Exploration and utilization of airborne bacteria for soil improvement using microbial induced carbonate precipitation

by

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ABSTRACT

With increasing awareness of energy and environmental crisis, novel technology as one of the countermeasures to the global crisis has been attracting more and more attention in a wide range of research fields. In the realm of geotechnical engineering, the past two decades have witnessed a rapid growth of Microbial Induced Carbonate Precipitation (MICP) technology, which achieves cementation of particles by carbonate from urea hydrolysis catalyzed by microbial urease precipitates at the presence of calcium ions. Despite the tremendous development, there are still some challenges that hinder the progress of upgrading this technology. On the one hand, the issue of cost reduction has received considerable critical attention since cost efficiency is a key determinant of further development of a novel technique. More and more research attempts now are focused on using waste materials as cementation reagents instead of raw materials for treatment. On the other hand, in order to cope with various complicated conditions in scaled-up field applications, the employed bacteria should be improved in terms of survivability and adaptability. To date, many studies have been devoted to addressing these issues to further improve MICP as an eco-friendly technique for the next generation. This dissertation focuses on addressing the challenges faced by bacteria used for biocementation. It aims to explore the potential of airborne bacteria as candidates for MICP applications. There are two primary objectives of this study: i) to investigate the occurrence of ureolytic bacteria in air samples collected from different sampling sites and characterize selected isolates after screening; ii) to further improve biocementation effectiveness through applying multiple strains selected from air isolates.

In the first stage of this investigation, airborne bacteria sampled using an air sampler were collected from three main sites with distinct climatic features in Japan, Sapporo, Tsukuba, and Okinawa. Sampling sites with higher vegetation density tend to have a higher population of airborne bacteria. Among tested colonies, 10-20% of ureolytic bacteria were identified using two culture media. After three rounds of screening, 30 isolates stood out from about 1500 isolates with good performance in precipitating carbonate and were then identified in gene analysis. Most isolates were found belonging to two orders, *Micrococcales* and *Bacillales*. They are from two of three major phyla that include most culturable bacteria, and many bacteria generalists. At the second stage, a series of characterization tests were conducted on selected strains, including temperature dependence (15-35 °C) of bacterial growth and bacterial urease activity, pH dependence (pH 4-9), and tolerance to various cementation media. The results revealed that many of these isolates are unique and resilient in many aspects. For instance, one strain showed a cold tolerant feature, and the other strain exhibited relatively high urease activity at acidic conditions. Among characterized strains, two strains with excellent performance were then evaluated in sand column solidification tests, revealing a high efficiency of carbonate precipitation in a two-week treatment compared to many existing studies.

At the third stage of this study, the mixing of two strains was evaluated in precipitation tests in order to further improve the effectiveness of cementation towards complicated conditions. Selected strains were mixed in different ratios before applying to cementation media under different conditions. The findings suggest that mixed strains can cope with less favorable conditions for cementation more flexibly than mono strains do, showing a stable and high efficiency of carbonate precipitation in varying situations. In a broad sense, it can be inferred that mixed strains might have more advantages than mono strains in the improvement of soils with complex properties. Finally, based on the findings on the influence of humic acid (HA) on bacteria and cementation process, HA was proposed as an amendment to the improvement of biocementation effectiveness. As a bio-stimulant, HA of small addition to the culture media can significantly increase the bacteria population in a short period of time, at the same time, increasing the urease activity. With remarkable cation exchange capacity, it can serve as nucleation sites for calcium carbonate precipitation and support the soil matrix as skeleton. Besides, its buffering capacity can significantly inhibit the emission of ammonia gas during the treatment process. This topic would be a fruitful area for further investigation.

As the first study to explore the potential of airborne bacteria for biocementation applications, the experimental work presented here provides some new insight into the improvement of cementation efficiency through selection and utilization of various bacteria. Due to time constraints, this work cannot provide a comprehensive understanding of how the proposed method can actually enhance the cementation efficiency of natural soils. Therefore, further investigations into several topics were recommended. Hopefully, it can contribute to the development of MICP as a real sustainable technology.

Keywords: Microbial induced carbonate precipitation (MICP), ureolytic airborne bacteria, urease activity, bacterial survivability and adaptability, humic acid (HA)

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Chapter 1 Introduction

1.1 Background

In the past centuries, lack of awareness of environmental issues on the civilization path has resulted in lots of irreversible damages to our mother planet, dominated by climate change, severe loss in natural resources, and environmental pollution (UNEP annual report, 2020). As time requires, the emerging technological innovation has been paving a path for the future generation to build a society of sustainability, which is far more important than saving limited existing resources. In recent years, there has been a dramatic increase in research efforts aiming to build a "greener" construction industry, however, traditional cement production has been in an unshakable position without alternatives appearing. As one of the green technologies, microbially induced carbonate precipitation (MICP) has become a well-investigated technique for soil improvement since it was first proposed by Stocks-Fischer, Galinat, and Bang (1999). In the realm of geotechnical engineering, it refers to utilizing microorganism function to induce the carbonate from urea to precipitate in the presence of calcium ions, filling the space in soils and bonding the loose particles to achieve an improvement in mechanical performance (DeJong et al. 2010).

Since it started receiving attention, biocementation has been attracting considerable interest for more than two decades. To further upscale its applications to various fields, there are some key factors taking into account. Of particular concern is the total cost of treatment, making it difficult to attract more investment on the improvement method. The existing studies on MICP have shown that the development of this technique has reached a bottleneck. On the other hand, to enhance the applicable range of the technology, there is an urgent need to address the problems caused by the weak bacterial performance in a less favorable environment. Few studies have investigated the changes of bacterial communities before and after the introduction of ureolytic bacteria into natural soils. It was reported that most introduced bacteria were outcompeted by native strains just after a few days of treatment (DeJong et al. 2022), which indicates that the cementation effectiveness is greatly affected by the native bacteria in the target soils. It directly impacts the effectiveness of biocementation, also indirectly determining the treatment cost and performance in a long term. Many studies have highlighted the importance of cost reduction for further development of technology, while most studies fail to specify the challenges caused by the weakened performance of employed bacteria in natural soils. This study tries to make contributions to the improvement of cementation efficiency by clarifying what kind of role the bacteria need to play and how the performance can be improved.

1.2 Objectives of dissertation

This dissertation seeks to investigate the potential of airborne bacteria for MICP applications, specially focused on the survivability and adaptability issues. To our knowledge, there is no study ever tried isolating bacteria from air for biocementation. As the first study to exploring airborne bacteria for biocementation applications, this study was exploratory and interpretative in nature.

Airborne bacteria were collected using an air sampler from Japan that is designed to monitor the environmental hygiene. Figure 1.1 below gives a brief idea about the scope of this research work. Firstly, airborne bacteria were sampled from different climate zones in Japan, including two sampling sites (Hokkaido, Tsukuba) with humid continental climate, one sampling site (Okinawa) with subtropical oceanic climate. The occurrence of ureolytic bacteria in each sample was studied to gain an understanding of the distribution features of ureolytic bacteria in distinct environments. After several rounds of screening, selected isolates were identified by gene analysis. Among these isolates, bacteria with excellent performance were characterized by precipitation tests and soil solidification tests. As a trial to enhance cementation efficiency, mixed strains were applied in precipitation tests under different conditions, and the calcium carbonate induced by different strains were discussed in terms of the morphological features. Finally, combing the previous findings of positive effects of HA, HA was proposed as amendment for improving MICP in some application cases.

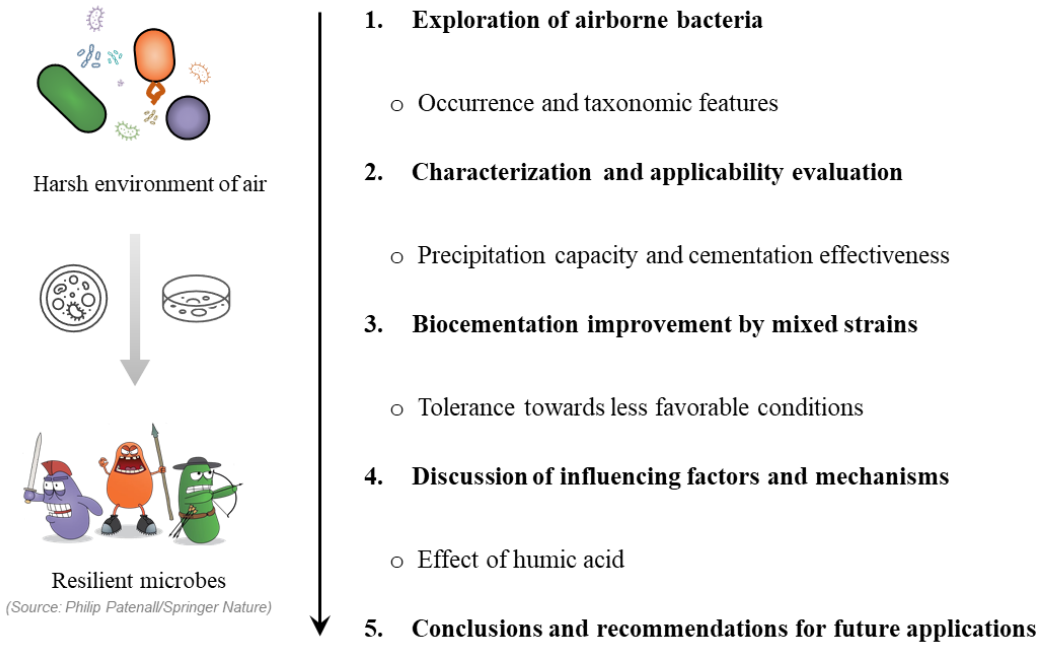

Figure 1. 1 Scope of this dissertation work

1.3 Novelty and significance of study

The present research explores, for the first time, the MICP potential of airborne bacteria. Generally, air is not considered as a source of microbes since it is less abundant in bioresources than other matrixes like soil and waterbody. On the other hand, since the technique aims to improve soil properties, bacterial isolation works focused on the soil. Studies of airborne bacteria have established that these bacteria are versatile and have immense potential for biotechnology applications. For long, their role has been underestimated. Therefore, this study makes a major contribution to research on the possibility of applying airborne bacteria for biocementation.

The airborne bacteria survive in a harsh environment, air, thus, they must have corresponding strategies to cope with various challenges. Being resistant to hostile conditions, bacteria with good survivability in MICP treatment can reduce the introduction of bacteria and enhance the cementation efficiency in the long term. According to previous research, it is highly possible that a small fraction of airborne bacteria is able to adapt to distinct environments in the transportation after being aerosolized. For MICP treatment, it is of great importance to apply bacteria with excellent adaptability considering the complicated properties of natural soils. In this case, this feature of bacteria contributes to widening the applicable range of technology. In a broader sense, the exploration airborne bacteria also contribute to an effective utilization of bioresources in the field of biotechnology, providing a new insight into the bacterial performance for engineering applications. The thesis focused on the investigation of bacteria and related experimental works, thus, a full discussion on the mechanical improvement of biocemented soils beyond the scope of this study. Future work of upscaled soil treatment could give us new information of the MICP potential of airborne bacteria.

1.4 Structure of dissertation

This work is described in seven chapters.

I. **Chapter 1** establishes the background, identifies the problems related to employed bacteria in the field of MICP research, and states the objectives and novelty of this research.

II. **Chapter 2** reviews the relevant literatures of challenges of MICP research and current research focus on airborne bacteria, proposing the introduction of airborne bacteria for applications.

III. **Chapter 3** explores airborne bacteria from different climate zones in Japan in terms of the occurrence of ureolytic bacteria and its taxonomic features.

IV. **Chapter 4** presents the characterization and applicability evaluation of selected airborne isolates after several rounds of screening.

V. **Chapter 5** demonstrates that the precipitation efficiency can be significantly increased by mixing two different strains in varying conditions.

VI. **Chapter 6** discusses the effects of HA on MICP and its potential as amendment to enhance the cementation effectiveness.

VII. **Chapter 7** summarizes the findings, restates the significant contribution of this work and makes recommendations for further investigation into the topic.

Chapter 2 Literature review

2.1 Introduction

Biocementation generally refers to the application of microbial activities in the field of environmental and geotechnical engineering to provide eco-friendly solutions to many problems (Dejong et al. 2013; H. Liu, Chu, and Kavazanjian 2023). To date, lots of environmental problems and corresponding MICP technique as solutions were investigated, including soil improvement, remediation of water and soil, enhancement of construction materials, carbon sequestration, etc., which greatly contribute to Sustainable Development Goals (K. Zhang et al. 2023). In nature, carbonate precipitation occurs through many metabolic pathways of microorganisms. Among these pathways involved in cementation, urea hydrolysis induced by bacteria is a major area of interest within the field of biocementation due to its high efficiency (Zhu and Dittrich 2016; Castro-Alonso et al. 2019). Since the initial development stage, MICP has been described as an ecofriendly technique that would greatly contribute to the sustainable development and is believed to be a versatile technology for next generation (DeJong et al. 2010; Achal and Kawasaki 2016). Actually, in terms of cost efficiency, "bio-cement" is incomparable with traditional cement products, as it requires a large consumption of pure chemical reagents. Therefore, recent developments in biocementation have heightened the need for incorporation with industrial waste. Existing research focuses on mainly low-cost calcium resources (recovered calcium from food waste, construction waste), carbonate resources (urea from animal husbandry), and complex culture medium from food industry (Fouladi et al. 2023).

