineral forming bacteria from dead mats sampled from Dohat Faishakh Sabka and from marine

sediments, sampled from Qatar offshore.

3. Analyzing the diversity of Qatari bacterial strains, isolated from sabkha mats and marine sediments. Analyze the protein profiles of the studied strains obtained by MALDI-TOF and combination between MALDI-TOF MS analysis and PCA.
4. Quantify the Carbonic anhydrase activity of all the strains investigated for a possible role on the cell surface in mediating biomineralization.
5. Study the influence of carbonic anhydrase on *Virgibacillus* capabilities to mediate carbonate minerals formation. Extrapolation to the isolated bacteria from marine sediments.

1.1 Rationale

Gulf countries, including Qatar, belong to the most arid coastal ecosystems of the world. The western coast of the Qatari peninsula known as sabkha is enriched with carbonate minerals formed during the Holocene, 4000–6000 years ago. The Inland sabkhas and the coastal sabkhas in the Qatari peninsula are characterized by highly saline water. Dohat Faishakh sabkha is a supratidal expanse. It passes laterally through algal mats near the intertidal region within the shallow marine area in the west of Qatar. Since 1965, this sabkha is being recognized as one of the few places on Earth where dolomite forms at ambient temperature. Dolomite, among others, is a common ancient carbonate rock. However, the rare occurrence of dolomite in recent environments and failure to synthesize it in laboratory at low temperature and pressure lead to an enigma frequently highlighted in literature as “dolomite problem”. Numerous recent studies have evidenced the role of microorganisms in the biomineralization process. In Dohat Faishakh sabkha, it was shown that bacteria, especially those belonging to the *Virgibacillus* genus were able to mediate mineralization process. This unique site in the gulf region, is drawing attention to understand the diversity of bacteria involved in biomineralisation, in decaying mats but also in living mats, to establish cause/effect

between metabolic activities and induction of mineral formation. The sabkha environments are transformed to locations where CO_2 is sequestered in forms of minerals. The merit of Qatari sabkhas is to allow to study the hypothesis of involvement of microorganisms in sequestration of CO_2. Providing carbonates in the environment is the crucial step to induce carbonates formation. While bacteria exhibiting urease activity can generate carbonate ions from urea, the question is still without answer regarding the source of carbonates for *virgibacillus*. The enzyme named carbonic anhydrase is now recognized as the principal tool to transform CO_2 into carbonite ions. The relationship should be established between the occurrence and the abundance of this enzyme in microorganisms and their involvement in biomineration by forming calcites and protodolomites

1.2 Hypothesis

The essence of this study on a larger perspective is linked the global issue of climate change and its environmental impacts. The primary gas emission and greenhouse gas CO_2 is the number one contributor to global warming. Today, the concern is on how to reduce the risks needed to be taken in order to sustainably address this environmental crisis. Several mitigation proposals arose due to the effects of climate change. Some were implemented through international agreements forcing countries to limit their GHG emissions. Others pictured scenarios taking into consideration the economic aspects of slowing down global warming. One of the proposed climate change mitigation scenarios is carbon capture, utilization, and storage. This approach can be implemented with the aid of technological advancements, and is categorized into three major groups for CO_2 sequestration: physical, chemical, and biological methods.

Both physical and chemical platforms have significant challenges of cost and environmental risks. However, microbial carbon fixation specifically using carbonic anhydrase enzyme remains a promising eco-friendly approach to offset emissions.

Carbonic anhydrase is a feasible biocatalyst not only for carbon capture and storage, but also for CO_2 precipitation to calcium carbonate.

In this study, we hypothesize an essential role for Carbonic Anhydrase in capturing a significant fraction of CO_2, which facilitates the formation of microbially-mediated carbonate minerals by *Virgibacillus* bacteria isolated from Qatari sabkhas and marine sediments. We expect specific carbonic anhydrase activity; as both esterase and hydration, to be evident in mineral-forming strains as opposed to non-mineral forming strains. If confirmed, these findings will partially explain the mechanism used in the process of microbially mediated carbonate precipitation. This role may be both in capturing CO_2 as source of carbonate, and partial solubilization of the formed minerals allowing incorporation of Mg instead of calcium, before catalyzing again the formation of more deposition of carbonates.

CHAPTER 2: LITERATURE REVIEW

2.1 Evaporitic Environments

The study of extreme environments has always been of great interest due to the vast information they provide on geochemical processes and the development of ocean and continental sinks (Caruso et al., 2015). Evaporitic environments that are ancient are excellent platforms for broadening the study and knowledge of the biogeochemical processes that have led to the current environments. It is the constant precipitation of minerals in arid places caused by the water evaporation that leads to formation of "evaporates" (Warren, 2016). Arid zones in which evaporates may precipitate are not restricted to high temperatures, but they can also be present in polar regions where salts are induced by the very cold temperatures to freeze out.

However, marine evaporitic environments have always taken much of the attention compared to non-marine. There are various features that allow the differentiation between minerals that are associated with the type of sedimentation, fossils, rocks and geochemistry (Manzi et al., 2016). Of all the sediments present in all the continents on Earth, 2% are composed of evaporates, covering an area of around 4.5 million km^2 (Al Disi, 2018).

2.2 Sabkhas

In so many different arid environments, sabkhas systems are found in fluctuating levels of complexities; however, significant abundance is noticed in arid coastal areas such as those present in Qatar (Al Disi et al., 2017). The ecosystem in arid coastal areas favors the occurrence of sabkhas, since they are typical saline environments. Extensive studies have been made in sabkha environments regarding the sedimentary structures. Probably the most interesting to study are the "supertidal sabkhas" which are also considered as marine; these unique sabkhas are present in coastal areas in the Mediterranean presenting extraordinary natural resources for the investigation of microbially induced

sedimentary structures (Brauchli et al., 2016). Along the Arabian Gulf as well, a significant occurrence of sabkhas is found along the coasts that provide the perfect environmental conditions being one of the hottest regions in the world. All year long, the average surface sea temperatures fluctuate between 20°C to 34°C, whereas, the surface temperatures of the sedimentary structures can even raise to 60°C in the summer days, characterized by hot and humid weather. In addition to temperature, high levels of salinity is also responsible a pivotal role in sabkhas for forming minerals. Regarding the Arabian Gulf, salinity can reach 30% (Brauchli et al., 2016).

2.2.1 Sabkhas as CO_2 sinks

Nowadays, sabkhas are vulnerable areas regarding future climate projections, with little rainfall, restricted freshwater supplies and a rising population (Cusack et al., 2018). Despite the harsh conditions, mangroves, sabkhas and seagrasses along the Arabian Gulf seem to serve as CO_2 sinks (Cusack et al., 2018), impacting the so-called "blue carbon," or the carbon stored in marine systems (Duarte et al., 2013). Until now, carbon storage in blue carbon habitats has been excessively studied in temperate and tropical climates (Cusack et al., 2018). The data for arid regions, including the adjoining Arabian Gulf, are very scarce, and the understanding of organic carbon storage in coastal ecosystems remains restricted (Rui Santo et al., 2019).

More research aims to investigate the driving mechanisms of biomineralization, and the CO_2 flux in the sabkhas of Qatar. It is hypothesized that organic matter mineralization will be impacted by fluctuating environmental conditions and salinity. Consequently, it is expected that CO_2 flux will be heterogenically distributed over the space in the sabkhas and will behave highly dynamically. Hence, a strong link between sedimentary structures of sabkhas, their function, and carbon sequestration exists.

2.2.2 Microbial mats in Sabkhas

A variety of environments are considered to be optimum for the formation of microbial mats (Bolhuis et al., 2014). One of which are coastal intertidal sediments and sabkhas due to the exquisite role they play in the development of sedimentary deposits. Microbial mats occurring specially in sabkhas serve as highly important natural laboratories for the investigation of the role played by the microbial community in evaporitic minerals formation (Rossi & De Philippis, 2015). The diversity of organisms in sabkha microbial mats is enormous, and it can only lead on to an even larger set of metabolic activities taking place in these mats. Hence, a microbial mat such as those present in sabkha environments are not only unique ecosystems, but also dynamic ones exhibiting a huge diversity of microorganisms that shape the assembly of its sediments (Iniesto et al., 2016).

The multilayered structure of microbial mats supports the availability of nutrient resources for the bacteria, archaea, fungi, and protozoans all together. Al-Thani et al (2014), have demonstrated that microbial mats in Um Alhool, Qatar are composed of several layers, each of which is dominated by a different class of *Proteobacteria*.

2.3 Biomineralization

Organic carbon production in the sabkhas is mostly driven by microbial mats activity; however, during its preservation, the presence of carbonates, clays and evaporates became an important factor. Supertidal zones of sabkhas undergo severe evaporation, contributing to a dramatic increase in the salinity and precipitation of gypsum and anhydrite. The permanent precipitation causes a state of supersaturation, and in turn uptakes all the calcium ions (Ca^{2+}). Recent studies in closer areas such as the Abu Dhabi sabkhas and Dohat Faishakh sabkha in Qatar as well, have highlighted the correlation between Exopolymeric Substances (EPS) secreted from microorganisms inhabiting sabkha mats and nucleation sites of one of the most interesting mineral, dolomite. The

release of EPS by microorganisms is a defense mechanism acquired for the protection form environmental stress condition such as salinity, UV radiation, and drought.

Nowadays, there is no doubt that microbes and EPS can induce the formation of carbonates, e.g. dolomites (Al Disi et al., 2017). However, there is still an ongoing debate regarding how organic molecules impact the crystal structure of dolomites and, in turn, how minerals affect the preservation of carbon. Evidence of these interactions from field studies in sabkhas is very rare.

2.4 Microbial Mg-Calcite formation

Amid the various process occurring in microbial mats the are mediated by microorganisms, the one of highest importance is that leading to the precipitation of magnesium rich carbonates (Plee et al., 2010). The particular interest around these carbonates comes from the fact that at moderate and low temperatures, the formation of Mg-carbonate minerals seems impossible due to kinetic limitations. However, microbes appear to have the ability to defeat such limitations (Bontognali et al., 2014).

2.5 Carbonic Anhydrase (CA)

Biomineralization based on urea hydrolysis is a process are widely reported in literature, but little efforts have been put into the activity of carbonic anhydrase in this matter. Few studies analyzed its role in $CaCO_3$ formation. Knowing that the arid hypersaline environment in Qatar defines the perfect place for the very common occurrence of carbonate minerals and sediments, and in reference to the previous work done on Dohat Faishakh by Al Disi et al. (2017), it is essential to further investigate the mechanism by which the isolated *Virgibacillus spp.* mediate mineral formation. Actually, a debate is still on the table regarding the mineralization process as a side effect of microbial metabolism under certain conditions or a direct effect based on the response of the microorganisms to environmental factors. The essential abiotic parameters that regulate precipitation of minerals are known to be: (1) concentration of

Ca, (2) pH of the medium/environment, (3) DIC, dissolved inorganic carbon, (4) nucleation sites availability. However, it is still questionable whether carbonic anhydrase is also part of the influence of microbes in the mineralization process.

The enzyme is known to be a zinc-containing metalloenzyme, catalyzing the reversible reaction between CO_2 and HCO_3^- (Rothenstein et al., 2012). Thus, this enzyme is able to accelerate both carbonate rock dissolution and CO_2 uptake at the same time. Previously, tests with rotating-disk or shaking flask as well as with soil columns were performed for the simulation and the investigation of several biological factors, including CA activity involved in dissolution of carbonate rock. However, the CO_2 capture was not clearly elucidated (Supuran, 2013).

Moreover, Xiao and Lian (2016) specifically studied the expression of carbonic anhydrase from *Bacillus spp.* in *E. cloi*. Their findings showed that microbial CA aided in the capturing of atmospheric CO_2. However, it is important to investigate to what extent microbial CA from *Virgibacillus* strains isolated from the Qatari environment.

2.6 The link between microbial activities and carbonate bio-precipitation

Several microbial activities known to promote the deposition of calcium carbonate, do so as part of their metabolic process. In such cases, CaCO3 acts as a by-product. Examples of such microbial activities include; (a) photosynthesis, specifically in many cyanobacteria species according to the following reaction $2HCO3 + Ca2+ \rightarrow CH2O + CaCO3 + O2$ (Kamennaya et al., 2012; Dupraz et al., 2009). (b) Denitrification in nitrate-reducing bacteria by a series of reactions: $CH2COO- + 4H+ + 2NO3 \rightarrow 2CO2 + N2 + 3H2O$

$Ca2+ + CO2(aq) + 2OH- \rightarrow CaCO3(s) + H2O$ (Karatas, 2008)

(c) Ureolysis in ureolytic bacteria, according to the following reaction $CO(NH2)2 + 2H2O + Ca2+ \rightarrow 2NH4+ + CaCO3$ (Mortensen et al., 2011; Helmi et al., 2010).

All of these and many others are of the reported microbial activities known to be involved in bio-precipitation of carbonates. It is with local supersaturation of the microenvironment that these microbial activities favor the precipitation of carbonate minerals. However in dolomite precipitation, overcoming the low-temperature kinetic barrier requires the possible association of EPS and the chemistry of the bacterial cell walls. This interaction is hypothesized to be an essential factor in the precipitation of dolomite.(Roberts et al., 2013; Voegerl, 2014; Qiu et al. 2107).

2.7 MALDI-TOF MS

With the increasing demand for research in all fields of science, there is a constant quest for methods of identifying organisms that is both reliable and quick. Although the most common method for organism identification is the molecular sequencing, it still requires substantial time, technical effort, and expenses. MALDI- TOF MS (Matrix Assisted Laser D Intensity- Time of Flight Mass Spectrometry) emerged in past 20 years as an alternative approach that simplifies the sample preparation process and allowed for output data in an exceedingly fast rate. Medically, MALDI-TOF was put in use around 16 years ago, specifically for the identification of pathogens in clinical medicine (Sandrin et al., 2013).

Many other types of MALDI have used as well for identification of nonbacterial organisms such as fungi and nematodes; however, MALDI-TOF remains the most commonly used due to its evident practicality and efficiency. Similar to many other techniques, identification by MALDI-TOF is strictly library-based, meaning a database of known reference bacteria need to be available for possible matching with unknown ones. The databases in many softwares vary according the geographic location, depending on the phenotypes and genotypes variability (Sandrin et al., 2013). There is no doubt, that advances in bioinformatics and the increased number of sequenced microbes have influenced researched to apply those bioinformatics approaches to better

identify protein peaks.

After analysis, MALDI-TOF is able to generate a useful protein spectra unique to each organism with a good resolution provided by adequate detectors (Yates, 1998). The collected spectral m/z ratios are selected between 2 to 20kDa by the instrument's operation software. In 1996, the log system present now in all MALDI-TOF devices was introduced for the very first time. The purpose behind the log system is to evaluate the level of similarity compared spectra share (Holland et al., 1999).