Most studies have only focused on the point of geoengineering to design approaches and examine the mechanical properties of treated materials, since the majority of researchers in the field of MICP have a geoengineering background. Such approaches, however, have failed to address the problems of bacterial survivability and adaptation to the natural soil environment. In a laboratory experiment, bacteria might have an excellent MICP performance for the cementation of standard sand materials in favorable condition, however, how they respond to a complicated soil in a natural environment is not predictable. So far, little attention has been paid to the bacterial role in the treatment process in field applications. To find a solution from the perspective of bacterial utilization, this chapter briefly reviews three treatment strategies based on what kind of bacteria are used, current challenges of MICP technology in the upscaling path, and then focuses on discussing diverse ways in which we can find solutions to the challenges faced by bacteria. Finally, airborne bacteria are proposed as a potential solution to some difficulties associated with weakened bacterial performance during treatment process.

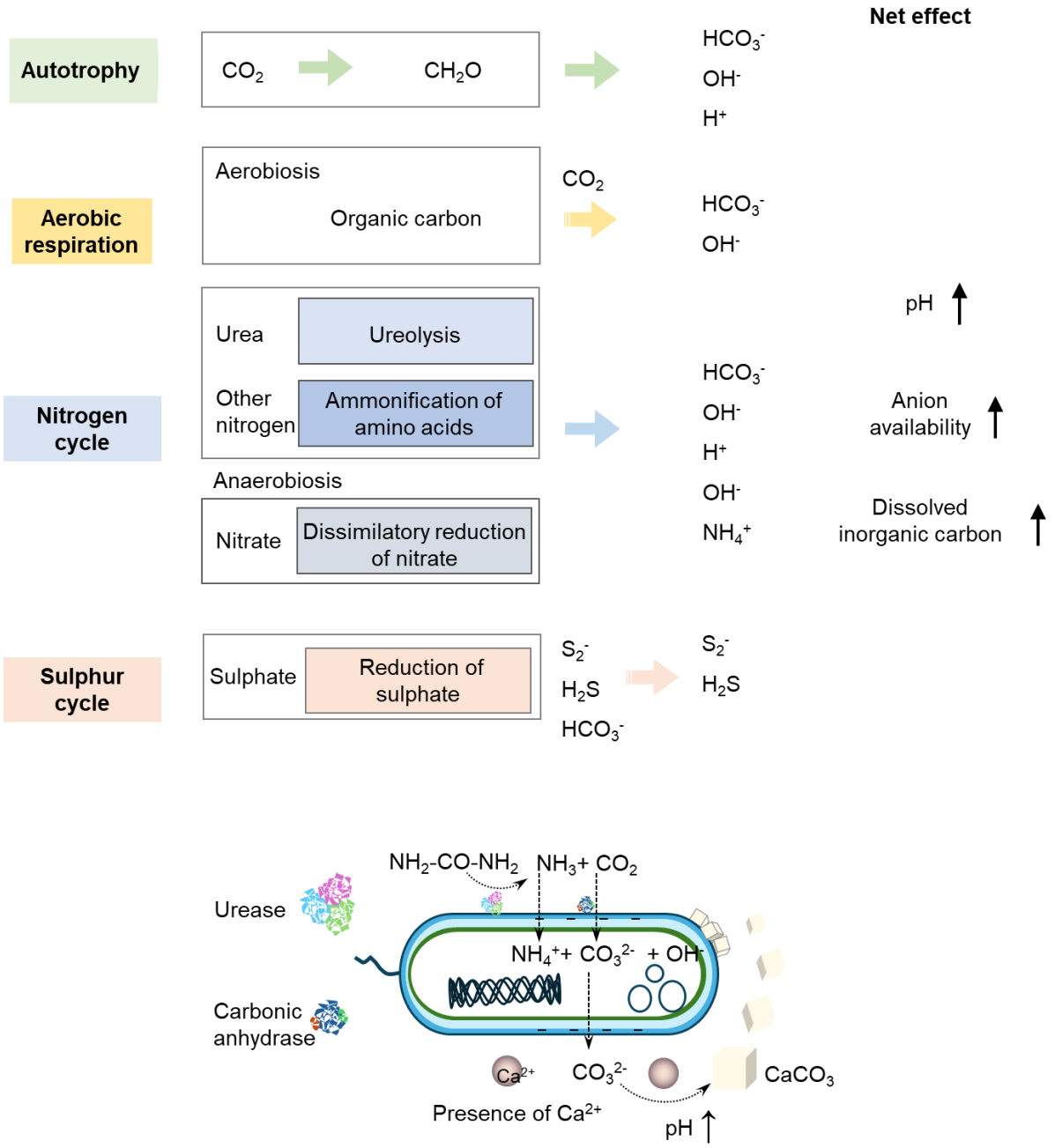

Figure 2. 1 Metabolic pathways associated with biocementation and mechanism of ureolysis pathway. Modified from Hoffmann, Reeksting, and Gebhard (2021)

2.2 Pathways and treatment strategies

2.2.1 Metabolic pathways associated with biocementation

In nature, there are many metabolic pathways contributing to biocementation, including autotrophic metabolic pathways, and metabolism of nitrogen cycle and sulfur cycle (see Figure 2.1). Products from these metabolisms lead to a typical increase in pH and anion availability, which favors the environment for carbonate precipitation (Castro-Alonso et al. 2019; Hoffmann, Reeksting, and Gebhard 2021). All these pathways have their drawbacks as technique for biocementation. Especially, undesired by-products are a controversial topic when considering their sustainability. For instance, carbon dioxide is emitted from the reduction of nitrate and sulfate. When the reduction of nitrate is incomplete, NO and N_2O are generated. Among them, the ureolysis in nitrogen cycle is the most researched one due to its simplicity and high efficiency of precipitating carbonate. Compared to precipitation induced through other pathways, it is relatively simple and efficient. The mechanism is illustrated in Figure 2.1. Generally, urease is frequently researched as the most important enzyme that induces precipitation, while another enzyme, carbonic anhydrase, also plays an essential role in the process. These two enzymes involved in this process are commonly synthesized by bacteria of great diversity. As for the by-products, many studies have been seeking solutions to reduce the negative effects. As a result, most MICP studies focused on the ureolysis pathway.

2.2.2 General treatment strategies

Based on what kind of bacteria and how the bacteria are introduced into soils, the treatment can be divided into three approaches: i) bio-augmentation, refers to supplementing sufficient exogenous bacteria; ii) bio-stimulation, refers to stimulating indigenous bacteria; iii) selective bio-stimulation, refers to selecting one or multiple candidates from local bacterial communities and introducing the bacteria back to the environment after enrichment (Dubey et al. 2022; W. Zhang et al. 2023).

a) Bio-augmentation

Among the three treatment strategies, bio-augmentation is the most researched method due to its simplicity and effectiveness in the treatment process. Bio-augmentation employs typical ureolytic bacteria of high performance, including “gold standard” *S. pasteurii* and some *Bacillus* species. These bacteria are ideal candidates for engineering applications because they usually have stable growth and high activity in laboratory conditions. More importantly, many of these strains are commercially available products in global bioresource centers, such as America Type Culture Collection (ATCC), and German Collection of Microorganisms and Cell Cultures (DSMZ). Following the instructions, these bacteria can be easily cultivated according to the well-developed culture method and applied for

treatment. However, despite the lack of sufficient supportive evidence, there's a risk of bio-contamination regarding the utilization of a large population of exogenous bacteria (Portugal et al. 2020). Furthermore, how the introduced bacteria respond during the treatment process differs greatly when being in a favorable condition laboratory and a less favorable or even hostile environment in a field-scale application, which means that the adaptability of applied bacteria is of foremost importance.

b) Bio-stimulation

In contrast, the adaptability of bacteria is not an issue for the bio-stimulation approach, as it activates native bacterial communities to induce precipitation. It should be noted that the effectiveness of treatment is greatly dependent on the composition of local bacterial communities. Compared with bio-augmentation, this approach is more time-consuming. More importantly, the introduction of high concentrations of nutrients into the target soil in the stimulation process might activate pathogens (e.g., *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Staphylococcus aureus*) at the same time, which would pose a threat to surrounding ecosystems (Naveed et al. 2020a; Dubey et al. 2022).

c) Selective bio-stimulation/bio augmentation

A compromise plan is to isolate and screen ureolytic bacteria with good MICP performance and introduce the bacteria back to their origins, which refers to the third treatment strategy selective bio-stimulation. It is also known as the bioaugmentation of indigenous bacteria (Gowthaman et al. 2019). Since the selected bacteria are native to the target soil, they are believed to have good adaptation to the environment and can survive till the target strength is achieved. In an ideal scenario, this approach can ensure the long-term performance of introduced bacteria.

Up to now, many studies have been working on the comparison of bio-augmentation and bio-stimulation in terms of cementation effectiveness. Gomez et al. (2017) conducted a large-scale comparison of bioaugmentation and biostimulation in 0.5 m high, 1.7 m diameter tanks for biocementation of sands, revealing that biostimulation can achieve comparable improvement to bioaugmentation using *S. pasteurii*. Dubey et al. (2022) investigated three treatment strategies comparing the performance of representative strains isolated from a riverbank with *S. pasteurii* in different concentrations of cementation media, which unravels the great potential of selective stimulation. In a recent study, Fan et al. (2024) investigated the effects of nutrients on bioaugmentation and biostimulation treatment. The authors concluded that the latter is a more eco-friendly and cost-effective method than the former one, however, its uniformity of cementation is usually one of the most concerned issues. Considering the drawbacks of two approaches, a growing body of literature focuses on employing indigenous bacteria after isolation and screening of bacteria with excellent performance, which was discussed in the flowing section.

2.3 Main challenges of MICP technique

Before mentioning the challenges of MICP technique, it is necessary to demonstrate what has been established in the past several decades. Up to now, numerable studies have elaborated on the influencing factors of biocementation effectiveness. Mainly, the properties of target soil, types and concentrations of cementation media, characteristics of applied bacteria, environmental parameters such as temperature, and treatment strategies were the focus of the experimental studies (Dejong et al. 2013; Mortensen et al. 2011; Al Qabany, Soga, and Santamarina 2012; Y. Wang and Konstantinou 2024). With the development of technology, numerical models are also gradually becoming one of the current trends in the field of biocementation, and they are also used to investigate the influencing factors and optimize MICP treatment. Treatment under a moderate temperature, weak alkaline conditions, and high concentrations of cementation media and bacteria culture can achieve a high cementation effectiveness on well-graded soils (Tang et al. 2020; Naveed et al. 2020; Armstrong I. Omoregie, Palombo, and Nissom 2021). In terms of treatment strategies, multiphase and electroosmotic grouting methods have been optimizing the grouting velocity and pressure to achieve high treatment efficiency (C.-S. Tang et al. 2020). Figure 2.2 illustrates three major parts of biocementation treatment. It should be mentioned that the process related to field scale application that deploys various methods is not discussed in this study. Challenges of biocementation treatment in three aspects were described accordingly.

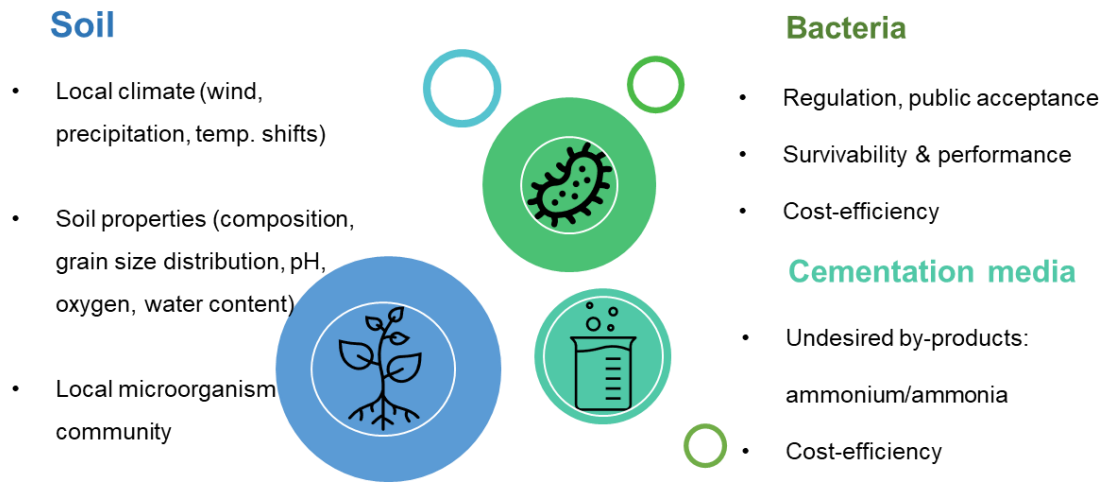

Figure 2. 2 General factors controlling the upscale applications of MICP technology

2.3.1 Raw materials used in the treatment-cementation and cultivation media

As discussed earlier, “biocement” is commonly reported as a real “green material”. However, few studies have been able to draw on any systematic research into assessing the cost and environmental benefits of the MICP treatment. Rahman et al. (2020) did a critical review work on MICP and discussed its sustainability with an assessment in three specific scenarios, revealing that extensive research is still needed to make it a cost-effective and eco-friendly technique. Using life cycle method that emerged in recent years, Deng et al. (2021) evaluated the energy consumption and carbon emissions of MICP technique in five application scenarios, the energy consumption and carbon emission is 15-23 times and 3-7 times higher than the conventional technologies, respectively. It was demonstrated that direct environmental impacts of the technique are actually less than the conventional one, whereas the indirect impacts from manufacturing the raw materials utilized in treatment are much greater than the conventional one. Finally, the authors recommended that more wastes materials could be reused in the MICP treatment to reduce the impact. Up to now, various alternatives have been studied to replace the utilization of some raw materials that greatly impact on the environment. Very recently, a comprehensive review work on the waste stream utilizations on MICP applications suggests that waste materials contribute to a more sustainable development of the technology (Fouladi et al. 2023). Some of these studies were explained in the following section.

a) Treatment cost

In terms of cementation media, the production of urea consumes relatively substantial amounts of energy in industry. In contrast, the cement products that have been accused of great CO_2 emissions have an increasing demand for industrial wastes being incorporated into production, which means the environmental impact of cement industry is actually lower than general expectation (de Brito and Kurda 2021). As one of the largest consumers of industrial waste, cement products are eliminating stigmatization by compensating for parts of the emissions. To compete with conventional cement products, “biocement” product should reduce the energy consumption and balance the cost and time efficiency of treatment. As for cost effectiveness, the cultivation of bacteria is usually believed to occupy the largest portion of the total cost, since the culture media and culture conditions should be well-controlled in order to harvest bacteria with stable performance. To solve this problem, more efforts to look for alternatives are necessary. For reducing the total cost of treatment, related studies mainly focus on alternative sources of calcium, urea, and nutrients for bacterial cultivation. Calcium sources are relatively accessible since they can be obtained from by-products/wastes from chemical industry (Solvay synthesis) and food industry (various shells), or other low-degrade chemicals, such as snow-melting agents for roads (Choi, Wu, and Chu 2016; Gowthaman et al. 2019). Several studies have confirmed the feasibility of using seawater as a free source of low-concentration calcium (L. Cheng,

Shahin, and Cord-Ruwisch 2014). Urine has been proved to be an alternative source of urea in many studies. This waste is usually from pigs and cows in animal farming (H. J. Chen et al. 2019), and human urine is also investigated as a urea source for manufacturing bio-bricks (Lambert and Randall 2019). Waste from the food industry is considered as an ideal alternative for lab grade chemicals using in culture medium, including molasses, lactose mother liquor, corn step liquor, dairy waste, Tofu wastewater, etc.(Amini Kiasari, Pakbaz, and Ghezelbash 2019; Armstrong Ighodalo Omoregie et al. 2019; Amiri and Bundur 2018; Fang et al. 2019). Other research directions are focused on the development of bio-stimulation technologies to reduce the cost of bacterial cultivation by supplementing less nutrients to activate bacteria with MICP potential.

b) By-products

Talking about the environmental impact of some applications, direct emission from the process is usually evaluated first. In the ureolysis process, the produced undesired by-product ammonium/ammonia, is one of the controversial parts of this technique when explaining its advantages from an ecological point of view. To tackle the harmful by-products from cementation reagents, there are three general strategies: transformation, avoidance, and posttreatment (Zhu and Dittrich 2016). The first method is transformation of by-products into a stable form of mineral to avoid emission of ammonia gas into air and percolation of ammonium into groundwater, which generally refers to precipitating ammonium as struvite ($NH_4MgPO_4 \cdot 6H_2O$). The second one refers to exploration of other pathways that do not produce such by-products. As mentioned in the introduction section, various metabolic pathways are contributing to the calcium carbonate precipitation in nature. Table 2.1 below summarizes reactions in five common metabolisms that induce carbonate precipitation, with the byproducts of each reaction bold. As shown in the table, various toxic bioproducts are produced from these reactions. Jain, Fang, and Achal (2021) reviewed more than 200 papers on the topic of MICP via denitrification process, and the authors evaluated different pathways based on factors such as rate of precipitation, intermediate product, product, cost, etc., revealing that the denitrification pathway outcompeted others on most factors. However, this comparison is based on an ideal scenario. In a real situation, reduction of nitrate is less controllable than the ureolysis process due to its relatively strict reaction conditions, thus, it is usually difficult to ensure long-term performance of bacteria and uniformity of cemented soils (Lin et al. 2021). Up to now, a number of studies have attempted to improve MICP through other metabolic pathways, while how effectively these pathways could meet technical requirement compared to the ureolytic pathway is still unclear (K. Zhang et al. 2023). As a result, there is a gradual increase in the number of studies on posttreatment strategies, including the microbial transformation of ammonium by anaerobic oxidation and utilization of adsorbents like zeolite. Although it may take a long time until the ammonia drops to an acceptable level, the former is a feasible method for in situ posttreatment. Recently, Lee and Gomez (2024) published a work on

removal of ammonium using rinse injection as a posttreatment strategy, and in this study, they achieved 95% total ammonium removal without significant impacts that negatively affect the cementation effectiveness of soil columns. Despite its limitation, these outcomes depict a promising future for the posttreatment of by-products.

Table 2. 1 Reactions and by-products in biocementation process through different metabolic pathways

Metabolisms	Reactions & **by-products**
Photosynthesis	$HCO_3^- + Ca^{2+} \rightarrow \mathbf{CH_2O} + CaCO_3 + \mathbf{O_2}$
Ureolysis	$CO(NH_2)_2 + H_2O + Ca^{2+} + Cell \rightarrow \mathbf{NH_4^+} + Cell\text{-}CaCO_3$
Ammonification	$Amino\ acid + O_2 + Cell \rightarrow \mathbf{NH_4^+} + HCO_3^-$ $Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$
*Denitrification	$CH_2COO^- + H^+ + NO_3^- \rightarrow \mathbf{CO_2} + \mathbf{N_2} + H_2O$ $Ca^{2+} + CO_2(aq) + OH^- \rightarrow CaCO_3 + H_2O$
Sulfate reduction	$SO_4 + CH_2O + OH^- + Ca^{2+} \rightarrow CaCO_3 + \mathbf{CO_2} + H_2O + \mathbf{HS^-}$

* Incomplete reduction of nitrate leads to the production of NO and N_2O.

2.3.2 Soil related challenges

a) Compatibility of target soil

From the perspective of target soil, it is of significant importance to understand its chemical and physical properties before designing the method of treatment. These are fundamental issues that should be taken into consideration before wide scale application in the field, while many studies have focused on the application of cementing soils with relatively simple characteristics, often sands, without considering about the complexity of natural soils. Figure 2.3 presents a group of soils that is generally considered as soils with good compatibility with MICP applications and gives a basic explanation about soil organic matter, an important composition in a soil profile (Collins and McGuire 2019). Generally, the technique is reported to be effective on cementing soils with grain size in the range of 10-1000 μm

without containing many organic matters (C.-S. Tang et al. 2020). Soils with excessive organic matter are usually considered as problematic soils that need to be treated with great consumption. And considering its relatively small coverage globally, the organic soils are not attracting many attentions in the field of biocementation. One of the main obstacles in the conventional method to stabilize organic soils is that humic substances in organic soils hinder the cementation process (Toda et al. 2020). Similarly, biocementation through ureolysis to stabilize such soils is also a tough task due to its cation exchange capacity and ability of enzyme inhibition. In order to widen the applicable range of MICP field, it is essential to understand how it works on other types of soil. Up to now, some researchers have made significant contribution to this direction. Portugal et al. (2020) did a critical review of MICP applications and probably first reviewed how this technique work on organic soils. Previous MICP studies on organic soil stabilization, including some of our studies, have found that cementation effectiveness is rather unsatisfactory (Canakci, Sidik, and Kilic 2015; Safdar et al. 2021; Gowthaman et al. 2021; M. Chen et al. 2021). Some researchers investigated the effects of organic matter on enzymatic biocementation by mixing inorganic soils with commercial organic soil for agricultural use (mixing organic content up to 11.3%), which revealed a complicated improvement mechanism (Venda Oliveira and Neves 2019; Oliveira, Freitas, and Carmona 2017). In our previous study, we tried to figure out the mechanisms from the point of soil humic substances, in which the results showed that humic acid (HA), a fraction of humic substance, can affect the bacterial growth and hinder the formation of calcium carbonate, interactions with ureolytic bacteria differ across species to species (M. Chen et al. 2022; M. Chen et al. 2023). Perhaps the most unexpected finding that stands out from our results reported earlier is that certain amounts of HA in soil matrix can enhance the effectiveness of enzymatic biocementation for fine soils, while the carbonate precipitation can be significantly hindered by excessive addition. From a unique perspective, this result revealed that HA can serve as an eco-friendly and affordable amendment to improve the efficiency of enzymatic biocementation, which would be a fruitful area for further work (Meiqi Chen et al. 2024). A study published very recently have observed similar effect of organic compound on the cementation effectiveness. C. Wang et al. (2024) compare the stabilization of fortified peat soil using MICP, EICP, and lignin aided EICP, revealing that the addition of lignin can contribute to a great enhancement of unconfined shear strength by creating nucleation sites for carbonate precipitation. One section in the following chapter describes the merits of HA as amendment for MICP applications. An insightful understanding of how cementation effectiveness is affected by various factors contributes to applications of MICP on a wider range of soils or other materials.

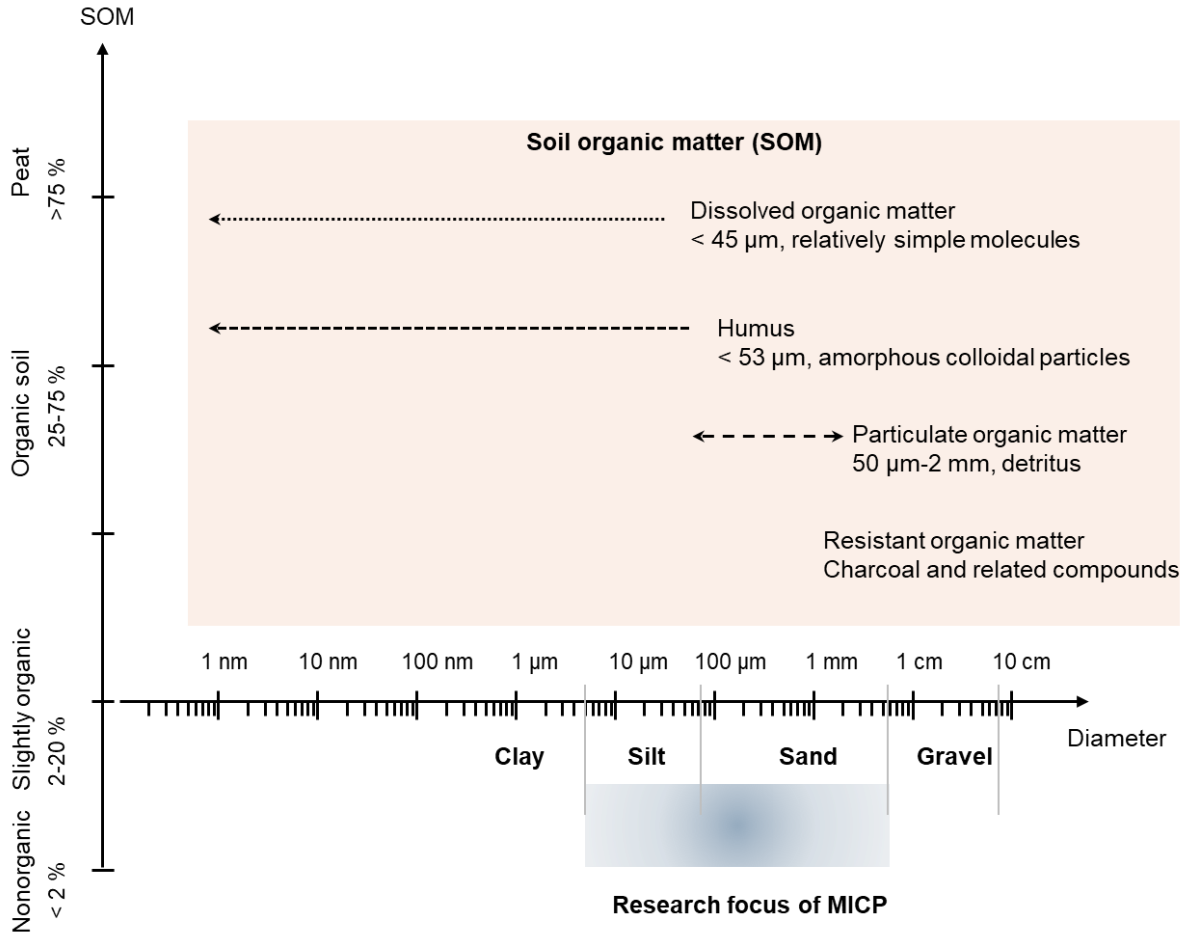

Figure 2. 3 Compatible range of soils in MICP research focus within a classification based on soil grain size and soil organic matter content

b) Durability of treated soil

Up to now, many field scale applications have proved that MICP can be an effective technology for soil stabilization. However, it should be noted that these applications have not been evaluated over their life span. There are many questions remain not answered, such as how long its total lifespan or service lifespan could be and what kind of strategies are there for restoration or demolition stage (D. L. de Melo, Kendall, and DeJong 2024). The long-term performance of bio-cemented soils under local climate should be taken into consideration, since chemical and physical weathering are always an on-going process in nature. To date, many researchers have been working on the long-term performance of treated soils through a series of durability tests, including wet-dry cycles, freeze-thaw cycles, wind erosion resistance tests and acid rain resistance tests. Donovan Mujah, Mohamed Shahin, and Liang Cheng (2016) examined the performance of biocemented sand under different environmental conditions,

including temperature changes, pH changes, and freeze-thaw cycles. It was found that the treated samples were able to tolerate up to 10 freeze-thaw cycles with certain reduction in the hydraulic conductivity values recorded. S. Liu et al. (2019) investigated the durability of MICP-treated sandy soils under wet-dry, freeze-thaw cycles, and acid rain conditions, revealing a significant reduction (50-80%) in unconfined compressive strength after the test. As an enhancement method, the authors have reported that synthetic fiber-reinforced MICP samples remained a relatively higher residual strength than cases without fiber. One of our previous studies using wastepaper also found a similar improvement of resistance against freeze-thaw cycles (Meiqi Chen, Gowthaman, Nakashima, Komatsu, et al. 2021). Depending on the cementation levels of treated soils, other studies also presented various levels of strength reduction after freeze-thaw cycles (Gowthaman, Nakashima, and Kawasaki 2020; Sharma, Satyam, and Reddy 2021). In a recent study, Sun et al. (2022) reported that with small addition of polyacrylamide (1.5 g/L), the weathering resistance under rainfall and freeze-thaw erosion of MICP treated loess slope surface was greatly enhanced. MICP treatment for wind erosion control is one recent research trend with a number of studies published (J. He et al. 2023). An interesting study investigated the effectiveness of suppression of sand dune wind erosion through two non-ureolytic pathways, oxidation of two organic salts of calcium (calcium acetate and calcium formate), using two species from *Bacillus* genus (Hemayati et al. 2023). The authors found that non-ureolytic bacteria can achieved significant cementation effectiveness that is comparable to the standard ureolytic bacteria.