2.8 Protein biomarkers

Protein biomarkers are exploited as biological features to evaluate the progression of pathogenic diseases, the succession of normal biological processes, or the biological reaction to certain therapeutic mediations. In pathology, biomarkers are used for the analysis of the degree of evolvement of diseases and its stages (Downing, 2001).

In all living organisms, the fundamental compounds responsible for the metabolic processes, cell-signaling, and stability of the organism are proteins. It is with the evolvement of mass spectrometry that is based on proteomics that novel biomarkers can be discovered and founded for clinical and research and development purposes (Rifai et al., 2006). Biomarkers research is mainly involving two major parts, starting with the discovery of the biomarker itself and then validating it. Discovering protein biomarkers is made possible using advanced proteomics, metabolomics or peptidomics and analysis of several samples. It also involves finding differences that are statistically different between protein profiles and mass fingerprints (Horvatovich & Bischoff, 2010).

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacterial strains from decaying mats used as reference

Twenty-three bacterial strains were used as reference strains (Table 1). They are aerobic, halophilic and heterotrophic bacteria that were previously isolated from decaying mats sampled from Dohat Faishakh sabkha, northwest of Qatar. Their identification was performed by ribotyping and the access numbers of their DNA sequences were published. 7 strains of *Virgibacillus marismortui*, 3 strains of *Virgibacillus salarius,* and 13 *Virgibacillus sp.* identified only at the genus level were tested in the current study.

Table 1: Reference strains previously identified by ribotyping and their corresponding strain codes.

Strain Code	Identification by Ribotyping
DF112	*Virgibacillus marismortui*
DF221	*Virgibacillus sp.*
DF231	*Virgibacillus marismortui*
DF241	*Virgibacillus sp.*
DF252	*Virgibacillus sp.*
DF281	*Virgibacillus salarius*
DF282	*Virgibacillus sp.*
DF291	*Virgibacillus sp.*
DF322	*Virgibacillus marismortui*
DF341	*Virgibacillus marismortui*
DF351	*Virgibacillus salarius*
DF411	*Virgibacillus marismortui*
DF431	*Virgibacillus sp.*
DF451	*Virgibacillus sp.*
DF461	*Virgibacillus salaries*
DF472	*Virgibacillus marismortui*
DF491	*Virgibacillus marismortui*
DF2102	*Virgibacillus sp.*
DF2121	*Virgibacillus sp.*
DF2131	*Virgibacillus sp.*
DF2141	*Virgibacillus sp.*
DF2161	*Virgibacillus sp.*
DF2172	*Virgibacillus sp.*

These strains were selected based on their significant role as aerobic microorganisms to mediate formation of Mg-rich carbonates. Bacterial cultures were preserved in 60% glycerol at −80 °C (Microbiology and Biotechnology lab, Qatar University, Doha) until use.

3.2 Mats and sediments Sampling

Samples in this study were obtained from different environments due to the importance of studying the microbial community in both sabkhas and marine sediments as well. In contrast with the reference strains used (Table 1) which were isolated from decaying mats, living mats were sampled from the same site, Dohat Faishakh in the north-west of Qatar. This site was chosen again due to previous studies that have highlighted the presence of minerals and different structures of sediments (Al-Disi, Brauchli et al., 2015).

Two other samples have been provided from coral sediments. This first was sampled in September 2018 from Al-Ashat. Almost a year after in December 2019, another coral sediment sample was provided from Sheraoha. Table 2 shows the description of every sample collected for this study.

Table 2: Detailed description of the 3 samples collected.

Sample	**1**	**2A**	**2B**
Type	Living sabkha mats	Coral sediments	Coral sediments
Date of sampling	Nov, 2017	Sep, 2018	Dec, 2019
Location (GPS coordinates)	24°38'44"N 50°57'31"E	24°74531"N 51°60062"E	25°03213"N 52°22474"E

All samples have been collected using appropriate aseptic techniques and preserved in complete darkness at 4°C until they were transported to the microbiology laboratory at Qatar University.

3.3 Media cultures

3.3.1 Enrichment cultures

The isolation of bacteria was performed by enrichment cultures. From each soil sample, our interest was focused on mineralizing bacteria, therefore specific liquid media cultures were used for this purpose. Sterile falcon tubes of 50 mL were filled with approximately 1 g of soil and liquid MD1 enrichment medium (g/L): (1 glucose, 5 peptone, 10 yeast extract, 35 NaCl, 8 Mg Acetate and 1 Ca Acetate). The pH of the liquid media was adjusted to 7.0 and then autoclaved for 21 minutes at 120 °C. The enrichment procedure was a modified one from the method used by Burbank et al., (2012). The tubes were incubated in a shaker set at 170 rpm and 37 °C during 3 days. Then, the solution was drained out and the soil re-treated for 2 days (48h). The media MD1 was used because it has been previously reported to induce mineral formation (Al-Disi et al., 2017).

3.3.2 Media cultures for bacterial isolations

Mineral forming bacteria, as mentioned earlier, have been shown to exhibit optimum growth and enhanced mineral formation activities when cultured on MD1 media. Therefore, for the isolation of mineral forming bacteria was done first by preparing serial dilutions of the enrichment mixtures obtained after 3 days of incubation. 100 µl of each diluted solution was plated on solid MD1 media having the same composition of the enrichment media used in section 3.3.1 (with 15 g/L Agar). The Petri-dishes were then incubated for 48 hours at 35°C. Differentiation between different strains was done based on morphological variation in shape, size, pigmentation, elevation, and appearance of the colonies in the mixture. Pure cultures of strains were obtained after a series of sub-cultures.

3.4 Investigations by MALDI-TOF MS

3.4.1 Protein extraction

The sample preparation of the newly isolated strains was performed in two different techniques in order to generate the most reliable results. Extraction procedure using ethanol/formic acid (v/v) was used as reported by Wang et al. (2012). Solid LB was used to grow the bacterial cultures. A colony was suspended in 500 µL sterile water in an eppendorf tube. Then the cells were re-suspended in 900 µL absolute ethanol. After centrifugation at 13,000 rpm for 2 min, the supernatant was discarded and the pellet mixed with 1mL 70% of formic acid and 1 ml 100% acetonitrile. The mixture was centrifuged for 2 min at 13,000 rpm, and from the supernatant, 1 µL was transferred onto a biotarget 48 sample spot. A total of 1 µl HCCA (α-Cyano-4-hydroxycinnamic acid) matrix solution (50% acetonitrile and 2.5% trifluoroacetic acid in pure water) is then added onto the sample spots for protein extraction. Analyses were performed in triplicates by spotting a colony into three different wells. Alternatively, since mass spectra obtained were not very clear, the whole cell method was performed. A volume of 0.5 µL was sampled from fresh colonies and transferred into the well of the target plate and mass fingerprints were obtained allowing for better results and detection of biomarkers.

3.4.2 Identification of bacterial isolates by MALDI-TOF

Similarities between individual mass spectra of the isolated strains and those of the database entries were expressed in the form of log (scores) obtained by default from the Biotyper software settings. The identification was performed using the Bruker Biotyper software, where a log scale from 0 to 3 defines the identification matching level with the database. The score of 2.300–3.000 is highly probable species level identification with high confidence, which shows the identification result is highly accurate up to the species level. Score 2.000 – 2.299 is genus and probable species level identification,

which shows the identification result is highly accurate up to the genus level, and probably correct at species level. Whereas scores between 1.700 and 1.999 indicates probable genus level identification, since the score is low, the result shows that the identification is probably correct at genus level. Polypeptides and proteins having a molecular weight in the range of 2000 and 20,000 m/z are utilized for identification of bacterial strains based on individual mass peaks corresponding to specific sequences and sizes of ribosomal proteins of distinct types of microorganisms.

3.4.3 Data processing

The protein profile of each bacterial isolate was obtained from the Bruker Flex Control software by using two sample spots. The Bruker Flex Control software was used to obtained mass spectra using linear and positive mode at 60 Hz laser frequency and intensity of 35%. The acceleration was and source voltages were set as 20 and 18.7 kV, respectively. using different areas of the sample spot, 240 laser shots in 40-shot steps for each spectrum were obtained and analyzed using default settings. The protein profiles were processed and analyzed using the Flex Analysis and Biotyper RTC 3 software.

The MALDI-TOF-MS and PCA techniques helped to acquire the differentiated bacterial strains. The obtained peaks generated from protein profiles were detected using default algorithms. The software detects consensus signals of mass spectra that occur at relatively high weights and in accordance to their specificities of available microbial spectra. These protein profiles with relative peak intensities ranging from 2,000–20,000 *m*/*z* were then separated into 3,600 classes with a width of 5 *m*/*z* for each class. The relative peak intensities were obtained by calculating the peak intensity to the base of the highest peak intensity and multiplying by 100.

Since the mass spectra generated from MALDI are regarded as multivariate data, in

which every mass signal represents a single molecular dimension, multivariate statistical methods are used for differentiation between bacterial species. Principle component analysis (PCA) was performed to decrease dimensionality of the data set and maintain the original information present. This method allows the formation of clustered groups of spectra having similar variation characteristics and the visualization of the differences between them. The data can be represented in either a 2D or a 3D coordinate system; however, it is usually adequate to use the 2D which plots PC1 against PC2, since it generally offers more than 80% of the total variance between the samples.

For a better visualization of the relationship between the isolates and known strains, dendrogram clustering was also carried out by the Biotyper analysis software using default settings according to the Manufacturer's instructions.

3.5 Identification of isolates by ribotyping

3.5.1 DNA extraction

Each pure isolate was grown on LB solid media at 35 °C 24 h prior to the extraction of DNA. In a sterile eppendorf tube, 500 μL of autoclaved distilled water was added. Then from each pure culture, a single colony is suspended in the water and mixed well using a vortex. To allow the cells to undergo a heat shock, the eppendorf tubes were placed in a hot water bath (100 °C) for 10 min and then immediately after transferred to an ice bucket for 1 min. The tubes were then centrifuged at maximum speed for 1 min. The supernatant containing the extracted DNA material was transferred to a new sterile eppendorf tube.

3.5.2 DNA amplification by PCR

Amplification of 16S rRNA gene fragments was performed by Polymerase Chain Reactions (PCR) in a PCR tube containing the following: 30 ng/μl DNA, 2.5 μl water,

15 µl master mix, and 1.35 µM of each the forward and reverse primer. The universal primers used were RibS74sp 5'-AAGGAGGTGATCCAGCCGCA-3' and RibS73sp 5'-AGAGTTTGATCCTGGCTCA-3'. The total volume of every PCR tube was 25 µl. The PCR reaction cycles were in order as follows; a 3-minute initial denaturation step done at 94°C, 45 seconds repeated over 35 cycles also at 94 °C, a 45-seconds annealing step done at 53°C, a 90-second elongation step at 72°C, then ending with 4-minute extension step at 72°C as well (figure 1).

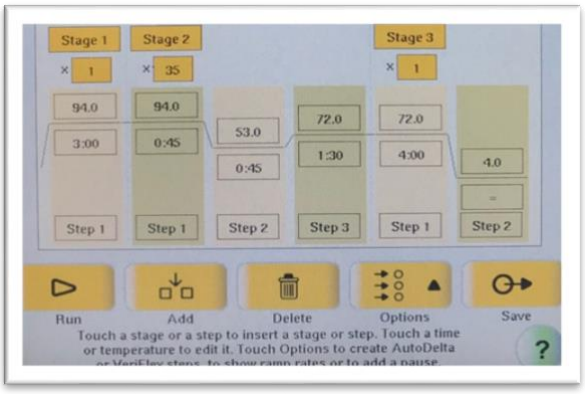


Figure 1. PCR running method showing details on the cycles run.

3.5.3 Gel Electrophoresis preparation

For the preparation of the gel, 0.5X of TAE (Tris-Acetate-EDTA) Buffer was first prepared. 1% agarose gel was then prepared: 1 gram of agarose was added to 100 ml of 0.5X TAE buffer and dissolved in a microwave and mixed very gently by swirling until the mixture became clear and homogenous. Very carefully, 0.5 µg/ml of Ethidium Bromide was added. Gently, the gel was poured into the plate, and the comb was placed on top of the gel and left to cool down and solidify. After approximately 15 min the tray with the gel was transferred to the electrophoresis apparatus, making sure the wells are directed towards the negative electrodes, and the comb was removed cautiously.

TAE buffer was added to cover the gel almost only 1mm above it.

In the first well a 100-bp DNA ladder was added. In the rest of the wells, the following was mixed on a parafilm paper and transferred with a pipette: 5µl autoclaved distilled water, 5µl sample DNA, and 2µl loading dye. The voltage of the apparatus was adjusted to 50V and the timer set to 50 min and the cycle started.

3.5.4 Purification of PCR products

The PureLink® PCR Purification Kit was used for PCR products purification. Following the protocol provided, the purification steps we done as follows:

1. 10 µl of each PCR product was placed in a sterile eppendorf tube. A volume of 40 µl of binding buffer 2 was added. The tube was mixed well.
2. The total of 50 µl was pipetted into a PureLink® Spin Column in a collection tube provided in the kit. The column was then centrifuged at maximum speed for 1 minutes before discarding the flow-through.
3. The column was inserted again into the collection tube and washed with 650 µl of washing buffer (W1) with ethanol. The column was again centrifuged for 1 minute at maximum speed before discarding the flow-through. The column was centrifuged for 3 min at 13,000 rpm.
4. The column was placed into a 1.7 ml Elution Tube provided within the Kit. 50 µl of the elution buffer was added in the very center on the column and the tube was kept at room temperature for 1 min before centrifuging the column for 2 minutes at maximum speed.
5. In the end the purified PCR products were contained in the elution tubes and were stored at -20 °C.

Before sending the samples for Sanger sequencing, a Nano-drop spectrophotometry was performed to quantify DNA and RNA concentrations.

3.6 Screening for mineral-forming bacterial isolates

On solid MD1 plates, each isolate was plated and incubated at 30 °C for 14 days. However, on regular basis the plates were constantly checked for the occurrence of precipitated minerals (crystals) under light microscopy. Upon the detection of any minerals at 40X or 100X, they were extracted from the plate and closely studied under 100X magnification by performing the "Acid test" (King, 2016) in which the presence of carbonate minerals is confirmed if the crystals dissolve and CO_2 bubbles of gas are produced when in contact with HCL (1M) added to the slide from underneath the cover slip.