2.3.3 Challenges related to employed bacteria

a) Public acceptance, regulations, and potential biohazard

There are still challenges faced by microorganisms, which need to be solved. Of particular concern is that mainstream biocementation research employs commercially available bacteria (e.g., *Sporosarcina* sp., *Bacillus* sp.) due to their high performance, which may lead to potential biohazard (Zhu and Dittrich 2016; Portugal et al. 2020). Although there is no comprehensive risk evaluation system established for this issue yet, general recommendations are to explore and utilize native strains, and then, control the bacteria population being applied. When considering the introduction of microorganisms into environments, public acceptance should be taken into consideration. Although biotechnology using bacteria has been developing for centuries, bacteria still impress people with its negative image as a cause of diseases. Further efforts are needed to increase the public acceptance of the utilization of bacteria. On the other hand, the utilization of microorganisms is under strict regulatory control in many countries. For example, the use of exogenous bacteria in Japan is prohibited. Owing to efforts from a variety of studies focusing on exploration of indigenous bacteria, it is now well established that these natives are able to perform MICP in a high efficiency which is comparable to the performance of some

popular commercially available bacteria, like *S. pasteurii* (Badiee et al. 2019) and *Bacillus megaterium* (Rajasekar et al. 2021). The exploration of bacteria is demonstrated in the following section.

b) Survivability and adaptability of bacteria

How local microorganism communities interact with the bacteria that we introduced into soils indirectly influences the cementation effectiveness in the long term. In a soil microbiome, life and death is happening every moment in the interactions with local communities, artificially introduced bacteria could die from grazing predation, viral lysis, bacterial predation, chemical warfare, etc. (Sokol et al. 2022). On the other hand, compared to the favorable conditions in laboratory-scale experiments, field-scale applications are quite harsh and are subject to constant changes, thus, bacteria may loss viability due to temperature shift, desiccation, osmotic shock, etc. As a result, applied bacteria may fail to achieve the desired outcomes. When considering the utilization of bacteria in large field scale, one of the concerns is whether these bacteria can survive till the desired requirements are achieved, as local bacterial communities are more likely to over compete foreign bacteria (Zhu and Dittrich 2016).

As mentioned before, alkalophilic ureolytic bacteria with high performance, such as *Sporosarcina* and *Bacillus* species are preferred in laboratory-scale studies. These bacteria are able to show excellent MICP performance in laboratory conditions that are favorable to their survival and growth, while whether these bacteria can adapt to the soils in the natural environment or not is still unclear. As most researchers with geoengineering background are focusing on the mechanical improvement of soil, few attentions were paid to checking the bacteria population changes after being introduced to the soil. Few studies have found that the added bacteria usually are eliminated by local communities after few days, leading to a decline cementation efficiency afterward (DeJong et al. 2022). It was reported that the interaction between added bacteria and indigenous bacteria might be competition for nutrients, leading to a decrease precipitation rate (P. Liu, Shao, and Huang 2019). These challenges faced by bacteria added difficulty in standardizing the application of MICP technique. On the other hand, for the borderless implementation of MICP, more options for microorganisms with desirable metabolic capabilities must be considered. Particularly, for the stabilization of problematic soils with complex properties, introduced bacteria might be eliminated soon after treatment.

There is an urgent need to address these issues to develop a more flexible technique towards a wide range of applications to various soils. It leads to a proliferation of studies on searching for local bacteria for applications. Many studies have concluded that selected native bacteria are able to achieve a higher cementation efficiency than the well-known commercial bacteria (Badiee et al. 2019; Mohammadizadeh et al. 2020; Rajasekar et al. 2021; Maureira et al. 2024). The following sections listed what have been established by researchers on addressing the issue of bacterial survivability and

adaptation and proposed a potential isolation source for identifying bacteria suitable for biocementation applications.

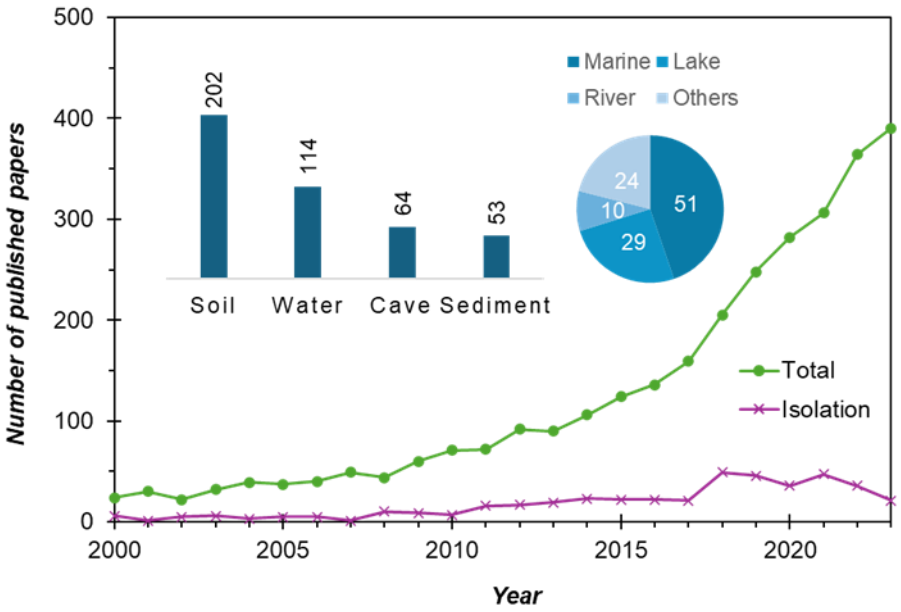

Figure 2. 4 Number of published research articles related to the topic of bacterial isolation in MICP research in Web of Science from 2000 to 2023. Research topics: (bacteria isolation, carbonate precipitation/calcite precipitation/calcification) and (soil/water/cave/sediment/marine/lake/river). The data were extracted in October 2024

c) Exploration of diverse bacteria in nature

Ammonium is one of the least expensive nitrogen sources that microorganisms can utilize, which is usually incorporated into organic compounds (Herrero, Flores, and Imperial 2019). As a simple organic source of nitrogen, urea can be assimilated by most microorganisms. For this reason, diverse bacteria species can produce urease enzymes to hydrolyze urea and make use of the products. These bacteria are ubiquitous in soil, marine, cave, and even extreme environments of hypersaline (Zhu and Dittrich 2016). So far, the isolation of bacteria from different environments for biocementation applications has been one of the topics that attract considerable interests. Figure 2.4 presents the paper number on the topic of bacterial isolation in MICP research since 2000. Up to now, approximately 10% of the MICP studies have been trying to isolate bacteria from different environments.

Most studies focused on screening ureolytic bacteria as a part of selective biostimulation for different purposes. Soil is the most common isolation source as this technique is usually applied for soil

improvement, bioremediation, biomineralization, etc. In particular, for stabilization of some problematic soils with unique physical and chemical properties, native bacteria are more preferred as they are adapted to the environment and are more likely to show good performance in cementation process. For instance, some studies did some trials to isolating ureolytic bacteria from acidic organic soils to stabilize soft ground (Phang et al. 2018; Safdar et al. 2021). A recent study isolated a ureolytic strain (*Pseudogracilibacillus* sp.) from a desert soil and employed the bacteria for the mitigation of aeolian erosion, which confirmed drastic improvements in the erodibility resistance of soil in a wind tunnel test (Dubey et al. 2021). Hata et al. (2020) isolated Sporosarcina sp. with excellent performance in deep-seabed environment from marine sediments for stabilization of methane hydrate-bearing layer. For stabilization of heavy metals, Li et al. (2022) found a ureolytic strain from *Glutamicibacter* sp. can tolerate significant concentration of heavy metal ions in growth media and precipitate carbonate of Pb, Cd, Cu, and Ni at low temperature and wide range of pH.

Some of these studies are targeted at looking for bacteria with specific features that are necessary for certain applications. Aiming to isolate halophiles from nature, ureolytic bacteria from different genera (*Bacillus, Porphyrobacter*, *Pseudomonas, Salinivibrio,* and *Halomonas*) were also isolated from water and sediment samples in hypersaline environments, and the distinguished halotolerant bacteria were confirmed with their applicability for biomineralization, suggesting a promising technique for water treatment (Arias et al. 2019). Other studies tried finding ureolytic bacteria with better performance and introducing the bacteria for broad MICP applications. The research group of A.I.Omoregie isolated and characterized ureolytic bacteria from a limestone cave located in Malaysia. The results suggested that these bacteria all belong to *Sporosarcina* sp. and *Bacillus* sp., some of them have a high urease activity that is comparable to *S. pasteurii* (Armstrong Ighodalo Omoregie et al. 2016; Armstrong Ighodalo Omoregie et al. 2019). Skorupa et al. (2019) investigated facultative and anaerobic consortia consists of haloalkaliphilic bacteria that are capable of inducing carbonate precipitation through ureolysis from lake sediments. The results showed that the bacterial consortia can achieve a higher ureolysis rates than *S. pasteurii* (ATCC 11859), indicating a great potential for MICP applications in anaerobic environments.

Although a considerable number of bacteria in nature are able to produce urease and induce carbonate precipitation, only a small group of *S. pasteurii* and some *Bacillus* species (*Bacillus sphaericus*, *Bacillus megaterium, Bacillus subtilis*) are contributing to the majority of MICP research. As these well-known ureolytic bacteria typically have a high urease activity and are widely available in bioresources center or natural environments. Chuo et al. (2020) reviewed more than 150 papers to gain insight into a trend in the use of bacteria, including ureolytic and non ureolytic bacteria. Without any doubt, the most popular bacteria strains are *S. pasteurii* and some groups in *Bacillus* species. More investigations are needed to widen the diversity of bacteria applied in MICP applications.

2.4 Potential of airborne bacteria for MICP applications

As mentioned above, MICP researchers have been continuously investigating ureolytic bacteria in various environments for a long time, aiming to find novel strains to improve cementation efficiency. These studies have been exploring ureolytic bacteria in diverse sources, such as soil, sediments, lake, ocean, etc., while no attention has been ever given to the biome in the air. Since the bacteria concentration in the air is 10 order magnitude less than that in the soil and ocean, air is not generally considered as an isolation source of bacteria (Šantl-Temkiv et al. 2022). Airborne bacteria are mostly investigated as a factor for environmental monitoring. In this section, the potential of airborne bacteria as candidates for MICP applications was discussed.

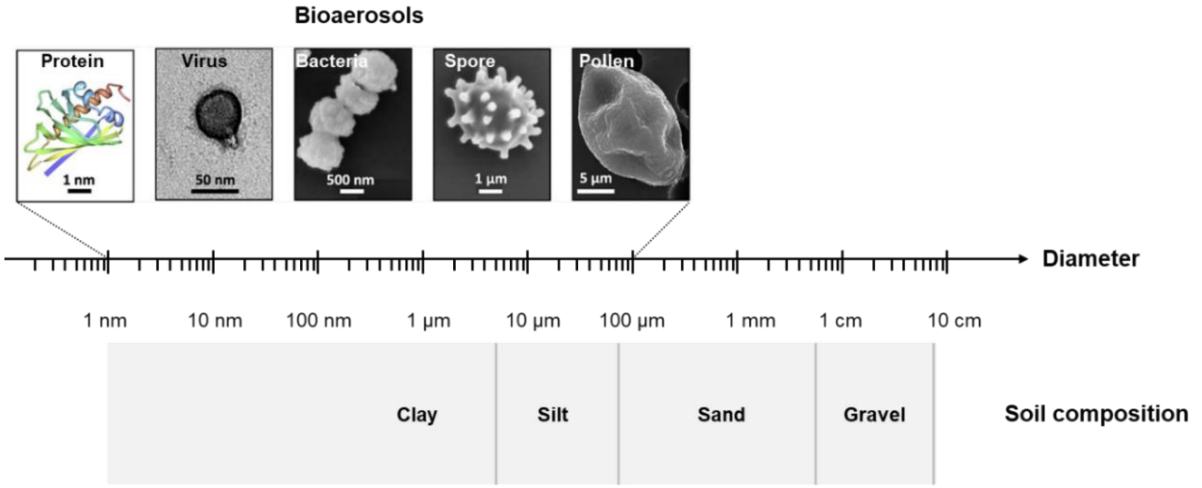

Figure 2. 5 Diverse bioaerosols and their size distribution. Modified from Fröhlich-Nowoisky et al. (2016)

2.4.1 Bioaerosols

In a broad sense, airborne bacteria are usually discussed as a component of bioaerosols. Therefore, it is important to understand what bioaerosols are before introducing airborne bacteria. Bioaerosols are airborne particles of biological origin, which are released from terrestrial and marine ecosystems into the atmosphere (Burrows et al. 2009). The biological origins include algae, fungi, pollen, bacteria, viruses, etc. Figure 2.5 compares the size of general bioaerosols and soil particles. Originally, they were emitted from common sources such as soil, rocks, plants, animals, and water in ecosystems by external forces, experience short-term or long-term transport, and finally deposit on surface. This cycle has a profound influence on the global environments in terms of plant and human health, biogeochemical

cycling, as well as long-distant genetic exchange (Després et al. 2012; Fröhlich-Nowoisky et al. 2016; Smets et al. 2016). Figure 2.6 illustrates emission sources in diverse ecosystems and some biological-driven processes during the transport period. Generally speaking, aerosolization is happening almost wherever there is a surface. Since the leaf surface is estimated to be 4 times larger than that of the ground surface, plants are believed to be one of the largest emission sources of microorganisms (Després et al. 2012). Other surfaces include soils (carried by wind), water bodies (bubble burst), animals. Up to now, it is still not explicit about how human activity affects bio-aerosolization, but some emission from industrial waste contribute to the transportation of bioaerosols to some degree. In a recent review work on atmosphere, Amato et al. (2023) described the aeromicrobiome as a selective and dynamic outer-layer of the earth's microbiome. In this review, biological and physical drivers in the atmosphere were well discussed. Basically, this emission-transport-deposition cycle has two major features, high turnover rate, and high selectivity. The first feature suggests that the system is highly dynamic, as it takes a relatively short time for most microbes to go from emission to deposition. It means that active cells either experience selection or evolution to survive in the air for long periods.

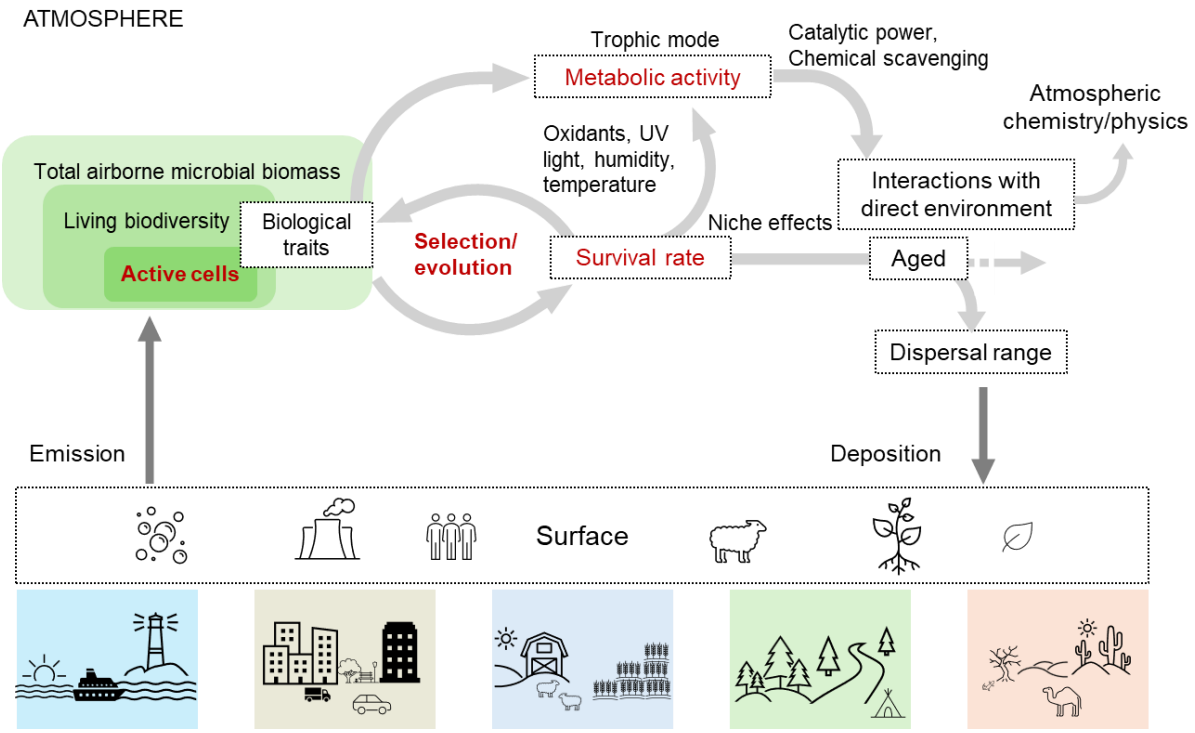

Figure 2. 6 Various emission sources of bioaerosols and biological-driven processes in the atmosphere. Modified from Amato et al. (2023)

2.4.2 Airborne bacteria

Airborne bacteria are one of the most investigated bioaerosols. Traditionally, they were characterized by the cultivation-based method, revealing a relatively small portion of culturable bacteria. With the development of technology, culture-independent molecular techniques have become a highly efficient technique to quickly identify the microbes in a sample. Undoubtedly, high-throughput sequencing has absolute advantages in the efficiency of characterizing large-volume samples. However, cultivation-based methods are still recommended for figuring out the metabolic features of bacteria. Although the development of mega genome analysis has enabled us to predict these features based on sequence reads, how these bacteria respond to various conditions is still unclear. For several decades, there has been a paradigm that less than 1 % of microbes in the environment are culturable in laboratory conditions. In a recent study, Martiny (2019) revealed that high proportions of bacteria are culturable across major biomes, and in the air, 50-80% of bacterial taxa had closely related cultured bacteria.

Extensive research has grown up around the topic of airborne bacteria in the built environment since it is one of the important risk factors associated with disease transmission (Prussin and Marr 2015; Fujiyoshi, Tanaka, and Maruyama 2017; X. Chen, Kumari, and Achal 2020). Much of these research works has been descriptive in exploring airborne bacteria, especially human pathogens, in terms of distribution, diversity, and characteristics (Gandolfi et al. 2013; Smets et al. 2016; Ruiz-Gil et al. 2020). To survive in a harsh environment like air, airborne bacteria need to have various survival strategies. For instance, to tackle nutrient deficiency, some bacteria can function at a low metabolic rate or enter into a dormant form (e.g., spores). To survive UV radiation, some bacteria are able to produce pigments in a light responsive manner, specifically for those who survive in the air of high altitude, and some are able to response to the DNA damage quickly and activate their repair system (Després et al. 2012; Smets et al. 2016; Tastassa, Sharaby, and Lang-Yona 2024). Studies have found many bacteria that stay viable in the air belong to generalist group which has a diverse nutrition, so they can make use of various resources for energy production (Amato et al. 2023). Due to their ability to adapt to extreme environments, some of them are frequently demonstrated as candidates for biotechnological applications, while these are still in the early stages with discussion focusing on feasibility. For instance, pigmentation is one of the features that is frequently identified in airborne bacteria. These bacteria can serve as a source of pigments for various applications purpose, including textile, food, and medicine industries (Smets et al. 2016; Ruiz-Gil et al. 2020). Airborne bacteria in clouds were found to synthesize proteins that have anti-freezing and ice-nucleating activities, and these versatile proteins are widely studied for different purposes in the field of biotechnology (Huang et al. 2021).

2.4.3 Airborne bacteria as candidates for MICP applications

Currently, microbes that are widely applied to industrial processes usually have certain features. Firstly, it should fall into a Bio-safety level one. On consideration of cost during the production process, fast growth with low requirements on energy consumption is preferred. Depending on the purpose of utilization, how long they can survive under certain storage conditions is essential to the commercialization of the product. For example, lactic acid bacteria were widely used in the food industry. As they are believed to be beneficial to human health, the storage requirements are strict to maintain their activity. For bacteria used in MICP applications, they are usually selected based on their capacity of carbonate precipitation, without much consideration on other biological features. According to existing studies, popular strains have a great similarity in terms of their behavior in the treatment process. These strains mostly have preference to a weak alkaline condition for cultivation and grow in a moderate temperature range, which limited their applicability to more diverse environments. Selecting bacteria with high resilience to harsh environments provides a solution to the survivability issue.

As described previously, airborne bacteria are from different emissions sources such as soil, water, animals, plant etc. Air is usually described as a harsh environment that kills microbes by UV radiation, desiccation, starvation, oxidants, etc. For instance, one result of evolutionary strategies is that some bacteria produce pigments to protect themselves from UV light exposure (Fröhlich-Nowoisky et al. 2016). Therefore, air could also be considered as a filtration system that exerts selective pressure to these wanderers, which leads to the survival of resilient species and the death of fragile ones. These survivals have enormous potential to be applied in environment with harsh physical conditions. On the other hand, bacterial generalists that are more likely to stay viable in the air might have better adaptation to nutrient deficient conditions as they make use of energy sources of great diversity. In a frequently disturbed system, bacterial habitat generalists (species are broadly classified as habitat generalists and specialists according to their niche breadth) are usually dominant owing to their metabolic flexibility (Y.-J. Chen et al. 2021). The atmosphere is such a kind of system. Based on the established studies, it is hypothesized that a fraction of airborne bacteria might be ubiquitously distributed (Ruiz-Gil et al. 2020). Theoretically, these kinds of bacteria generalists are ideal candidates for board less applications of MICP technique. Since they are ubiquitous, there is less concern about biohazard caused by the strains, and they should be more acceptable to the public compared to the exogenous strains. And more importantly, these bacteria can adapt to various harsh conditions. In an ecosystem, bacteria habitat generalists are generally believed to be versatile but not as efficient as specialists (Y.-J. Chen et al. 2021). However, for MICP applications, these generalists might contribute to a long-term performance than the specialists as they are able to survive for a longer period.

Overall, there are many potential merits of introducing airborne bacteria to MICP applications for soil improvement. As discussed above, firstly, after being screened in the harsh environment, air, these resilient bacteria have immense potential to enhance the possibility of survival and adaptation to various conditions of natural soils. It can directly contribute to a highly efficient cementation through airborne bacteria for long term. On the other hand, as mentioned earlier, the regulatory issues of utilization of microorganisms are strict in some areas, which limit the applications of MICP technology. In the case of applying airborne bacteria that are ubiquitous, the public acceptance can be enhanced, and the treatment can be conducted under regulation. In particular, the predicted existence of a small fraction of airborne bacteria inhabits most environments on the earth can contribute to a borderless application of these bacteria. Considering their features as habitat generalists, they can adapt to various problematic soils. Furthermore, these bacteria are more likely to be nutritionally versatile, which means they can utilize various sources as energy sources. This would contribute to a cost reduction in the culture media if they can efficiently make use of waste materials. It is worth mentioning that this is the first study to make an original contribution to exploring resilient bacteria for MICP application from the air, the indispensable yet sometimes disregarded existence.

Chapter 3 Exploration of ureolytic airborne bacteria for biocementation applications

3.1 Background

What has been discussed in the literature review highlights the need to address these issues to establish a more flexible technique aiming for application to various soils. Most researchers augmented alkalophilic ureolytic bacteria, to produce calcium carbonate in laboratory-scale MICP studies. It is worth mentioning, however, for the borderless implementation of MICP, more options for microorganisms with desirable metabolic capabilities must be considered, and this research work has made some efforts to overcome these obstacles by seeking solutions from microorganisms. As discussed in the previous chapter, selecting bacteria with high resilience to harsh environments provides a solution to the survivability and adaptability issues. Air is such a cruel environment that screens out bacteria with great resistance. On the other hand, since airborne bacteria are ubiquitous, there is less concern about biohazard caused by the strains, and they should be more acceptable to the public compared to the exogenous strains.

The ureolysis is the most energy-efficient pathway of MICP, and that has been the primary focus of geotechnical researchers to date. Therefore, this study aims to explore ureolytic airborne bacteria in an easily accessible elevation and investigates whether they could be effectively applied in MICP for soil improvement. Firstly, samplings were conducted using an air sampler to collect airborne bacteria from three climate zones in Japan. Two sampling sites (Hokkaido, Tsukuba) have a humid continental climate. Hokkaido is the typical cold region in Japan with long, very cold winters and warm, cool summers. Tsukuba has a moderate climate with warm summers and cool winters with light snowfall. The third sampling site, Okinawa, has a subtropical oceanic climate with long summer, and mild winters. The occurrence of ureolytic bacteria in each sample was studied to gain an understanding of the distribution features of ureolytic bacteria in distinct environments. There are 10- 20 % ureolytic bacteria in the air based on our findings using ammonium-yeast media that was designed to cultivate *S. pasteurii*. Many of these isolates have shown an excellent performance in precipitating carbonate in a short period. The results of gene analysis revealed that the collection from Sapporo samplings has a high similarity in terms of taxonomy with the collection of Okinawa and Tsukuba samples. Most identified ureolytic bacteria were found belonging to two orders, *Micrococcales* and *Bacillales*. The culture media used for sampling narrowed down the range of ureolytic bacteria collected in this study. As the first stage of exploring airborne bacteria for MICP applications, this chapter demonstrates that there are abundant ureolytic bacteria in the air that can induce significant amounts of carbonate precipitation.

3.2 Materials and methodology

3.2.1 Sampling sites

All samplings described in this thesis were conducted in some cities with typical climate features in Japan (see in Figure 3.1). Most sampling sites were mountains, forest parks, farms, and campuses. These sites are chosen due to the dense vegetation, as it has been reported that the leaf surface (4 times larger than that of the ground surface) is a large emission source of microorganisms (Després et al., 2012). On the other hand, some spots with penned animals were believed to have a high possibility to inhabit microorganisms with urease activity since these animals provide a source of urea.