3.7 Spectroscopic Analysis

3.7.1 Scanning electron microscopy, Energy-dispersive X-ray spectroscopy (SEM/EDS)

Scanning electron microscope was used in order to analyze the precipitates formed by the isolated strains, and to examine the morphological differences existing between them. The samples were prepared first by carefully scrapping the upper layer formed on the petri-dishes with the help of sterile scalpels. Much of the precipitated crystals were embedded in the media itself, therefore it was extensively cut into tiny pieces and introduced into 50 ml tubes. Then the samples were purified and homogenized by standard washing methods with water, then centrifugation for 15 min at 5000 g. Before allowing the samples to dry, the crystals were again observed under light microscopy to insure that the recovery of the minerals was successful. The samples were then dried in air at 40°C for 24 h. For SEM/EDX analysis the samples were coated with carbon followed by a gold layer according to the required protocol.

3.7.2 X-ray Diffraction (XRD)

The same procedure described in section 3.7.1 was used for the preparation of the minerals for XRD analysis. This analysis allowed for the investigation of the

mineralogical composition of the studied mineral samples, by using a Rigaku model-Miniflex II Desktop X-ray Diffractometer, equipped with a Scintillator NaI (TI) detector and selecting the targeted molecules.

3.8 Determination of Carbonic anhydrase enzymatic activity

3.8.1 sample preparation

The set of reference *Virgibacillus spp* strains (table 1) previously isolated and preserved in our laboratory were selected for testing of the carbonic anhydrase activity along with the newly isolated mineral forming isolates. Enrichment cultures were prepared from freshly cultured isolates on MD1 media and incubated at 30° for 5 days. At 24, 48, 72, 96 and 120 hours, 1 ml was transferred from the enrichment culture into a sterile Eppendorf tube. The tube was centrifuged for 5 min at 8000 rpm, the supernatant transferred into a new tube, and the pellet was washed twice with 300 µl 0.1M phosphate buffer, Then the pellet was suspended in 1 ml of the same buffer.

To evaluate the carbonic anhydrase activity inside the cells, 1 ml of the culture was centrifuged for 5 min at 8000 rpm, the supernatant discarded, and the pellet washed twice with 300 µl of 0.1M phosphate buffer and centrifuged. Then the pellet was re-suspended in 1ml of buffer with lysozyme and incubated for 30 min in a water bath at 37°.

The supernatant, cells, and lysed cells were tested separately to perform the assay. LB, buffer, buffer and lysozyme were used as a control for the supernatant, cells, and intracellular material respectively.

3.8.2 Esterase assay

A carbonic anhydrase assay was performed, following Ramanan et al. (2009). The reaction is based on a reversible hydration and was experimented as follows:

CA enzyme reacts with p-nitrophenyl-acetate (p-NPA) to produce p-nitrophenol and acetate, which produces a yellowish color. Hence, bacterial strains' activity was

investigated at the level of both the cells and the supernatant.

250 μl of the tested sample was added to 750 μl of 0.1M PBS buffer (Kordel et al., 1991). At the moment of the addition of the substrate NPA, which is the beginning of the reaction, the absorbance is measured over the course of 10 minutes at 405nm.

NPA stock solution for esterase activity determination was prepared by dissolving 63 mg in 10 ml of ethanol with strong agitation and stored at 4°C. Later 1ml of this stock solution was added to 100 ml of distilled water for experimental use and was constantly checked for precipitation hat would interfere in the results.

3.8.3 CO_2 hydration assay (Wilbur–Anderson assay)

The WA assay was carried out by mixing 100 μl of the solution containing the enzyme, 12.0 mL Tris-HCl buffer solution (20mM, pH 8.3), and 8.0 ml of CO_2 saturated water. The timer was set the moment the CO2 water was added to the solution (start of the reaction), and the time taken for the pH to drop by 1 unit (to pH 7.3) was recorded. The control solution used was Tris–HCl buffer (pH 8.3) without any enzymatic solution. Three replicates were recorded, and the average of all was used in the rest of the calculations.

The formula used to quantify CO_2 hydration activity is:

$(T_c - T_{test}) / T_{test}$

Where:

T_c: Time needed for one unit pH drop in the absence of CA.

T_{test} : Time needed for one unit pH drop in the presence of CA.

The hydration activity was then quantified as follows: each unit of CO*2* hydration activity corresponds to the amount of CA required to decrease the pH of the buffer by 1 unit from 8.3 to 7.3, which is expressed as WA units per unit volume.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Isolation of aerobic, halophilic, heterotrophic bacteria from living mats

4.1.1 Identification by MALDI TOF MS

It is known that the key factor influencing the formation of Mg-rich minerals does not in fact lie in the replacement process, but in the microbial EPS found in decaying and living mats (Brauchli et al., 2016). Indeed, SEM analysis of living microbial mats from Dohat Faishakh have succeeded in finding a protruding organic matrix containing the EPS very much similar to that of dolomite in living mats, but failed to reach the same outcome when decaying have been investigated (Brauchli et al., 2016).

Twelve bacterial isolates were isolated from the living mat sampled from Dohat Faishakh sabkha in Qatar by enrichment culture on the mineral inducing medium MD1 (Table 3). The study of living mats in this research intended to highlight the microbial community present in it as opposed to that in the decaying mats, of which 23 strains where used as reference (Table 1).

The 12 newly isolated strains were subjected to identification by MALDI-TOF MS. The twenty-three strains, previously isolated from decaying mat sampled from the same sabkha and identified by ribotyping as *Virgibacillus* bacteria, were used as reference for possible identification by MALDI-TOF mass spectrometric protein profiling, by matching their scores in the available database.

The results showed that the method based on ethanol/formic acid extraction of proteins before MALDI-TOF MAS analysis was not effective, since none of the isolates was reliably identified, although mass spectra were obtained. In fact, Wang et al. (2012) proceeded with extraction, because identification with whole cells of their bacteria was not effective. Here, the last method was effective to identify *Bacillus* strains. Table 2 shows the list of *Virgibacillus* strains used as reference and the 12 isolates from the living mat, with their corresponding code in the MALDI TOF MS protein profiling

employed for the data analysis by PCA and dendrogram. All strains identified by ribotyping as *Virgibacillus* exhibited MALDI TOF MS scores less than 1.70 that cannot provide their reliable identification, according to the available database in the used machine. Of course, this incongruence was because the identification using the MALDI-TOF MS approach is dependent on the MALDI BioTyper database (Krakava et al., 2018). The key component in all the platforms that have been commercialized for identification purposes in several markets worldwide is strictly the database of organisms (Singhal et al.,2015). However, interestingly, a reproducible protein profile obtained by MALDI TOF MS profiling was obtained for all these *Virgibacillus* isolates. From the 12 newly isolated isolates, four were identified at the level of *Bacillus* genus *(*two *Bacillus cereus,* one *Bacillus circulans* and one *Bacillus Licheniformis)* with corresponding scores of 1.87, 2.25, 1.76, and 1.81 respectively. The others exhibited scores less than 1.70, which cannot identify them, reliably.

The significance of using this method for identifying bacterial isolates or closely related ones lies in the fact that masses and signals of proteins serving as markers, interlinked representatives of their corresponding expressed genes. Moreover, mass spectra and protein profiles were established for each isolate.

Table 3: Identification of the 35 bacterial strains isolated from decaying and living mats of Dohat Faishakh sabkha (Qatar), using MALDI-TOF MS. ND: Not determined. NR: Not reliable.

Strain Code	Identification by Ribotyping	MALDI TOF Score	Identification by MALDI TOF
DF112	*Virgibacillus marismortui*	< 1.70	NR
DF221	*Virgibacillus sp.*	< 1.70	NR
DF231	*Virgibacillus marismortui*	< 1.70	NR
DF241	*Virgibacillus sp.*	< 1.70	NR
DF252	*Virgibacillus sp.*	< 1.70	NR
DF281	*Virgibacillus salarius*	< 1.70	NR
DF282	*Virgibacillus sp.*	< 1.70	NR
DF291	*Virgibacillus sp.*	< 1.70	NR
DF322	*Virgibacillus marismortui*	< 1.70	NR
DF341	*Virgibacillus marismortui*	< 1.70	NR
DF351	*Virgibacillus salarius*	< 1.70	NR
DF411	*Virgibacillus marismortui*	< 1.70	NR
DF431	*Virgibacillus sp.*	< 1.70	NR
DF451	*Virgibacillus sp.*	< 1.70	NR
DF461	*Virgibacillus salaries*	< 1.70	NR
DF472	*Virgibacillus marismortui*	< 1.70	NR
DF491	*Virgibacillus marismortui*	< 1.70	NR
DF2102	*Virgibacillus sp.*	< 1.70	NR
DF2121	*Virgibacillus sp.*	< 1.70	NR
DF2131	*Virgibacillus sp.*	< 1.70	NR
DF2141	*Virgibacillus sp.*	< 1.70	NR
DF2161	*Virgibacillus sp.*	< 1.70	NR
DF2172	*Virgibacillus sp.*	< 1.70	NR
K011	ND	1.81	*Bacillus licheniformis*
K1031A	ND	2.25	*Bacillus cereus*
K103B	ND	1.87	*Bacillus cereus*
K9-3-1	ND	1.76	*Bacillus circulans*
K012A	ND	< 1.70	NR
K012B	ND	< 1.70	NR
K9-1-1	ND	< 1.70	NR
K9-1-2	ND	< 1.70	NR
K9-1-4	ND	< 1.70	NR
K915A	ND	< 1.70	NR
K9-2-1	ND	< 1.70	NR
K9-2-2	ND	< 1.70	NR

4.1.2 Analysis of protein profile

TOF (time of flight) mas analyzers have been in use for a variety of applications especially those in the field of microbiology because the information they generate about the analyzed samples is in the form of a characteristic spectrum named peptide mass fingerprint (PMF). Upon the analysis by MALDI-TOF MS, identification of microorganisms is done by matching and comparisons done between the tested sample's PMF and those found in the database.

Hereby, MALDI-TOF-MS provides a platform for the comparison of mass spectra of the 35 studied isolates from Dohat Faishakh sabkha, analyzed using the data analysis software. The obtained peaks revealed admissible resolution using the whole cells, whereas proteins extraction method revealed no separate peaks. For each strain, an average spectrum was obtained from duplicated spectra of triplicated colonies (six in total). Examples of the spectra obtained are shown in figure 1. Growth media and conditions were similar to all isolates, to reduce culturing impact on the protein profiles, biomarkers and reproducibility of the profiles. The mass spectra demonstrated complex collections of discrete ions of m/z ratios ranging between 2,000 and 10,000. The range 2-20 kDa is selected is because that is the range the generally represents the ribosomal proteins which comprise 60 to 70% of the dry mass of a microbial cell (Murray, 2012).

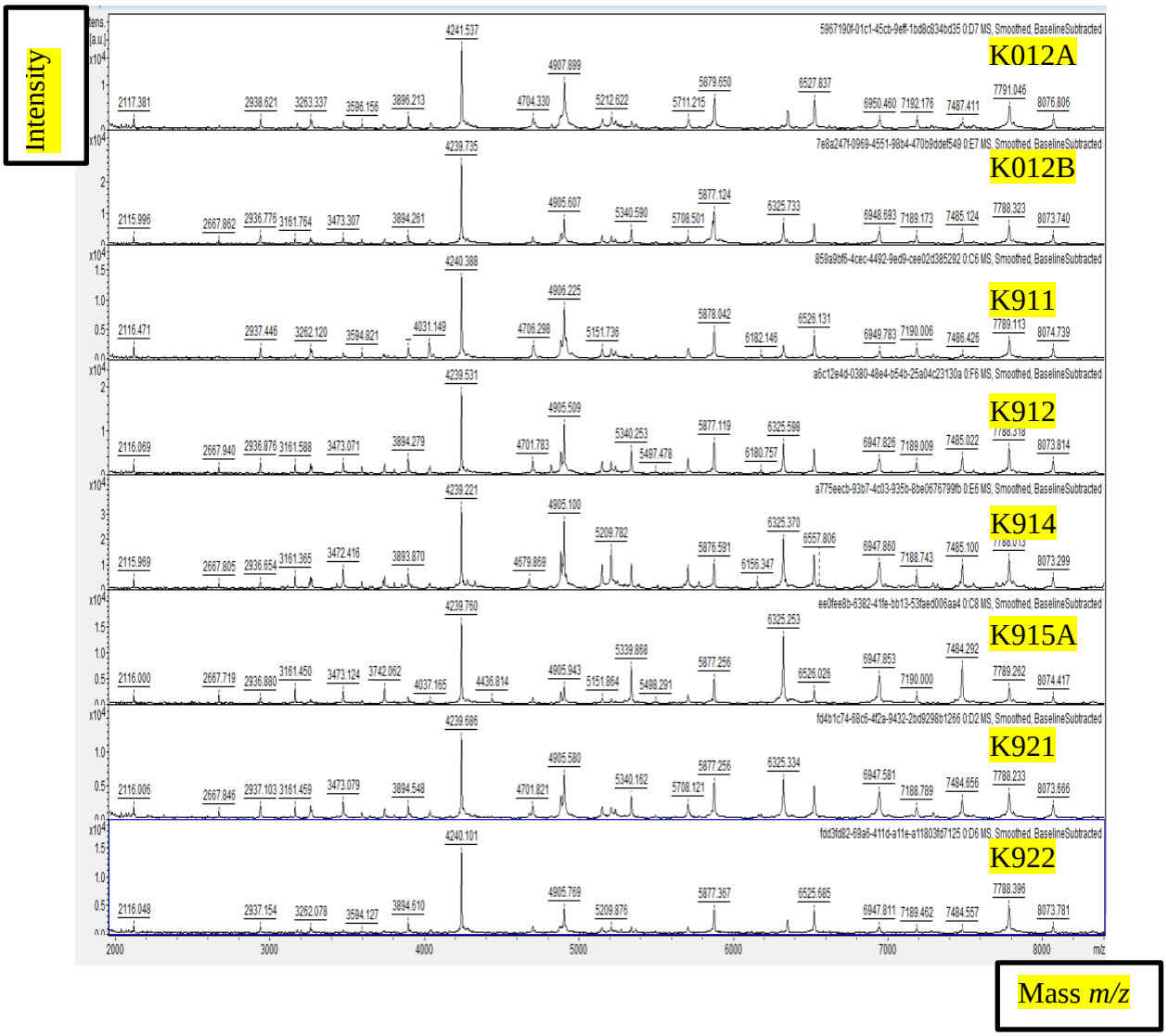


Figure 2. Examples of MALDI-TOF spectra of the group of the newly isolated strains of the used in this study. Mass spectra analysis of the obtained *m/z* values ranging between 2000 and 8500.