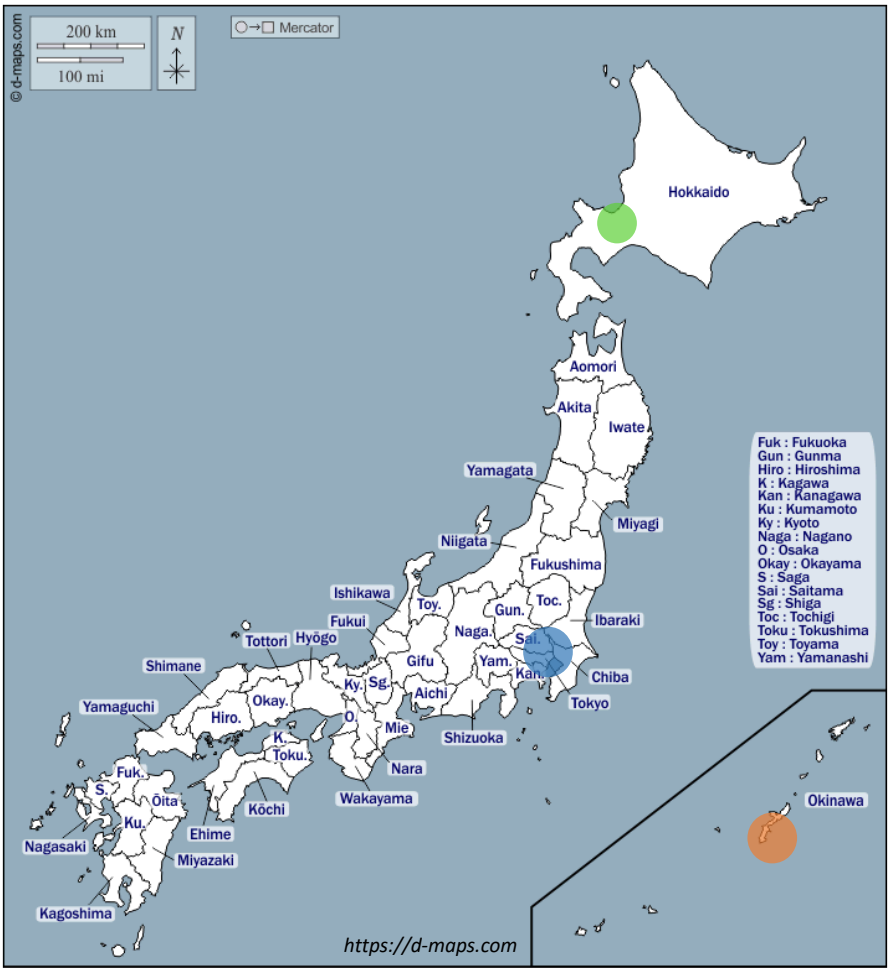

Figure 3. 1 Three main sampling sites of this study *(source: https://d-maps.com)*

a) Sapporo sampling

The sampling sites are in urban terrestrial areas of Sapporo, Hokkaido, Japan (see in Figure 3.2). Hokkaido, a cold region with an annual mean temperature of about 10°C, is located at the northern tip of Japan and surrounded by the Pacific Ocean, the Sea of Japan, and the Sea of Okhotsk. Sapporo is the capital city, with a low population density of 67 persons/km^2 (MLIT of Japan). Table 3.1 presents the details of sampling sites. The sampling was conducted on sunny days in the fall season of 2022. The temperature was mostly in the range of 15°C–25°C and the average wind speed is about 6.33 km/h. Based on the data provided by Japan Atmospheric Environmental Regional Observation System and Sapporo atmospheric environment observation data breaking system, the average air quality in the sampling month was good (PM2.5: 25–50 μg/m^3; PM10: 0–25 μg/m^3; O_3: 25–50 ppm; NO_2: 0–25 ppm; SO_2: 0–25 ppm; CO: 0–25 ppm).

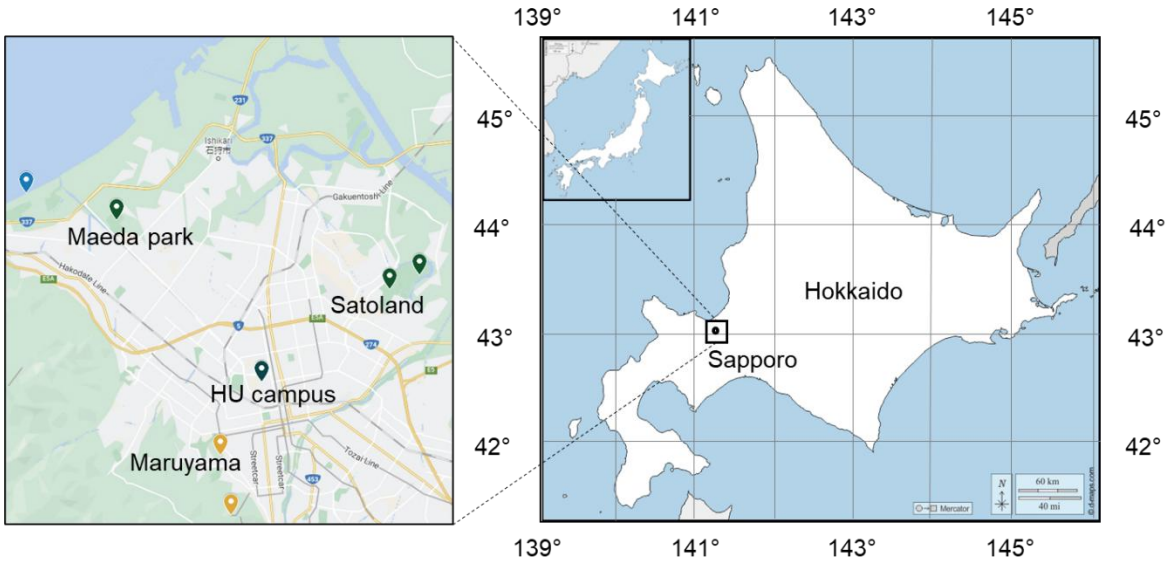

Figure 3. 2 Sampling sites in Sapporo City *(source Google map &: https://d-maps.com)*

Table 3. 1 Information on sampling sites in Sapporo City

Location	Site ID	Latitude (N)	Longitude (E)	Altitude	Note
Hokkaido University	HU	43°4'28"-43°4'44"	141°19'59"-141°20'15"	10-20 m	Field, dairy farm, forest
Dream beach	DB	43°9'3"-43°9'26"	141°11'15"-141°12'21"	0 m	Beach
Aso beach	AB	43°14'56"-43°15'7"	141°21'2"-141°21'8"	0-10 m	Beach
Moerenuma	MN	43°7'12"-43°7'20"	141°25'34"-141°25'50"	10-60 m	Wetland Park
Mt. Moiwa	MW	43°1'18"-43°1'20"	141°19'21"	510-520 m	Observation deck, forest
Mt. Maruyama	MY	43°2'5"-43°3'9"	141°18'31"-141°18'59"	60-220 m	Mountain, forest
Maeda	MD	43°8'39"-43°8'52"	141°15'11"-141°15'23"	10 m	Forest Park
Satoland	SL	43°6'58"-43°7'10"	141°24' 48"-141°25' 3"	10 m	Park with penned animals

b) Okinawa sampling

The second sampling was conducted in Japan's southernmost prefecture, Okinawa, a chain of islands with its own history as an independent kingdom (see in Figure 3.3). Okinawa, with a distinctly subtropical climate, its mean temperature prevailing in the city is recorded as 22.9°C. Table 3.2 presents the details of sampling sites. This sampling consists of two sampling trials, as the first trial did not find sufficient candidates for analysis. The first trial was conducted on sunny days (July 18th-21st) in the summer season, and the second trial was from September 27th - 28th of 2023. The temperature was in the range of 26°C–33°C and the average wind speed is about 13.53 miles per hour. Based on the data provided by Japan Atmospheric Environmental Regional Observation System, the average air quality in the sampling week was good (PM2.5: 18–22 μg/m^3; PM10: 13–23 μg/m^3).

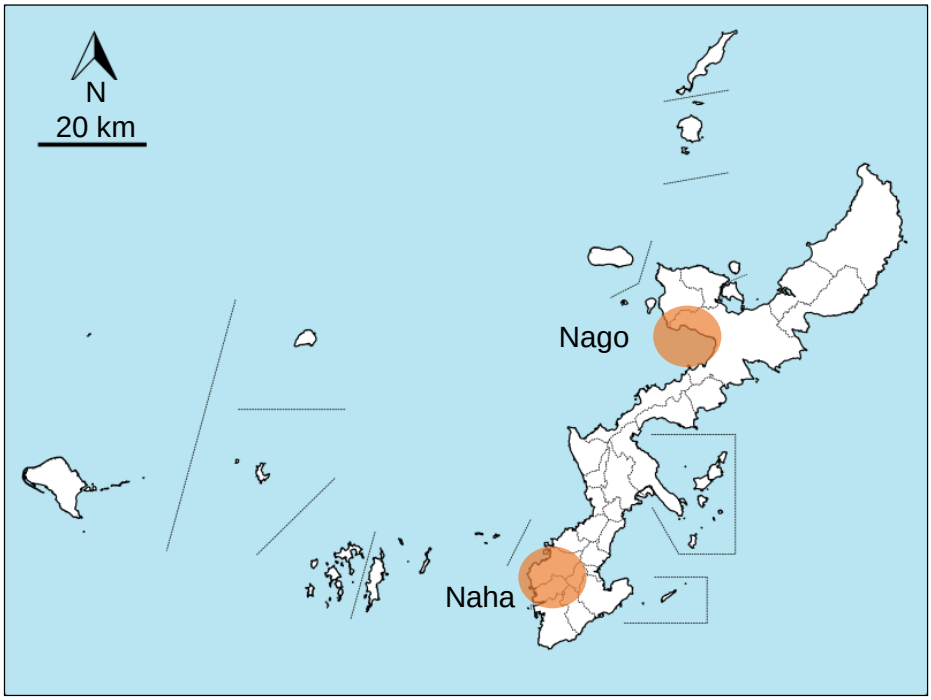

Figure 3. 3 Sampling sites in Okinawa. *(source: https://d-maps.com)*

Table 3. 2 Information on sampling sites in Okinawa

	Location	Site ID	Latitude (N)	Longitude (E)	Altitude	Note
First trial	Shurijo	SR	26°13'2"	127°43’2"	120 m	Historical heritage
	Kouriohashi	KU	26°40’44"	128°0’47"	0-10 m	Beach
	Nakijinjo	NK	26°41’31"	127°55’39"	80 m	Limestone wall
	Churaumi	CR	26°41’31"	127°52’39"	40 m	Aquarium
	Bise	BS	26°42’13"	127°52’47"	0-10 m	Beach
	21C forest park	21C	26°35’29"	127°58’20"	0-10 m	Coastal Park
	Kakazu	KK	26°15’31"	127°44’13"	100 m	Observation deck
	University of the Ryukyus	UR	26°15’4"	127°45’57"	120 m	Forest
Second trial	Shurijo	SR	26°13'1"-26°13'7"	127°43’2"-127°43’6"	110-130 m	Historical heritage
	Kakazu	KK	26°15’30"-26°15’32"	127°44’8"-127°44’15"	70-100 m	Observation deck
	Urasoe	US	26°14’48"-26°14’53"	127°43’54"-127°43’57"	110-130 m	Historical heritage

c) Tsukuba sampling

Tsukuba is in the southwest of Ibaraki Prefecture (east of Japan mainland). It has a humid continental climate with warm summers and cool winters. The average annual temperature is 14.3 °C. The sampling was conducted from September 17th - 18th of 2023. The temperature was in the range of 24°C–34°C.

Based on the data provided by Japan Atmospheric Environmental Regional Observation System and Ibaraki Prefecture EPA, the average air quality in the sampling week was good (PM2.5: 20–50 μg/m^3; PM10: 0–25 μg/m^3). The details of sampling sites are presented on the table below.

Table 3. 3 Information on sampling sites in Tsukuba

Location	**Site ID**	**Latitude (N)**	**Longitude (E)**	**Altitude**	**Note**
Matsumi park-1	TF1	36°5’28"	140°6’26"	70 m	Observation tower
Matsumi park-2	TF2	36°5’28"	140°6’26"	75 m	Observation tower
Matsumi park-3	TF3	36°5’30"	140°6’24"	30 m	Grove
Tsukuba-Agricultural center-1	TF4	36°7’11"	140°5’43"	30 m	Cow house
Tsukuba-Agricultural center-2	TF5	36°7’6"	140°5’37"	30 m	Field
Doho park-1	TF6	36°3’41"	140°7’20"	30 m	Pond
Doho park-2	TF7	36°3’34"	140°7’25"	30 m	Grove, horse
Mt. Tsukuba-1	TS1	36°13’34"	140°6’6"	800 m	Observation deck
Mt. Tsukuba-2	TS2	36°13’34"	140°6’6"	800 m	Observation deck
Mt. Tsukuba-3	TS3	36°12’52"	140°6’1"	350 m	Cable car station
Tsukubasan Shrine	TS4	36°12’48"	140°6’5"	250 m	Stone wall
Haneda T2-1	TS5	35°30’58"	139°47’23"	10 m	Observation deck
Haneda T2-2	TS6	35°30’58"	139°47’23"	10 m	Observation deck

3.2.2 Sampling device and sampling methods

An air sampler (MAS-100 Eco®, Merc Millipore) that is designed for monitoring environmental hygiene was used to collect airborne bacteria at an airflow of 100 L/min (±4%) for 500 s per sampling (see the mechanism in Figure 3.4). Before sampling, the perforated lid was cleaned with ethanol for disinfection to reduce contamination. After each sampling, the samples were sealed with PVC tape and stored in sterile Ziplock bags temporarily. All samples were then incubated at 25°C or 30°C (depending on isolation sites) for 48 h before the isolation and screening process.