4.1.3 Characteristic mass peaks

Growth media and conditions were similar to all isolates, to reduce culturing impact on the protein profiles, biomarkers and reproducibility of the profiles. The mass spectra demonstrated complex collections of discrete ions of m/z ratios ranging between 2,000 and 10,000. However, comparisons made based on only PMFs of the isolates is not enough to clearly detect the differences that lie between each. Hence, a comparison was done between the mass peaks generated by MALDI-TOF for every strain, and based on

the common peaks, the 35 isolates was divided into 3 groups. Table 4 shows peak masses of 17 *Virgibacillus* reference strains and Table 5 shows those of 7 isolates all belonging to the living mats. And Table 6 comprises the other 6 *Virgibacillus* reference strains along with 4 newly identified *Bacillus* isolated strains (K011, K1031A, K1031B, and K931). Peak highlighted in bold are peaks obtained by most of strains.

The intensity of the generated peaks allows the detection of some unique proteins considered as biomarkers among highly related isolates. Indeed, peak of 4905 and 9815 m/z are only obtained with *Virgibacillus* strains, they can be considered as biomarkers of the genus *Virgibacillus*. However, peak masses of 4240, 6430 and 7765 m/z are found commonly in most *Bacillus* and *Virgbacillus* strains. This results is not surprising, since *Virgibacillus* genus is strongly similar to the *Bacillus* genus. It was reclassified fom *Bacillus* genus in 1998 based on analysis of the species *Virgibacillus pantothenticus* (Heyndrickx et al., 1998). However, at the cultural conditions favorable to minerals formation, *Virgibacillus* strains produced much more proteins than *Bacillus* strains.

Therefore, these indicated peaks are regarded as genus specific biomarkers for the known genus. Thus, it can be used for future identification of newly isolated bacterial strains belonging to these genera. Moreover, the mass spectra of *Virgibacillus* appear to be highly similar, at the species level. Indeed, the peak at 3880 m/z was detected in five *V. marismortui* strains in addition to the peak at 4240 m/z in four of them. This suggests that these two protein peaks could be possible species-specific biomarkers of *V. marismortui*. On the other hand, some common peaks were present in most of the isolates, but none was shown to be species specific in that sense.

In a third group of *Virgibacillus* isolates (DF221, DF282, DF341, DF351, DF491 and DF2141) the peak at 3265 m/z is specific (Table 6). None of the other reference

Virgibacillus strains revealed this peak, which suggests that the peak 3265 m/z is specific to some *Virgibacillus*, not to all. In addition, the biomarker 3265 m/z was not found in the mass spectra of any of the *Bacillus* strains grouped in this cluster. Six other peak masses were detected in most *Virgibacillus* strains (m/z 3880, 4240, 4905, 6430, 7765, and 9815), showing high similarity among all studied *Virgibacillus sp.*

Table 4: Characteristic peak masses of 17 *Virgibacillus* reference strains. The values represent the arithmetic means of the m/z values of three replicates of the corresponding strains. Peak masses corresponding to are genus specific are highlighted in bold.

Strains	DF411	DF2121	DF322	DF472	DF461	DF451	DF2102	DF241	DF112	DF2172	DF231	DF291	DF2131	DF2161	DF281	DF252	DF431
Characteristic Peaks							2855				2855	2855					
	3210		3210				3210					3210					
	3880		3880	3880		3880	3880	3880	3880	3880	3880	3880		3880	3880		3880
	4240		4240				4240		4240		4240	4240		4240	4240		4240
	4905	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**
						5240	**5240**				**5240**	**5240**					**5240**
	5910						5910		5910		5910	5910					5910
		6180	6180										6180	6180		6180	
	6430	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**	**6430**
		6750	6750										6750				
			6905		6905								6905	6905			
	7765	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**	**7765**
	9815	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**	**9815**
	10485						10485		10485		10485	10485					

Table 5: Characteristic protein peaks of the unidentified bacterial isolates. The values represent the arithmetic means of the m/z values of three replicates of the corresponding strains. Peak masses corresponding to are genus specific are highlighted in bold.

Isolates	K012A	K012B	K911	K912	K914	K915A	K921	K922
Characteristic peaks	2115	2115	2115	2115	2115	2115	2115	2115
		2667		2667	2667	2667	2667	
	2940	2940	2940	2940	2940	2940	2940	2940
		3160		3160	3160	3160	3160	
	3260		3260					3260
		3470		3470		3470	3470	
	3595		3595					3595
	3880	3880		3880	3880		3880	3880
			4035			4035		
	4240	4240	4240	4240	4240	4240	4240	4240
	4700		4700	4700	4670		4700	
	4905	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**	**4905**
	5880	5880	5880	5880	5880	5880	5880	5880
			6180	6180	6180			
	6525		6525		6525	6525		6525
	6950	6950	6950	6950	6950	6950	6950	6950
	7190	7190	7190	7190	7190	7190	7190	7190
	7490	7490	7490	7490	7490	7490	7490	7490
	7790	7790	7790	7790	7790	7790	7790	7790
	8070	8070	8070	8070	8070	8070	8070	8070
	9400	9400	9400	9400		9400	9400	9400
	9800	9800	9800	9800	9800	9800	9800	9800
	10420	10420	10420	10420	10420	10420	10420	10420
	11415	11415	10415	11415	11415	11415	11415	11415

Table 6: Characteristic peak masses of 6 *Virgibacillus* reference strains and 4 newly isolated strains of *Bacillus* genus. The values represent the arithmetic means of the m/z values of three replicates of the corresponding strains. Peak masses found to exist in all strains of the group are highlighted in bold.

Isolates	DF282	DF341	DF491	DF2141	DF351	DF221	K1031B	K1031A	K931	K011
Characteristic Peaks	3265	3265	3265	3265	3265	3265				
	3880		3880	3880		3880		3880		
	4240		4240			4240				
	4905	4905	4905	4905	4905	4905				
	5240	**5240**	**5240**	**5240**	**5240**	**5240**	**5240**	**5240**	**5240**	**5240**
	6430		6430	6430	6430	6430	6430	6430	6430	6430
	7765		7765	7765	7765	7765	7765			7765
	9815	9815	9815	9815	9815	9815				

4.1.4 Similarity percentage

In order to confirm the differences in term of biomarkers at the level of species, the intra- and interspecific percentage of similarity was established for strains identified at the species level using Composite Correlation Matrix. Results are shown in Table 7. The differentiation showed existence of a wide range of similarity between strains belonging to *Virgibacillus* species which goes under “intraspecific similarity”, as well as similarity between different *Virgibacillus* species known as “interspecific similarity”. Thus, *V. marismortui* and V. salaries are shown highly related species with 70.1-99.5% interspecific similarity. Similar results were reported in 2015, but with phylogenic trees generated based on16S rRNA sequences of *Virgibacillus* species (Khelaifia et al., 2015). Here, evidences were simply provided by the rapid and reliable MALDI-TOF MS proteins profiling. However, *B. cereus* and *B. lechinforms* showed only 13-15% interspecific similarity. *B. circulans* was highly different from other *Bacillus* strains with extremely low similarity inter and intra-specifically.

Table 7: Intra- and Interspecific percentage of similarity between species studied.

Similarity Percentages (%)					
	Virgibacillus marismortui	*Virgibacillus salarius*	*Bacillus cereus*	*Bacillus licheniforms*	*Bacillus circulans*
Virgibacillus marismortui	100	70.1-99.5	0.5-18.4	0.2-14.7	0.2
Virgibacillus salarius	70.1-99.5	100	0.9-16.2	3.8-5.2	0.2
Bacillus cereus	0.46-18.4	0.9-16.2	100	13.2-15.3	0.1-0.3
Bacillus licheniforms	0.2-14.7	3.8-5.2	13.2-15.3	100	0.2
Bacillus circulans	0.2	0.2	0.1-0.3	0.2	100

In order to establish the similarities at the genus and species level, the interspecific percentage of similarity of the unidentified isolates shows that the unidentified strains should be classified in a group distinct to *Virgibacillus* strains (Table 8). The results show existence of a wide range of similarity with strains of *Virgibacillus* identified at the genus level, as well as similarity with different *Virgibacillus* species. Generally, similarities fluctuate between 20% to 40%, with the exception of the isolate K012A which shows 12% and 7% only with *Virgibacillus* DF2131 and *Virgibacillus* DF2102 respectively. This study clearly shows that the unidentified strains should be classified in a group distinct to *Virgibacillus* strains.

Table 8: Similarity percentages between unidentified isolates of group C and reference *Virgibacillus* sp. strains.

	Unidentified strains of Group C							
	25	**32**	**34**	**35**	**23**	**17**	**16**	**9**
Virgibacillus sp.	29.6	21.6	21.2	24.9	28	16.4	22.06	33.2
	30.8	23.8	26.5	28.1	27.4	23.3	26.1	35.15
	36.7	29	30.1	28	33	24.2	0.29	37.9
	37.5	32.4	35.1	38.3	35.2	30.2	34.9	41.2
	33.8	28.8	28.4	30.7	31.5	21	28.2	38.03
	32.7	25.02	28.4	11.7	31.3	25.9	29.3	37.6
	37.2	34.8	36.4	36.4	37	32.1	37.2	43.24
	31.2	28.3	28.4	32.4	28.7	24.1	29.7	35.8
	37.8	32.6	34	7.3	34.5	28.5	33	41.5
	32.2	30	30.6	34	32.5	26	31.7	38.93
	35.5	26.1	25.8	26.2	32.3	20.8	26.7	33.8
	34.9	25	28.7	28.7	31.4	26.7	29.5	37.4
	30.7	24.5	26.3	26.3	29	23.8	27.6	38.8
Virgibacillus marismortui	40.8	39.6	41.5	35.9	41.7	35.7	41.0	39.9
Virgibacillus marismortui	36.2	29.2	33.8	34.1	33.5	30.0	33.2	39.6
Virgibacillus salarius	30.9	25.1	27.6	25.5	29.0	26.6	29.3	38.2
Virgibacillus marismortui	35.6	32.2	32.7	31.6	33.8	25.9	32.2	38.4
Virgibacillus marismortui	33.7	27.3	23.7	27.1	29.5	18.3	24.5	35.7
Virgibacillus salarius	28.6	23.2	23.7	28.5	28.5	20.3	25.3	35.5
Virgibacillus marismortui	30.5	24.4	24.3	26.3	29.8	17.9	23.8	36.5
Virgibacillus salarius	33.7	28.0	29.4	29.7	31.5	24.6	29.2	38.8
Virgibacillus marismortui	37.6	34.9	34.9	32.8	34.6	25.9	32.8	40.8
Virgibacillus marismortui	30.9	26.4	27.1	28.6	30.3	22.6	28.2	36.9

4.1.5 Relationships and clustering by PCA

Although the information about the relationship between bacterial isolates can be obtained by comparison of the protein m/z fingerprints in MALDI-TOF MS profiles, however, differentiation between the closely related isolates can be revealed by combination between MALDI-TOF MS analysis and PCA. PCA provides a rapid qualitative assessment tool for determining the association among the studied isolates and evaluating large data sets (protein profiles). MALDI-TOF MS instrument employed in this research has a built-in software for statistical analysis in which Protein profiles can be directly analyzed (after baseline subtraction and smoothing). The values used in the PCA are exact m/z values and their relative intensities. Principle component analysis (PCA) is one of the oldest methods used by statisticians in order to interpret large datasets (Jolliffe & Cadima, 2016). This method analyzes matrices of variance-covariance and correlations of data. Its main objective is to use principal components as a way of reducing the dimensions of objects being studied. Furthermore, this reduction creates linear combinations of variables representing the objects being studied. The combinations are known as principal components. Table 9 shows the PCA codes that were used in the clustering diagrams below.

Table 9: PCA codes corresponding to each strain ID.

Strain ID	Strain Identity	PCA CODE
DF112	*Virgibacillus marismortui*	31
DF221	*Virgibacillus sp.*	19
DF231	*Virgibacillus marismortui*	1
DF241	*Virgibacillus sp.*	18
DF252	*Virgibacillus sp.*	27
DF281	*Virgibacillus salarius*	21
DF282	*Virgibacillus sp.*	3
DF291	*Virgibacillus sp.*	4
DF322	*Virgibacillus marismortui*	7
DF341	*Virgibacillus marismortui*	14
DF351	*Virgibacillus salarius*	8
DF411	*Virgibacillus marismortui*	11
DF431	*Virgibacillus sp.*	30
DF451	*Virgibacillus sp.*	5
DF461	*Virgibacillus salaries*	2
DF472	*Virgibacillus marismortui*	15
DF491	*Virgibacillus marismortui*	20
DF2102	*Virgibacillus sp.*	22
DF2121	*Virgibacillus sp.*	12
DF2131	*Virgibacillus sp.*	13
DF2141	*Virgibacillus sp.*	28
DF2161	*Virgibacillus sp.*	29
DF2172	*Virgibacillus sp.*	10
K011		26
K1031A		33
K103B		6
K9-3-1		24
K012A		35
K012B		9
K9-1-1		16
K9-1-2		25
K9-1-4		34
K915A		32
K9-2-1		17
K9-2-2		23

The results of PCA are shown in Fig 3. From Figure 3-a it is clear that the strains exhibit large biodiversity at the protein level. The total variance of the 10 PCA's is shown in Figure 3-b. It shows that the three principle components, that is PC1, PC2 and PC3 each carrying percentages of 32, 21.5 and 12.5 respectively, combine to show 66% variability in the data. These three clusters have been used and resulted in the grouping of three clusters of isolates. The distance between the clusters shows the variation at groups level, while the distance between the strains (within the cluster) shows the differences in protein profiles at strain level. Cluster 1, which has positive correlation to PC1 and negative correlation to PC2 and PC3, include unidentified strains (K012B, K911, K921, K922, K914, K912, K915A, and K012A). Whereas, cluster 2 has positive correlation to all three components and is mainly comprised of *B. cereus* and *B. licheniformis* (K1031B, K011 and K1031A) demonstrating variation in their protein profiles in comparison to other studied strains. These results are in complete agreement with previous studies showing that these two species give well detectable and easily distinguishable band pattern profiles. Nevertheless, differentiation between *B. cereus* and *B. lichenifroms* (cluster II). Remaining strains (DF231, DF291, DF2172, DF2131, DF2161, DF281, DF431, DF241, DF2102, DF461, DF112, DF252, DF322, DF451, DF472, DF411, DF2121) can be categorized separately, in which the variations between the strains are also higher since they are located relatively far from each other e.g. strains DF291 and DF2121 of respective PCA codes 4 and 12, as clearly shown in Fig. 3-a.

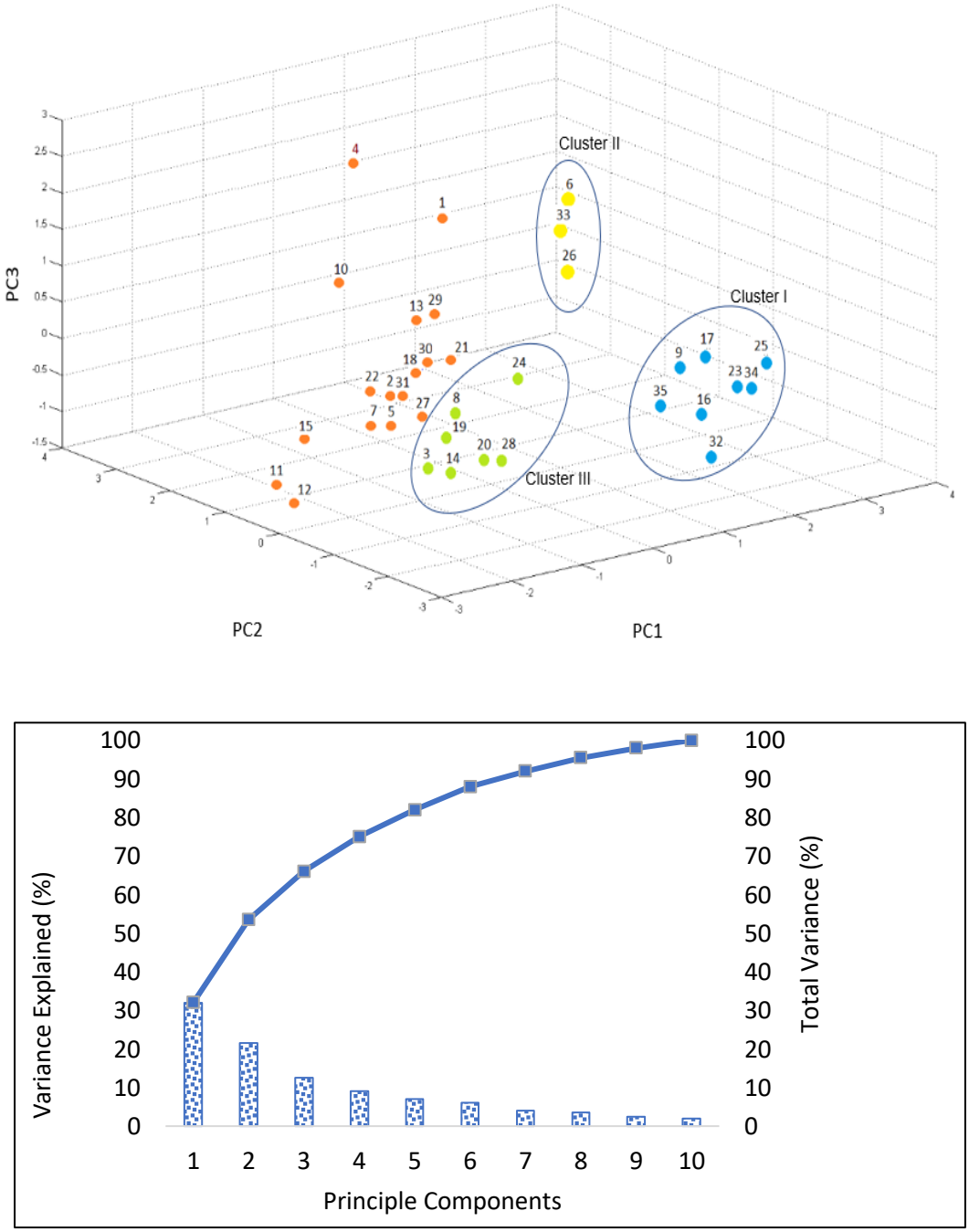


Figure 3. Classification of strains using PCA (a) PCA plot (b) Percentage of variance explained.

4.1.6 Clustering by a Phylloproteomic tree

The PCA results lead to the establishment of a dendrogram as a phylloproteiomic tree (Fig. 2). It shows that the studied strains may be categorized into three distinct groups

based on their similarity matrix. The 23 *Virgibacillus* which are used as reference, are clearly divided into two separate groups (A and B) in the phylloproteiomic tree. The isolates *V. marismortui* (DF112, DF231, DF322, DF411 and DF472), *Virgibacillus sp.* (DF 241, DF252, DF291, DF451, DF2121, DF2102, DF2131, DF2161 and DF2171), *V. salarius* (DF281, DF461 and DF431) were grouped together (group A). On the other hand, the other reference strains *Virgibacillus sp.* (DF221, DF282 and DF2141), *V. marismortui* (DF341 and DF491) and *V. salarius* 351 were categorized separately into another group (group B) along with four newly isolated strains that have been identified by MALDI-TOF, *Bacillus cereus* (K1031A and K1031B), *Bacillus licheniformis* (K011), and *Bacillus circulans* (K931). The eight remaining strains were categorized in one group (group C). Those strains are the ones isolated from living mats which could not be identified by MALDI-TOF (K012A, K012B, K911, K912, K914, K915A, K921, and K922). Attempts to show any similarities between these strains and other *Virgibacillus* or *Bacillus* species have failed. Clustering analysis by neither PCA nor dendrogram could show any significant relationship between isolates of group A and those of groups B and C; therefore, it can only be deduced that they have no resemblance with any *Bacillus* or *Virgibacillus* strains in this study.

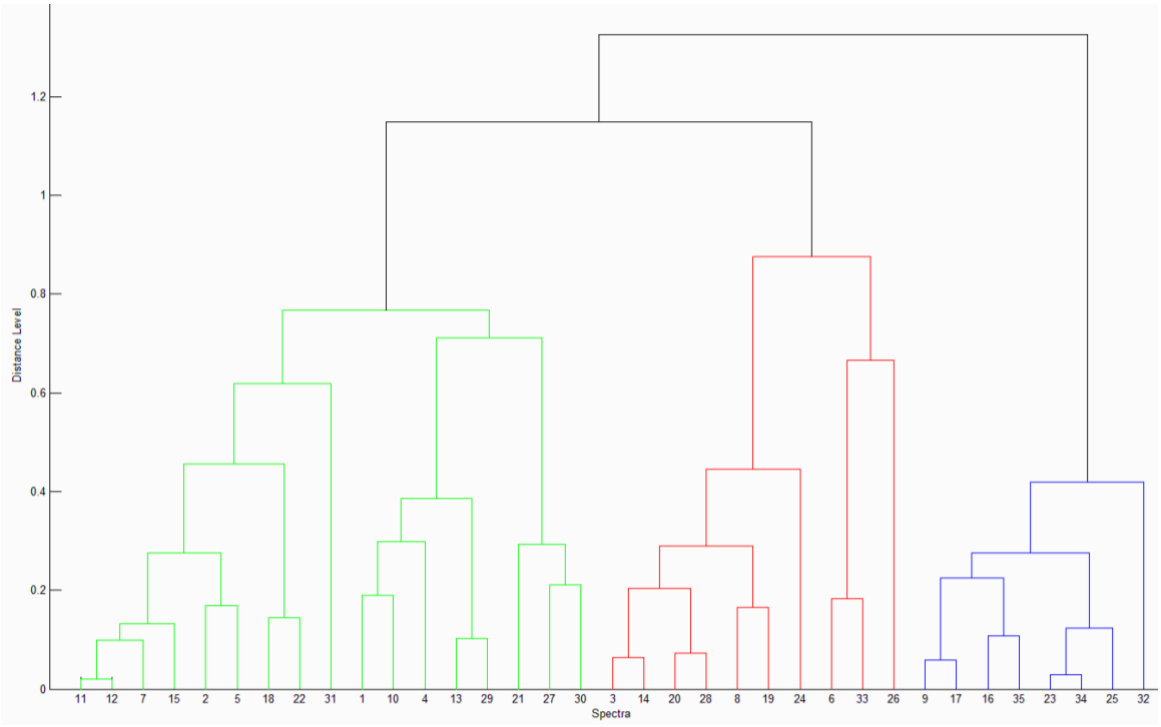


Figure 4. Phylloproteomic tree illustrating the relationship among the strains. The scale on the y axis represents the relative distance using during the analysis.

In comparison to each other, both approaches of clustering; PCA and dendrogram, have showed quite similar results. In fact, both approaches allow clustering of isolated and reference strains, which means that the data obtained by MALDI-TOF MS could, orient the prediction of the identity of unknown isolates belonging to any of the clusters. Nevertheless, strains are differentiated using the presence/absence of one or more discriminating peaks without presenting any hierarchical relationship between them in the PCA approach, but it is only using the dendrogram that hierarchical clustering of samples is made possible, in which the relationship between the strains in the same group and those in different ones are presented.

4.1.7 Molecular identification by 16S rRNA ribotyping

The newly isolated bacteria have also been identified as described in section by molecular ribotyping since the MALDI TOF technique have failed to match 8 of the isolates from the living mats to any of its database protein profiles. The sequences obtained have been matched with identical sequences in the Gene Bank by BLAST (Table 10).

Table 10: List of bacterial strains isolated from living mats identified by ribotyping.

Sample ID	**Identity**	**Accession No.**	**Similarity Score**
K1031A	*Bacillus cereus*	MN647516.1	99.63%
K912	*Virgibacillus dokdonensis*	MK622389.1	100%
K011	*Bacillus licheniformis*	MN865990.1	100%
K922	*Virgibacillus chiguensis*	MK622392.1	100%
K1031B	*Bacillus cereus*	MF407480.1	97%
K911	*Virgibacillus dokdonensis*	MK488064.1	99.51%
K012A	*Virgibacillus pantothenticus*	MF449183.1	98.75%
K914	*Virgibacillus dokdonensis*	MK488064.1	98.56%
K931	*Virgibacillus dokdonensis*	MK488064.1	99%
K915A	*Virgibacillus dokdonensis*	MK488064.1	98%
K921	*Virgibacillus chiguensis*	MK622392.1	99.97%
K012B	*Virgibacillus pantothenticus*	MF449183.1	100%

The certain identity of the strains obtained by molecular techniques serves as a tool for determining the level of reliability of the proteomic identification done using MALDI-TOF MS. Comparing the 4 strains previously identified by MALDI; K1031A, K011, K1031B and K931, we find that the matched species are in congruence with those matched by BLAST for 3 out of the 4 strains. MALDI-TOF gave strain K931 a relatively low score of 1.76 when identified as *Bacillus circulans,* which was hence found to be a *Virgibacillus dokdonensis* strain molecularly. Therefore, although MALDI-TOF classifies scores below 1.7 as non-reliable, in our study a score between 1.7 and 1.8 has also not been found to be correct. Nevertheless, clustering analysis

based on the PMF's of the studied strains was more accurate since cluster I comprised isolate K931 along with the other *Virgibacillus dokdonensis* strains (Figure 3).

Another interesting finding lies in the fact that although many strains of the genus *Virgibacillus* have also been isolated from living mats, none of them was found to be have similar protein profiles to *Virgibacillus* strains isolated previously from the decaying mats. This is strange since it is expected that strains belonging to the same genus would exhibit similar PMFs. But when it comes to the ability of these strains to induce mineral formation, the differences they showed becomes quite explainable. All the reference strains have been selected due to their cited ability to precipitate carbonates specifically with high Mg: Ca ratios; however, none of the *Virgibacillus* strains isolated from the living mats have showed any precipitation capacity on MD1 medium (Al Disi et al., 2017).

In this study, MALDI-TOF MS has clearly demonstrated that differences in the significant activities of microorganisms is reflected upon their protein profiles, which renders the classification using clustering analysis and PCA along with detection of protein biomarkers an efficient and rapid method of categorization.

4.2 Isolation of mineral forming bacteria from coral sediments

In many of the early geological process that have shaped the earth and its structures, microorganisms have played a significant role in the $CaCO_3$ precipitation. In particular, it has contributed and still contributes to the global carbon cycle (Li et al., 2011). A large portion of these processes has participated in the early diagenesis of marine sediments. Consequently, they have been the center of attention of much research for the sake of investigating the $CaCO_3$ precipitation mechanisms. Despite it being well established that an important role is played by bacteria in the promotion of $CaCO_3$ precipitation, the mechanism acquired by organisms remains vague. Nevertheless, the proposed mechanisms after multiple research studies are varied between EPS,

involvement of microbial mats, photosynthetic microorganisms in upper layers, metabolic pathways involving urease, ammonification and denitrification, all of which lead in a way or another to the generation of carbonates in all layers from upper oxic to deep anoxic (Castanier et al., 2000; Bachmeier et al.,2002).

Deposits of $CaCo_3$ in marine sedimentary structures are ubiquitous and could hold a record of the earliest forms of life on planet Earth. In the past decade, numerous evidence have been found suggesting that morphology of $CaCO_3$ and sediments is not in fact altered by bacteria, but in fact it is the other way around; it is the composition and polymorph selection of sediments and calcium carbonates that affecting the precipitation of the microbial community surviving in it (Wei at al., 2015).

Hereby, we aim at investigating the microbial biodiversity in Qatari marine costal sediments and identifying $CaCO_3$ mineralizing bacteria. As described in section 3.2, 2 sampling sites from marine sediments have been studied. The isolated bacteria from each are presented in tables 11 and 12. The isolates have been identified by MALDI-TOF mass spectrometry.

Table 11: Bacterial isolates from marine sediments sample "2A".

Strain ID	Identified organism by MALDI	Score
1	*Exiguobacterium arantiacum*	1.74
2	*Exiguobacterium arantiacum*	1.90
3	*Vibrio alginolyticus*	2.09
4	*Photobacterium damselae*	2.30
5	*Pyschrobacter sp*	1.73
7	*Vibrio alginolyticus*	1.98
8	*Vibrio alginolyticus*	2.05
9	*Vibrio alginolyticus*	1.86
10	*Vibrio alginolyticus*	2.23
11	*Exiguobacterium arantiacum*	2.06
12	*Exiguobacterium arantiacum*	1.90

Table 12: bacterial isolates from marine sediments sample "2B".

Strain ID	Identified organism by MALDI	Score	Molecular identification	Accession No.
S1	NR	<1.70	*Pseudomonas cedrina*	MN758770.1
S2	NR	<1.70		
S3	*Vibrio alginolyticus*	2.08		
S4	NR	<1.70		
S5	*Pyschrobacter sp*	1.73		

Sample “2A” was dominated by two bacterial species, *Vibrio alginolyticus* and *Exiguobacterium arantiacum* with 5 and 4 species each, respectively. There is a possibility that these isolates belonged to the same strain; however, they have been sub-cultured based on distinct morphologies. The biodiversity is significantly less in sample “2B” with only 5 different isolates identified, three of which received a low score in MALDI-TOF and therefore were not identified. Molecular identification of these strains was not yet performed at the time this thesis was submitted due to unexpected circumstances.