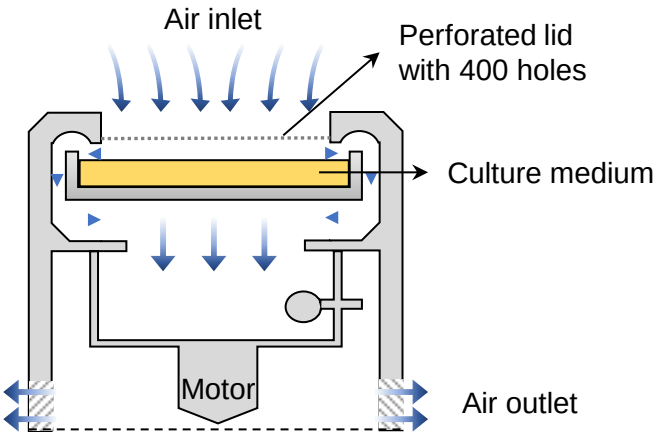

Figure 3. 4 Sampling mechanism of air sampler MAS-100 Eco®

3.2.3 Culture media and chemicals

The culture media used in this study were summarized in the table below. From the second sampling of Okinawa, a fungicide (Kabicidin, SHIOTANI M.S. CO., LTD) was added to the culture media to avoid growth of fungi, and 0.1 mM nickel chloride was added to improve concentration of nickel source for urease synthesis.

Table 3. 4 Culture media used for different purposes in sampling

Media type	Formulation
NH_4-YE	15.75 g/L of tris-aminomethane, 10 g/L of ammonium sulfate, 20 g/L of yeast extract, and 20 g/L of agar
Zobell	5 g/L of poly-peptone, 1 g/L of yeast extract, 0.1 g/L of $FePO_4$, and 15 g/L of agar (final pH 7.4-7.8)
MRS broth	10 g/L of proteose peptone no. 3, 10 g/L of beef extract, 5 g/L of yeast extract, 20 g/L of dextrose, 1 g/L of polysorbate 80, 2 g/L of ammonium citrate, 5 g/L of sodium acetate, 0.1 g/L of magnesium sulfate, 0.05 g/L of manganese sulfate, 2 g/L of dipotassium phosphate, and 15 g/L of agar
R2A	0.5 g/L of peptone, 0.5 g/L of yeast extract, 0.5 g/L of casamino acids, 0.5 g/L of glucose, 0.5 g/L of soluble starch, 0.3 g/L of K_2HPO_4, 0.05 g/L of $MgSO_4 \cdot 7H_2O$, 0.3 g/L of sodium pyruvate, and 15 g/L of agar (final pH 7.0-7.4)
B4	0.04 g/L of yeast extract 0.05 g/L of dextrose, 0.05 g/L of calcium acetate, and 15 g/L of agar
CPA	5 g/L of nutrient broth, 6.006 g/L of urea, 11.098 g/L of calcium chloride, 10 g/L of NH_4Cl, and 15 g/L of agar

3.2.4 Isolation and purification of bacteria

After 48-hour culture at 30℃, single colonies from the plate were inoculated on new plates using a platinum loop. The purified strain was obtained after 48- hour cultivation. Then the bacteria were cultured for another 24 hours to prepare for a simple urease activity test. Figure 3.5 below presents a simple illustration of the isolation process.

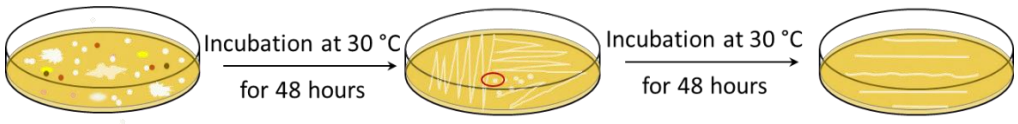

Figure 3. 5 Isolation and purification of isolates

3.2.5 Screening of ureolytic bacteria

The screening procedures could be simplified as three rounds of screening, as seen in Figure 3.6. Simple urease test could quickly identify ureolytic bacteria, then the precipitation test confirms the bacterial ability to induce carbonate precipitation in cementation media, and finally gene identification confirms the biosafety level of the isolates.

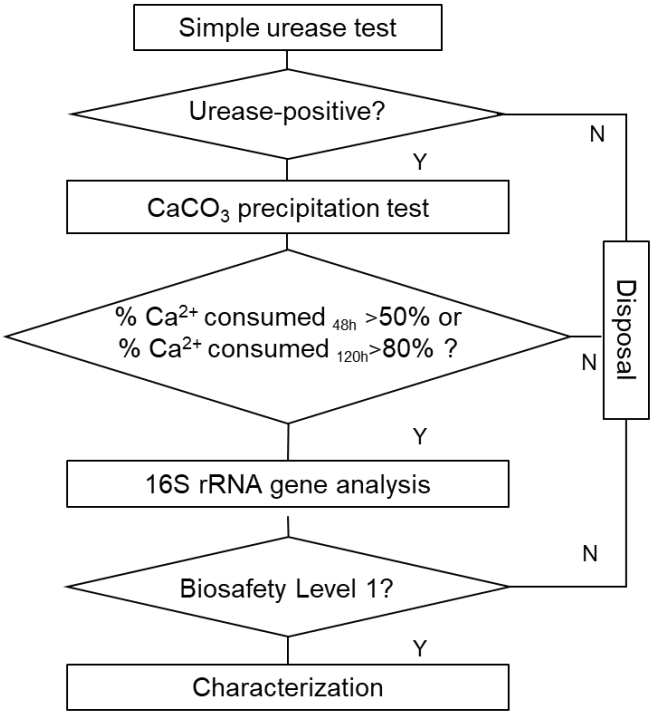

Figure 3. 6 Isolation and screening of candidates for MICP applications

a) Simple urease test

To begin this process, a simple and rapid test using cresol red is adopted to identify ureolytic bacteria efficiently. During the process of urea hydrolysis, the pH of the solution increases over time. A simple urease activity test using cresol-red could confirm urease activity by a qualitative observation of the color change from yellow to purple, indicating an increase in pH. 100 mL test water solution consists of 2.5 g of urea and 2 mL of 0.04% cresol-red ethanol solution. The bacteria were added to the testing solution, then incubated at 45°C for 2 h. Species that changed color into purple were identified as urease activity positive (Danjo and Kawasaki, 2016).

b) Calcium carbonate precipitation test

Bacteria were prepared in liquid culture and evaluated in calcium carbonate precipitation tests under second-round screening which also rules out bacteria that fake the first-round test by other metabolic activities. The tests were conducted at room temperature to confirm the bacterial ability to induce calcium carbonate precipitation. For each test tube, 2 mL of bacterial main culture was added into 8 mL of 0.5 mol/L of $CaCl_2$ and urea solution. The calcium meter used in this measurement is a compact Ca^{2+} meter B-751 (HORIBA, Ltd., Kyoto, Japan), with a measurement range from 4 to 9,900 ppm. For each sampling, 100 μL of the solution was taken and diluted before measurement. To select both fast and slow precipitators, two lines were set up to select potential candidates.

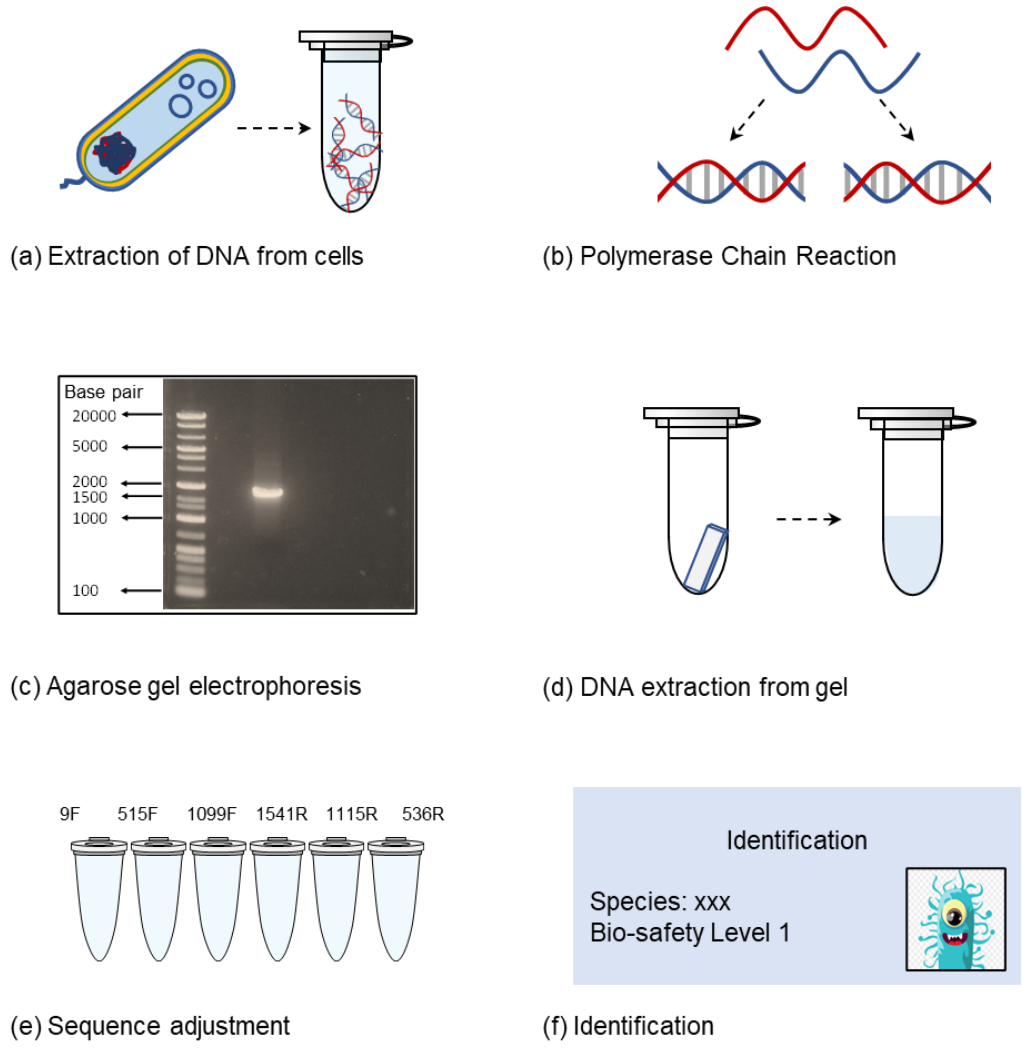

Figure 3. 7 Identification of bacterial species by sequencing 16S rRNA

c) 16S rRNA gene analysis

The DNA of selected isolates was extracted using MightyPrep reagent for DNA (TAKARA Bio Inc.) following the instructions provided by the manufacturer. The PCR components include 25 μL of Primestar Max (TAKARA PrimeSTAR Max DNA Polymerase), 1.5 μL of forward primer 9F (5′-GAGTTTGATCCTGGCTCAG-3′), 1.5 μL of reverse primer 1541R (5′ AAGGAGGTGATCCAGCC-3′), 1 μL of DNA template, and 21 μL of sterile distilled water. The thermal cycler (T100 Bio-Rad Laboratories, Inc.) started the PCR with a 30-s initial denaturation at 94℃, and the thermal cycling process consisted of 30 cycles of 10-s denaturation at 98°C, 5-s annealing at 55°C and 8-s extension at 72°C. Agarose gel electrophoresis was then conducted to visualize and purify the PCR amplicons. Bands that appear around 1,500 base pairs were cut from the gel and DNA was extracted from the gel using FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd.). Six primers were used in the sequence adjustment process: 9F, 515F (5′-GTGCCAGCAGCCGCGGT-3′), 1099F (5′-GCAACGAGCGCAACCC-3′), 1541R, 1115R (5′-AGGGTTGCGCTCGTTG-3′), and 536R (5′-GTATTACCGCGGCTGCTG-3′). Adjusted samples were then sent to Eurofins Japan to analyze the sequence. Finally, BLAST (Basic Local Alignment Search Tool) analysis was performed using the database DB-BA17.0 (Techno Suruga Laboratory, Japan). Under Japanese laws as to microorganism utilization, it is necessary to examine the bio-safety level (BSL) of bacteria before applying the microorganism to a field scale. The isolates confirmed as Biosafety Level one species were stored at −80°C in glycerol stock for further characterization. The process is illustrated in Figure 3.7.

3.3 Results and discussion

3.3.1 Representative pictures of collected samples

a) Sapporo sampling

Figure 3.8 provides images of representative airborne bacteria sampled from four sites. There are some notable differences between these samples. For instance, more undesired fungi colonies grew on plates sampled from sites with penned animals compared to that on other samples. It also depicts some samples (Moerenuma park and Mt. Moiwa) covered by fungal mycelium, and some samples from coastal areas. Four Hokkaido sampling samples from Hokkaido University, Maeda Park, Maruyama Park, and Sato Land obtained sufficient colonies to analyze.