In the further investigaton, *Photobacterium damselae* was excluded from the study due to its severe pathogenicity (Romalde, 2002; Fouz et al., 2000). Not only does this species cause infection in fish, sharks, and other marine animals, but it is also pathogenic to humans. *P. damsalae* has been reported to cause severe necrotizing faciitis, and if not treated in time may lead to fatality (Rivas et al., 2013).

4.2.1 Investigation of mineral-forming potentials

Using different media cultures, the ability of all the marine bacterial isolates to form carbonate minerals was investigated. Since growth media MD1 has been previously reported to mediate precipitation by *Virgibacillus* species from decaying sabkha mats, it was the first media on which the isolates were sub-cultured and incubates for 20 days at 30°C (Al Disi et al., 2017). Subsequently, modifications on the media were made and

the occurrence of solid phase minerals was closely assessed under optical microscopy every day. Media composition MD1 was used as described in section 3.3. MD1- is another modified media that was sued for screening, it has the same composition as MD1 but without any glucose, MD2 is excluding yeast, and MD3 is excluding peptone. The growth and precipitation results are represented in table 13.

Table 13: Results of the investigation of mineral forming bacteria isolated from marine samples 2A and 2B. (NG) no growth; (-) growth with no precipitation; (+) growth with precipitation; (++) growth with significantly higher precipitation.

Strain/Isolate ID	MD1	MD1-	MD2	MD3
***Exiguobacterium arantiacum* (1)**	-	-	-	-
***Exiguobacterium arantiacumi* (2)**	-	-	-	-
***Exiguobacterium arantiacum* (11)**	-	-	-	-
***Exiguobacterium arantiacum* (12)**	-	-	-	-
***Vibrio alginoliticus* (7)**	-	NG	NG	NG
***Vibrio alginoliticus* (8)**	-	-	NG	NG
***Vibrio alginoliticus* (9)**	-	+	-	-
***Vibrio alginoliticus* (10)**	-	NG	NG	NG
***Vibrio alginoliticus* (S3)**	+	(++)	-	-
S2	+	+	-	-
S4	+	+	-	-
***Psychrobacter sp.* (5)**	+	+	+	+
***Pseudomonas cedrina* (S1)**	(++)	+	-	-

None of the *Exiguobacterium arantiacum* isolated strains have shown any level of mineralization on neither of the media tested, although other species belonging to the genus *Exiguobacterium* also isolated from marine environments have been recently reported to biomineralize and exhibit enzymatic activities that induce precipitation under high salt stress conditions (Bansal et al., 2016). However, this does not exclude

Exiguobacterium arantiacum capability to mineralize since it belongs to psychrophiles and consequently may have enhanced abilities at lower temperatures (Ehrlich et al., 2017).

Vibrio alginoliticus isolated strains showed a variation of results with the different culturing media used. *Vibrio alginoliticus* (9) of site 2A and *Vibrio alginoliticus* (S3) of sight 2B were the most interesting, and were able to crystalize minerals significantly on MD1- medium.

Pseudomonas cedrina (S1) from sample site 2B exhibited better mineralizing ability on MD1 medium in the presence of glucose. Whereas, isolates S2 and S4 from the same sample showed no difference in their mineralizing abilities with or without glucose in the media; i.e. MD1 and MD1- .

Moreover, the only bacteria that showed consistent results of precipitation in all 4 media tested is *Psychrobacter sp.* (5) from sample site 2A. Another *Psychrobacter sp.* SHUES1 isolated from soil samples in China has also showed very remarkable activity in the precipitation of metals at low temperatures, and when sequenced it its DNA showed the presence of a Carbonic anhydrase gene (Xuezheng et al., 2010; Li et al., 2016).

Figure 5 shows examples of different morphologies of precipitated crystals observed at 40X magnification by mineral forming bacterial strains. Most of the crystals formed appeared to be spherical in shape, and were formed after a minimum of 5 days of incubation.

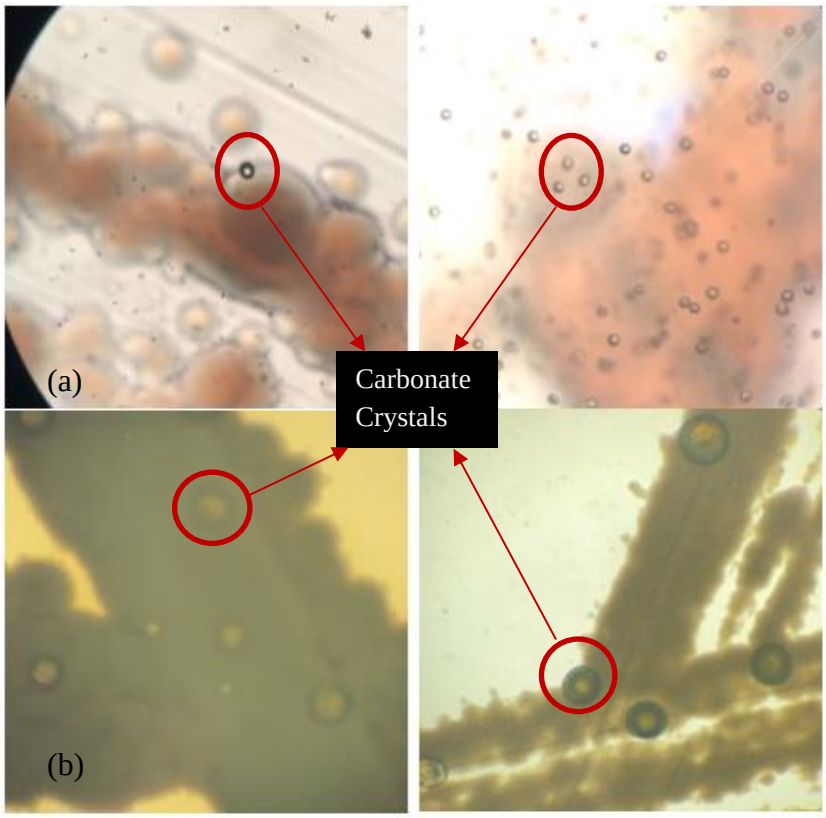


Figure 5. Observations of precipitates under light microscopy. (a) Deposits by *Psychrobacter sp.* (b) Deposits by *Vibrio alginoliticus.*

4.2.2 Determination of mineral composition

After identification of the mineral-forming bacteria from the collection of strains isolated from both marine sediments samples, an association between the growth of each and the composition of the formed crystals was determined using the analysis by SEM/EDS.

Vibrio alginoliticus (9) on MD1- formed crystals indicated in Figure 6, with very low Mg incorporation. Although the crystal is embedded within the media, it appears to be a spherical calcium carbonate. Similar findings have been found with *Vibrio alginoliticus* (S3) of sample site 2B. However, a higher Mg:Ca ratio has been found in minerals precipitated on MD1- as presented in Figure 7. EDS analysis show a bigger Mg peak corresponding to nearly half the atomic % of Ca. Although bacteria of the genus *Vibrio* have not been reported to have a correlation with biomineralization

potential activities; however, several of its species are known to cause infections in fish and other marine species. Interestingly, *Vibrio alginolyticus* species were reported to cause PAWS (*Porites andrewsi* White Syndrome) in 2013 in the South China Sea. When infected, *Porites andrewsi* corals undergo coral bleaching and its white calcium carbonate skeleton becomes visible (Zhenyu et al., 2013). To date, little is known regarding about the mechanism of infection of *V. alginolyticus*; however, it is suggested that development of further detection methods of this species could possibly help in avoiding the loss of corals. It was hypothesized earlier, that *Vibrio* species are able to adhere to the corals and somehow penetrate its skeletal tissues (Ben-Haim & Rosenberg, 2022). With these findings regarding the ability of *V. alginolyticus* to form calcium carbonates, it is possible that it is a mechanism linked to the infection it causes in coral reef species.

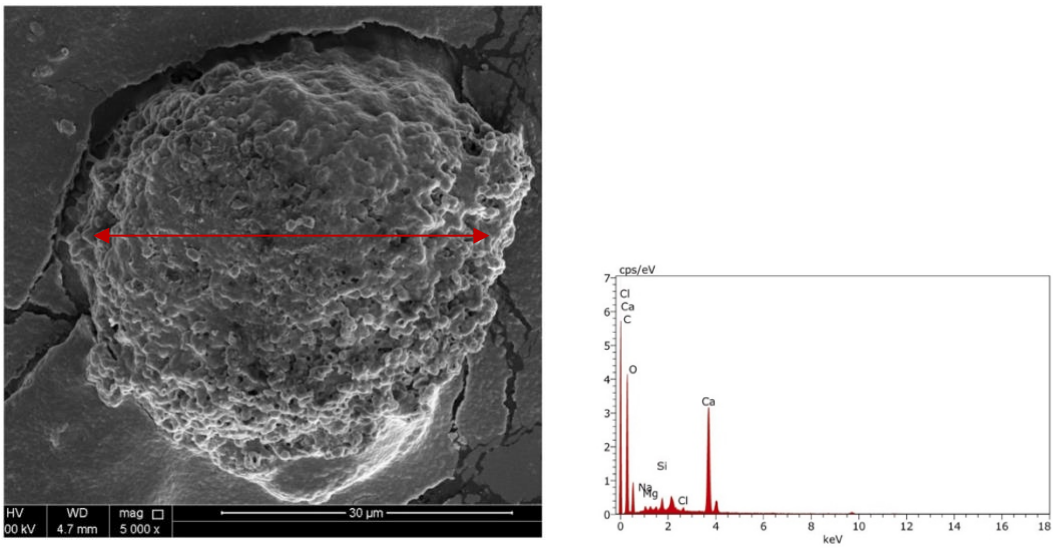


Figure 6. SEM/EDS analysis of crystals formed by Vibrio alginoliticus (9) on MD1-. A close up of a cacium carbonate crystal of rough surface.

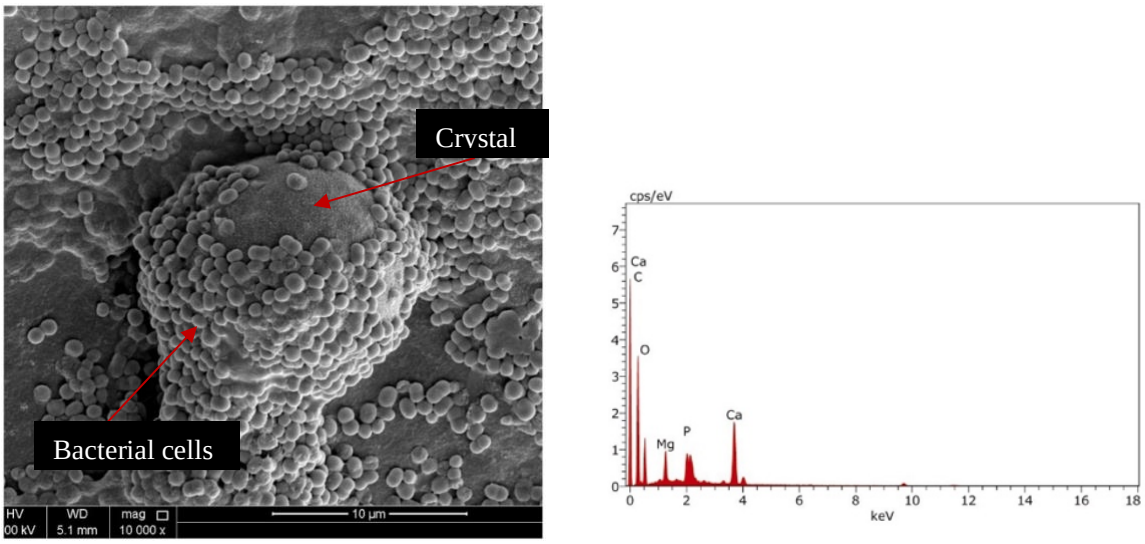


Figure 7. SEM/EDS analysis of crystals formed by Vibrio alginoliticus (S3) on MD1-. Bacterial cells are shown to be surrounding the crystal with inly part of it appearing to be smooth and spherical.

Since *Psychrobacter sp.* showed interesting mineralizing activity on both MD2 and MD3, SEM/EDS analysis on the recovered crystals was performed (Figures 8 and 9).

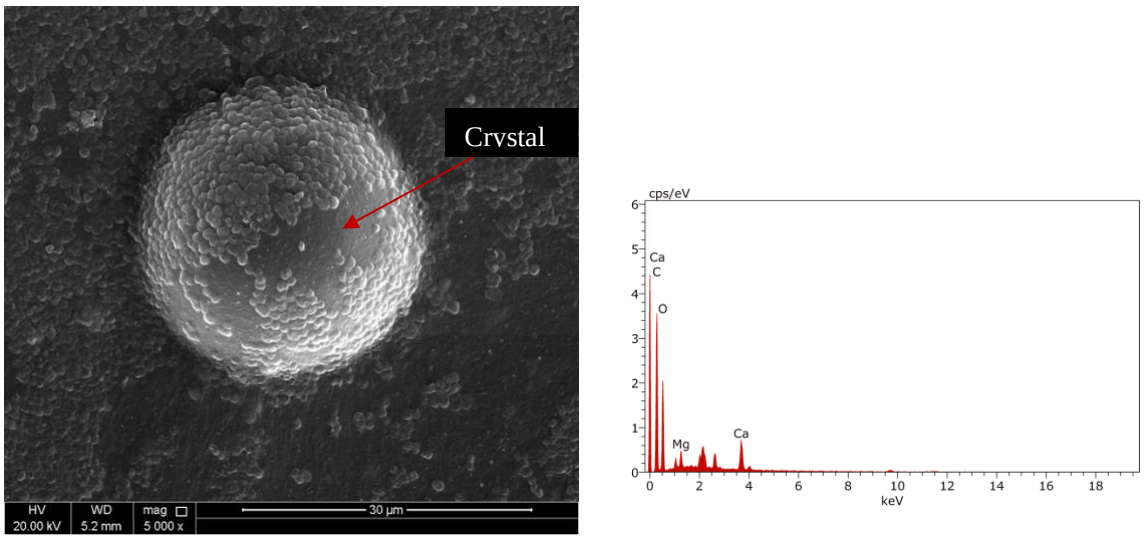


Figure 8. SEM/EDS analysis of minerals formed by Psychrobacter on MD3. Morphologically of the crystal formed appears to be a perfect sphere, partially covered with bacterial cells.

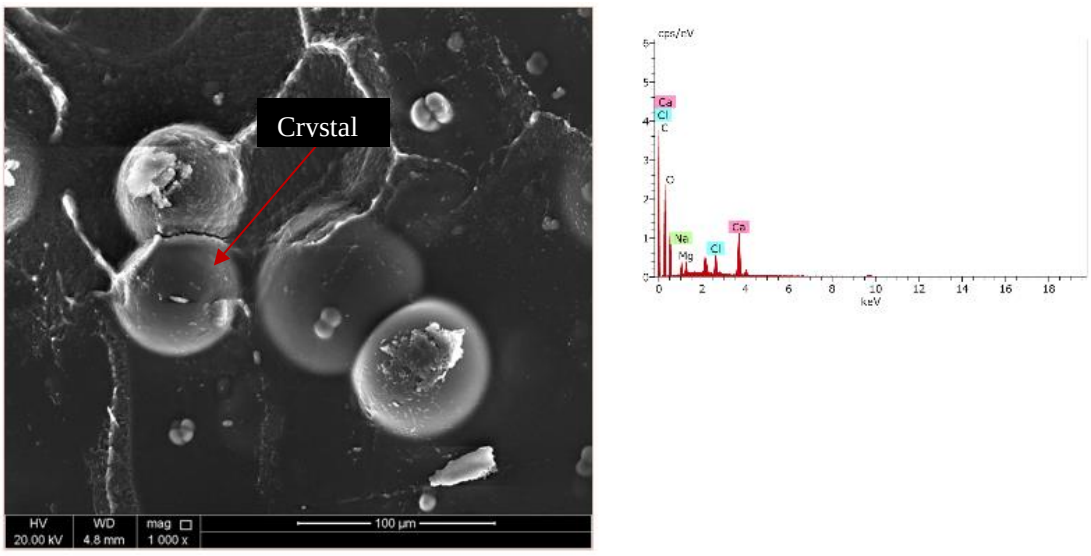


Figure 9. SEM/EDS analysis of minerals formed by Psychrobacter on MD2. Representative images of calcium carbonate crystals.

EDS analysis clearly demonstrated that Mg incorporation on MD3 media, i.e. without any addition of peptone, was significantly higher than that on the other media tested. On MD3 the Atm % of Mg and Ca was 0.28 and 0.57 respectively. Whereas, on MD2 media the Atm % reported was 0.23 and 1.35 respectively. Both SEM images indicate crystals with smooth surfaces.

XRD analysis of pure carbonate crystals revealed that the most abundant carbonate precipitates are monohydrocalcites. Comparisons between the XRD patterns of minerals from both media are presented in figure 10. A magnesium calcite peak is only visible in the sample formed on MD3. Therefore, different compositions of carbonates deposits are dependent on the growth media compositions.

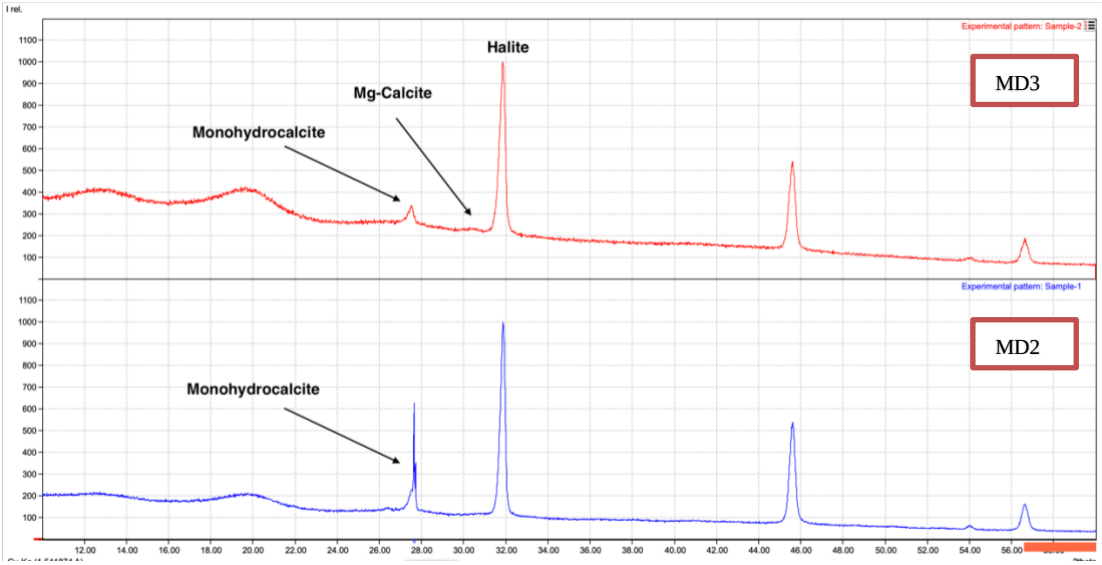


Figure 10. XRD patterns of minerals formed by *Psychrobacter sp.* on MD2 and MD3 media.

4.3 Carbonic anhydrase activity

The super groups of CA are known to be ubiquitous in the catalysis of a variety of process necessary for the survival of cells, organs, and organisms. CA, like other enzymes plays an essential role in maintaining homeostasis. It regulates the pH balance, aids in gas exchange, and detoxify the body (Abbas et al., 2018).

Biomineralization that takes place using the carbonic anhydrase metabolic pathway, is a naturally found process in a range of organisms, especially marine organisms. Microbial strains possessing this activity may belong to many types of bacteria, be it cyanobacteria, sulfate reducing or others. Carbonic anhydrase activities are not even strict to bacterial organisms, and that is due to the ubiquitous role that the enzyme plays in bio-calcification. CA activity can be evaluated either by assessing its esterase activity, or by investigating its ability to release H^+ upon the production of bicarbonate. In this research, we addressed both activities.

4.3.1 Screening for Esterase activity

CA acts on carbonyl-compounds, such as ester, by catalyzing the hydrolysis of ester in

a mechanism that is not quite known yet. The active site at which the ester substrate binds is yet to be identified (Lindskog & Silverman, 2000). However, there is enough evidence of the esterase activity exhibited by CA (Elleby et al., 1999).

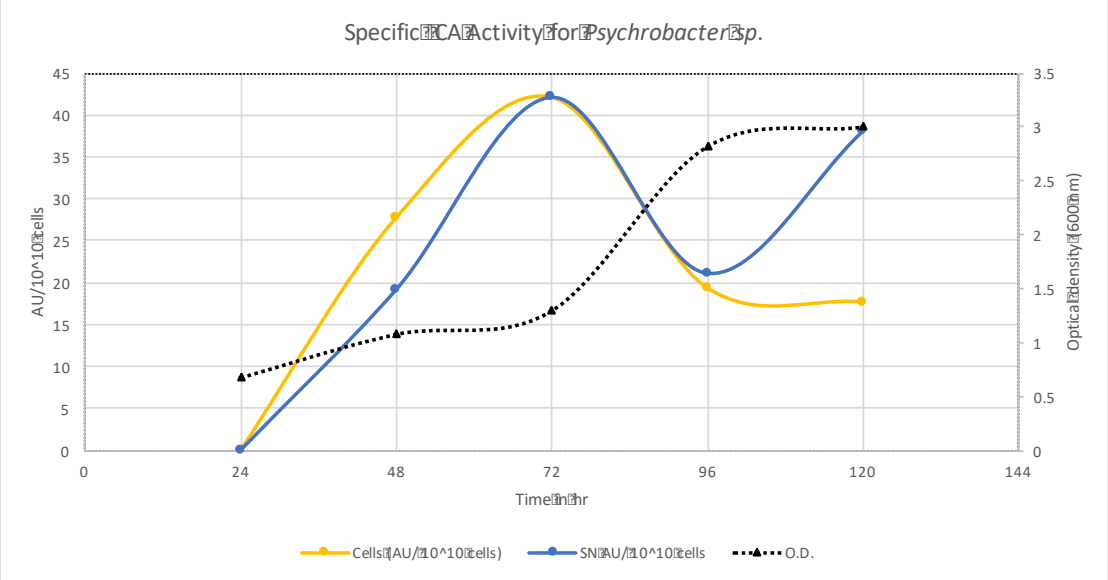
The esterase activity of mineral forming strains from this study was determined using the NPA protocol. It was evaluated in the cells and supernatant of some of the studied strains every day during an incubation period of 5 days. In addition to the mineral forming bacteria isolated in this work, one strain has been selected from the reference strains stated in section 3.1, which is DF2141 based on its significant mineralizing potentials. From the collection of mineral-forming strains, *Psychrobacter sp., Vibrio alginolyticus (9),* and *Vibrio alginolyticus (S3)* were tested for esterase activity, in addition to *Exiguobacterium arantiacum (1)* as one of the nonmineral-forming strains. A growth curve for every strain was constructed for a reliable kinetic study of the activity of CA, and the NPA protocol was followed every 24 hours of the 5-day incubation period. Each 24 hours, 1 ml of the liquid cultures was withdrawn and tested for esterase activity. The activities were calculated as arbitrary units AU/ 10_{10} of cells in both the supernatant and the cells separately (Figure 11).

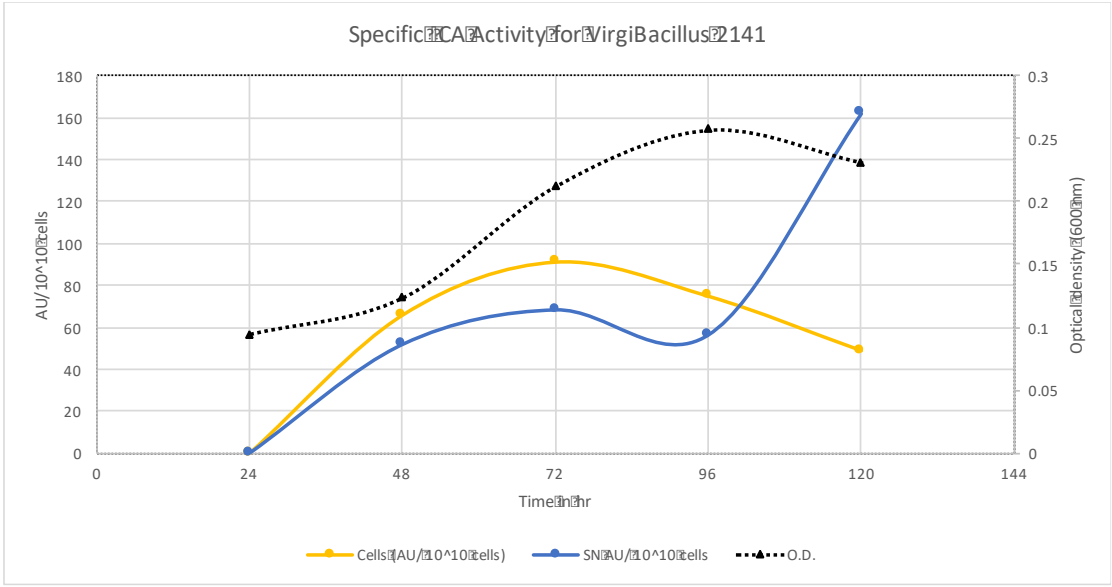
The trend in all kinetic curve seems to be similar in such that at 24 hours, only one day after incubation period started, the CA activity is almost null. Both intracellular and extracellular activity in all strains increased with time, and recorded a maximum activity at 72 hours (3 days of incubation), during which all strains were in their exponential (log) phase of growth. As expected, after the peak, the enzymatic activity decreases gradually almost in the second half of the exponential growth phase in all strains. CA activity in the cells continues to decrease, and sometimes reaches a constant value at 96 and 120 hours, as seen in *Psychrobacter sp., Vibrio alginoliticus (3) and Exiguobacterium antarcticum (1). Virgibacillus 2141, Vibrio alginolytocus (9)* and

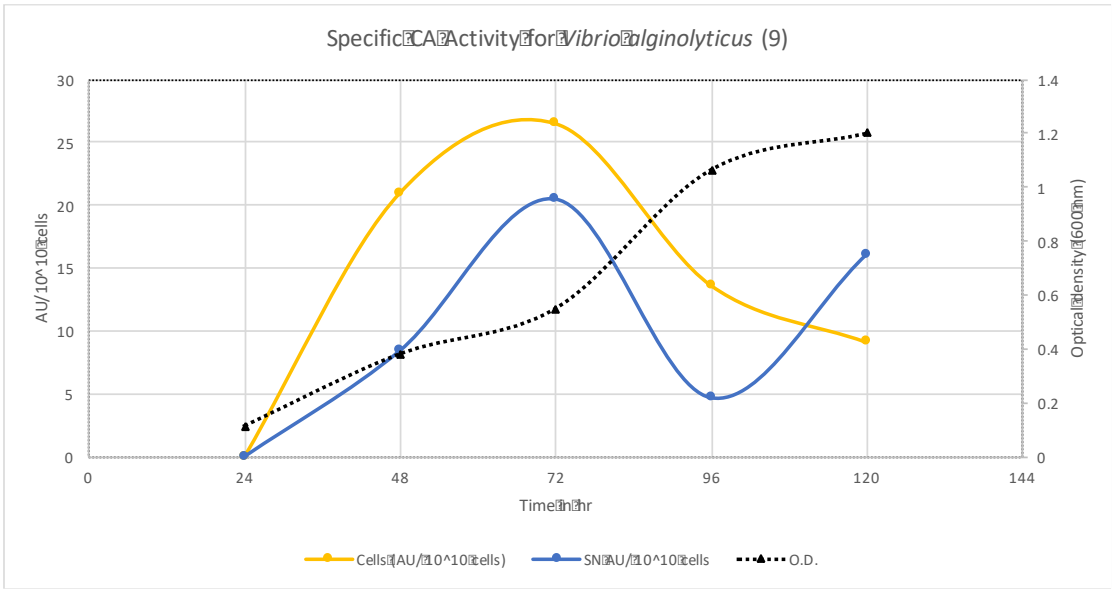
Psychrobacter have showed a significant increase in the enzymatic activity in the supernatant as opposed to the cells. The patterns suggest that at 120 hours of incubation, all tested strains were in the stationary phase of growth; therefore, cell death already started. Since CA's activity was detected at maximum in the cells at 72 hours, the results propose that after cell lysis the enzyme gets released. Hence, the activity gets transferred from the cells to the supernatant. Similar patterns in all mineral forming bacteria support this hypothesis.

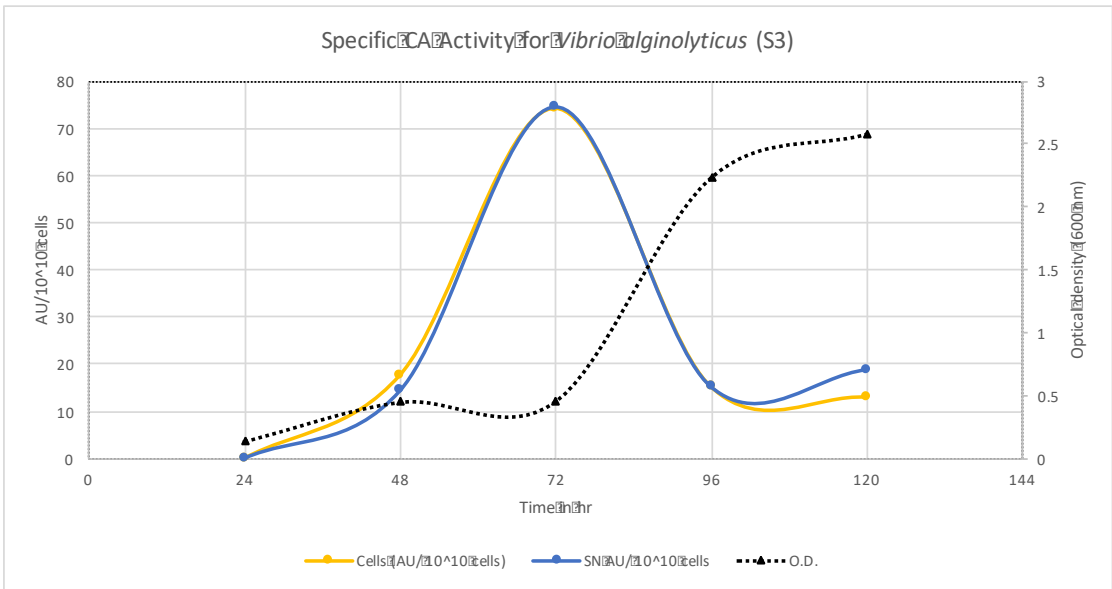
The results are also in coherence with the mineralization results. *E. arantiacum*, a non-mineral forming bacteria as per our findings in section 4.2.1, was tested as a negative control. Although its CA kinetics curve follow the same trend as the rest of the studied strains, at 72 hours the maximum activity was reported to be 0.01155 AU/10_{10}cells intracellularly, a number when compared to the activity of mineral forming strains is negligible (table 14).

In literature, none of these bacterial species was previously investigated for esterase activity, making this data very novel and open to further studies.




Specific CA Activity for VirgiBacillus 2141
AU/10^10 cells
Optical Density (600nm)
Time in hr
Cells (AU/10^10 cells)
SN AU/10^10 cells
O.D.


Specific CA Activity for Vibrio alginolyticus (9)
AU/10^10 cells
Optical Density (600nm)
Time in hr
Cells (AU/10^10 cells)
SN AU/10^10 cells
O.D.


Specific CA Activity for Vibrio alginolyticus (S3)
AU/10^10 cells
Optical Density (600nm)
Time in hr
Cells (AU/10^10 cells)
SN AU/10^10 cells
O.D.

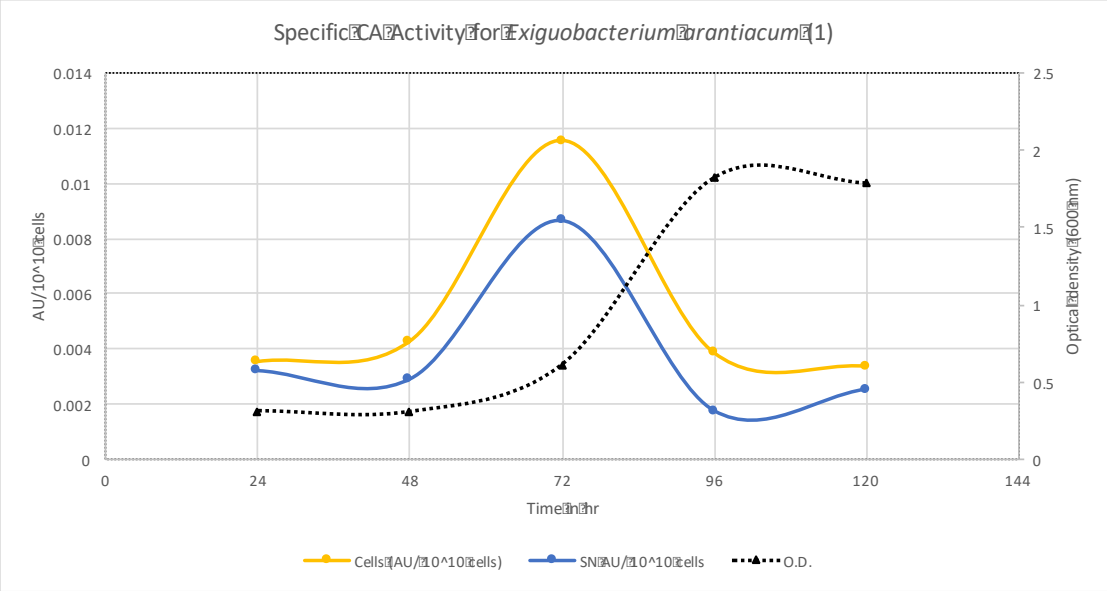


Figure 11. Carbonic anhydrase enzyme kinetics in 5 studied bacterial strains at 5 different time intervals. Specific activity is measured in AU/10^10 of cells on the left y-axis. Optical density at 600 nm is plotted on the secondary y-axis on the right.

Table 14: Highest CA activity recorded at 72 hours of incubation.

Bacterial strain	**Highest CA activity in Cells**	**Highest CA activity in SN**
Virgibacillus 2141	90.97	68.2
Vibrio alginolyticus (9)	26.4	20.5
Psychrobacter sp.	42.1	42.09
Vibrio alginolyticus (S3)	74.1	74.3
Exiguobacterium arantiacum (1)	$1.6*10^{-2}$	$8.6*10^{-3}$

4.3.2 Screening for hydration activity

In most cells of a variety of organisms and forms of life, essential metabolic reactions require two pivotal substrates, carbon dioxide and its hydration product bicarbonate (CO_2 and HCO_3^-). The constant interconversion between the two is essential for maintaining cell homeostasis. The hydration reaction of CO_2 and HCO_3^- when occurring without any catalyst can take place at a substantial rate. However, at physiological conditions of temperature and pH, it requires a catalyst that increases the rate of interconversion between the two. Consequently, the spontaneous enzymatic interconversion of CO_2 and HCO_3^- is essential for the support of the different cellular functions and metabolic reactions in all cells. CA found in all animals and microorganisms, catalyzes the reversible hydration of CO_2 to maintain correct flow of physiological processes:

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$$

In carbonate-precipitating microorganisms, such as the ones isolated in this study, CA does not only ensure the survival of bacterial cells, but it is also involved in their ability to deposit carbonate minerals.

Since the quantification of the CA enzymatic activity requires not only the study of the esterase activity of CA, but also its important hydration activity, the same strains investigated in section 4.3.1 have been screened again for hydration CA activity following the Wilber-Andersont (WA) analysis method (ref). WA assay is a electrometric procedure which tracks the time it takes CA enzyme to catalyze he hydration of CO_2. The hydration reaction is monitored by the release of protons (H^+) during the reaction, which significantly lowers the pH of the solution. Therefore, WA unit is calculated by measuring the time taken to drop the pH of the solution by 2 units. In ours experiments, it was challenging to fix the pH of the initial solution to 8.3 as

indicated in the assay's protocol. This is why the method was slightly modified and the initial pH was regarded differently in each trial, consequently every trial had a slightly different T_0 depending on the pH of the solution initially. The enzymatic activity in WA units/ml was first calculated according to the equation (section 3.8.3). And the Specific activity was then calculated as WAU/ 10_{10} cells for all 5 strains, table 15 shows the results in both units in *Virgibacillus* 2141 an example, and graphical representation of the kinetics as a function of the growth curve in Figure 12.

	Cells		**Supernatant**	
Time	Enzyme Activity (WAU/ml)	Specific Activity (WAU/10^{10} cells)	Enzyme Activity (WAU/ml)	Specific Activity (WAU/10^{10} cells)
24	58	143.9716312	49	121.6312057
48	35	4573170.732	35	4573170.732
72	58	4396900.27	37	2804919.137
96	25	1563368.538	26	1625903.28
120	28	1948051.948	27	1878478.664

Table 15: Evaluation of CA specific activity using WA assay in *Virgibacillus* 2141 strain.

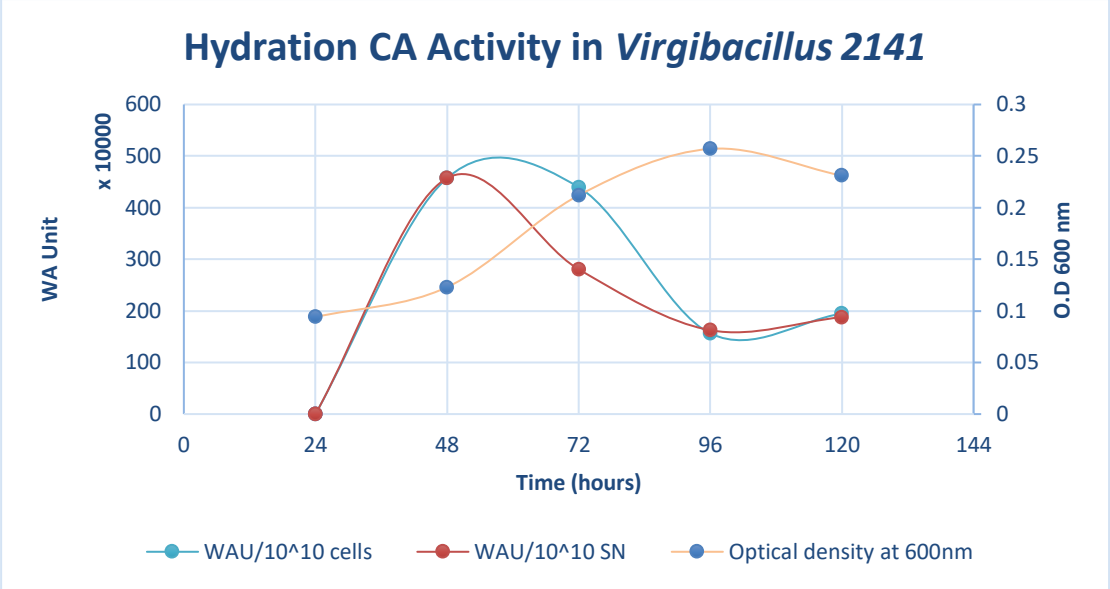


Figure 12. Carbonic anhydrase hydration enzyme kinetics in *Virgibacillus* 2141.

The results of the hydration activity of carbonic anhydrase in the studied samples were in consistency with those of esterase. The highest activity is recorded between 48 and 72 hours, and declines radically as the cells go into the stationary phase of their growth curve, i. e. at 96 hours and beyond. Table 16 shows a comparison between all the strains tested, it can be denoted that similarly to the esterase activity in the section above, the highest activity was recorded in the cells as opposed to the supernatant, although the difference was sometimes not very significant. *Virgibacillus* showing the highest hydration activity among the strains, remarkably did not exhibit a much less activity in the supernatant ($45*10_5$ WAU/10_{10}cells) than in the cells ($43.9*10_5$ WAU/10_{10}cells).

Table 16: Highest recorded hydration activity of CA in the 5 studied strains both intracellularly and extracellularly.

Bacterial strain	Highest hydration activity in Cells (WAU/10_{10}cells)	Highest hydration activity in SN (WAU/10_{10}cells)
Virgibacillus 2141	$43.9*10_5$	$45*10_5$
Vibrio alginolyticus (9)	$50.6*10_4$	$20.8*10_2$
Psychrobacter sp.	$40.1*10_4$	$33.5*10_4$
Vibrio alginolyticus (S3)	$27.6*10_4$	$17.1*10_3$
Exiguobacterium arantiacum (1)	$4.6*10^{-3}$	$2.2*10^{-5}$

When compared with hydration CA activities reported in literature, it is deduced that the separation of the intracellular and extracellular matter of the cells is rarely ever done. In fact, analysis is done on the crude enzyme after purification, and then the activity is expressed as WAU/ mg of protein (Hou et al., 2019). Although, investigation on the purified enzymes clearly assesse the determination of their potential hydration or esterase activities, it is suggested that when the investigation aims to understand the effect of microorganisms, particularly mineral-forming, on the deposition of carbonates and the establishment of the ancient sedimentation structures, studied on whole bacterial cells serve the purpose better (Ineisto et al., 2016). Carbonic anhydrase and all the enzymes yet to be researched that are partially or fully responsible for the precipitation of carbonates and the alterations of Earth's sedimentary compositions do not exist in nature in the pure form. They are part of much more complex metabolic pathways and activities that need only to be addressed as such.

CHAPTER 5: CONCLUSION

Results in this study confirmed several aspects of biomineralization and the tools utilized for that purpose. It was demonstrated that mineral-forming bacteria in Qatari environments are not restricted to decaying mats, for even marine sediments revealed a group of bacteria with similar abilities. It was also established that MALDI-TOF when not able to provide an updated database for the rapid and efficient identification of newly isolated bacterial strains, can be used for classification of bacteria, detection of specific biomarkers to aid in future closely related species, and drawing correlations between characteristic peaks and significant enzymatic activities. The most accurate classification that allows for grouping of strains based on similar protein mass fingerprints was performed by the Principle Component Analysis clustering.

Moreover, the study highlighted the pivotal microbial biodiversity in marine costal sediments along the Qatari coast. It was also found to house carbonate-depositing bacteria whose precipitates were analyzed using SEM/EDS and XRD. To date, this is the first study of marine sediments in Qatar for the investigation of biominerals. The quantification of the two mostly describes activities of the enzyme Carbonic anhydrase; esterase and hydration was performed, and it was measured using arbitrary units (AU). Mineral-forming strains were shown to exhibit high CA activities as opposed to those that do not form crystals, which evidently confirms the relation between the presence of active CA enzymes and the biomineralizing potential of bacteria.

The novelty of this research extends to utilizing MALDI-TOF MS and PCA as a comparative tool for the differentiation between the protein profiles of *Virgibacillus* strains featuring a diversity in the biomineralization potentials. These tools helped in understanding the involvement of microbial mats. It also highlights the importance of monitoring the changes that occur during the time during which a mat evolves from a

living to a decaying one.

Isolations of mineral-forming bacteria from Qatari sediments along with previous findings on carbonates in microbial mats in Qatari sabkhas, together can serve as the basis of special distribution analysis of biomineralization mechanisms combining both laboratory and field experiments. Such analysis is of great interest for the study of the ecology of the deeper layered sediments and possible fossil records.

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