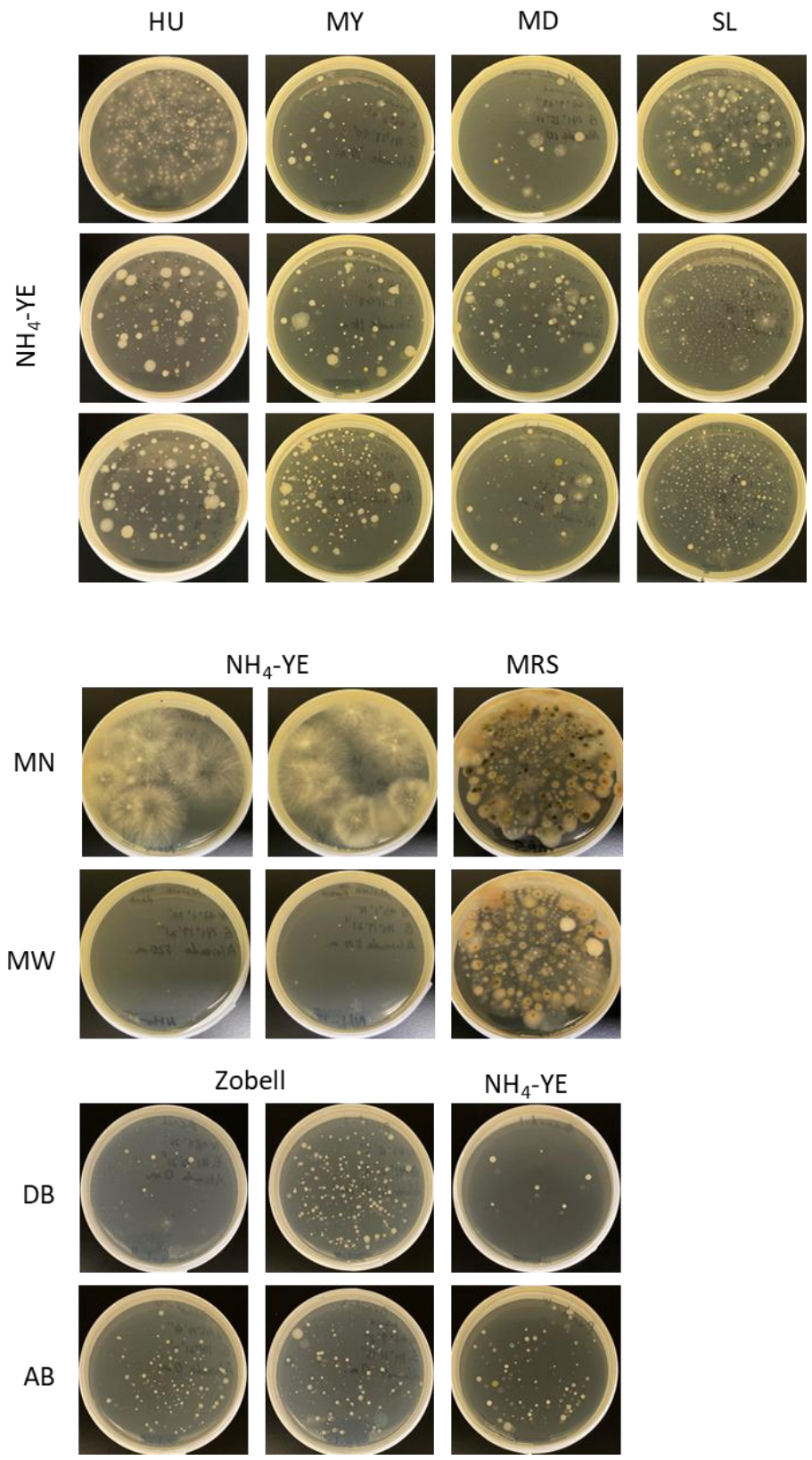

Figure 3. 8 Representative samples collected from Sapporo sampling

b) Okinawa sampling

Samples collected from the first trial in Okinawa are presented in Figure 3.9. Unfortunately, most agar plates were covered by fungal mycelium, regardless of what type of culture media was used for sampling. In particular, some media with high concentrations of sugar and peptone grew no visible bacterial colonies. A possible explanation for this phenomenon is that the high humidity in the air of subtropical areas favors the growth of fungi and the spread of fungal spores. Although populations of bacteria in the air are not a small number, their growth might be inhibited. For this sampling, two carbonate precipitation culture media, CPA and B4, were used to identify calcifying species from collected colonies directly. Unfortunately, affected by the fungal growth, these samples did not make a quick identification of ureolytic bacteria as we expected. An explanation is that growth of calcium carbonate crystals takes long time, making it difficult to identify the calcium carbonate aura by naked eyes.

To overcome the problem caused by fungi, a fungicide was added to all the culture media used for the second sampling in Okinawa. As can be seen in Figure 3.10, there are still some fungal colonies growing, but these colonies are not as aggressive as they were in the previous sampling. Probably, the fungicide undergoes degrading when being kept at room temperature for long, so these fungi grow slowly but are not completely inhibited by fungicide. In general, the addition of fungicide could increase the survival of bacteria and improve the isolation efficiency of the first stage of this work. For this sampling, R2A, as a general culture media for heterotrophs, was used as a reference. As Figure 3.10 shows, there is a significant difference between the R2A samples and NH4-YE samples in terms of the bacterial population, which means that the NH4-YE, as a selective culture media, filtered the majority of the airborne bacteria and grew a small group of the airborne bacteria that tolerate high concentrations of ammonium salt. To get a better understanding of the difference between bacteria from these two types of media, colonies on R2A media were also isolated and evaluated in the following tests.

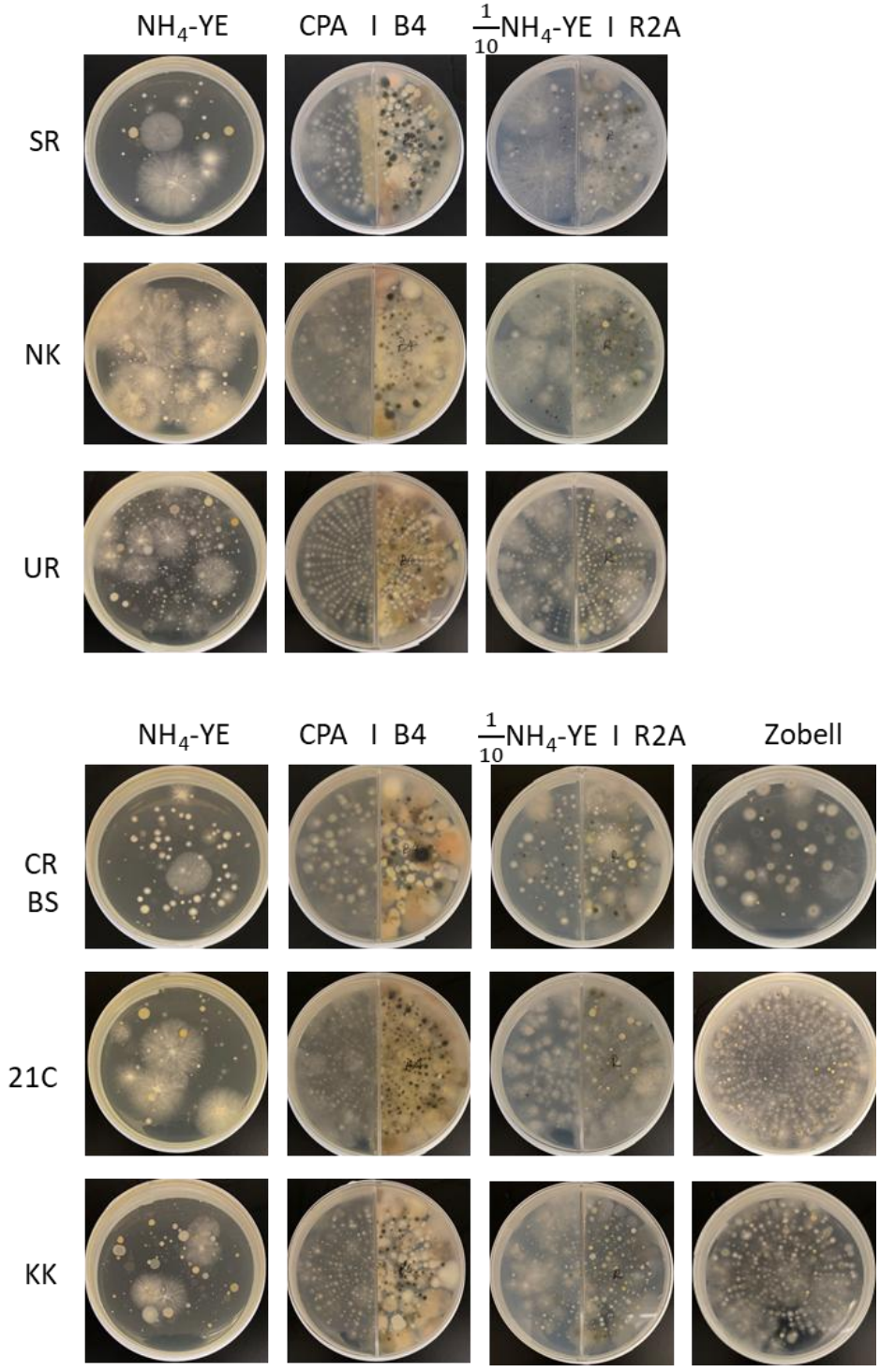

Figure 3. 9 Representative samples collected from the first Okinawa sampling

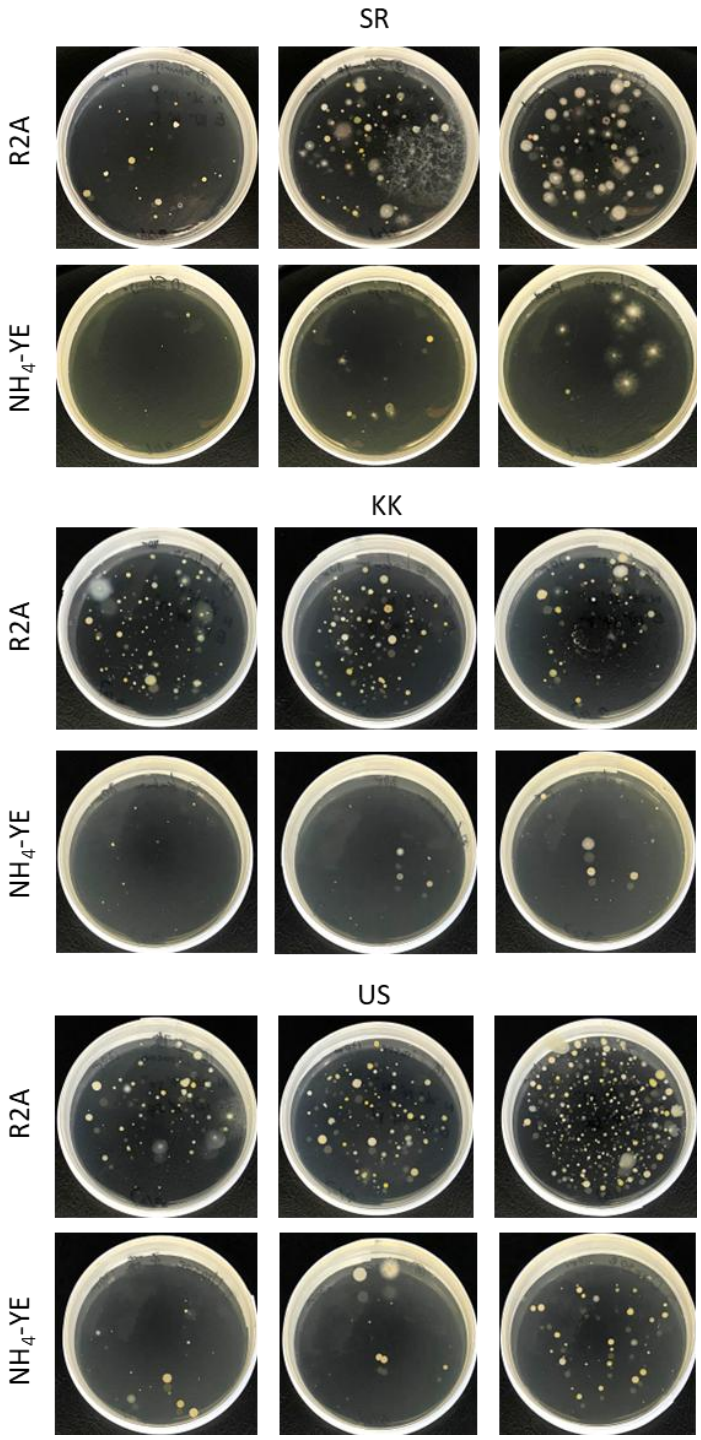

Figure 3. 10 Representative samples collected from the second Okinawa sampling

c) Tsukuba sampling

Figure 3.11 illustrates representative samples collected from Tsukuba. Similar to Sapporo sampling, we went to Tsukuba University, Tsukuba Mountain, and several parks in the city. Unexpectedly, the air was cleaned up by sudden rainfall before the sampling. For this reason, less bacterial colonies were obtained this time. Samples obtained from Tsukuba University did not catch as many bacteria as we expected. Sampled on the same day, samples from two parks show quite distinct colonies in terms of population. Matsumi Park has a wide range of ponds but less vegetation, while Doho Park is mostly covered with trees, and more importantly, there is a horse house for riding experience in the park. These big mammals are urea sources for microorganisms, which explains why a higher population of airborne bacteria grew on the NH_4-YE sample from this park than samples from other sites. On the other hand, the colonies from Doho Park shared a great similarity in terms of bacterial morphology. Based on their colony morphology, many of them possibly belong to the *Sporosarcina* genus or *Bacillus*-related genus.

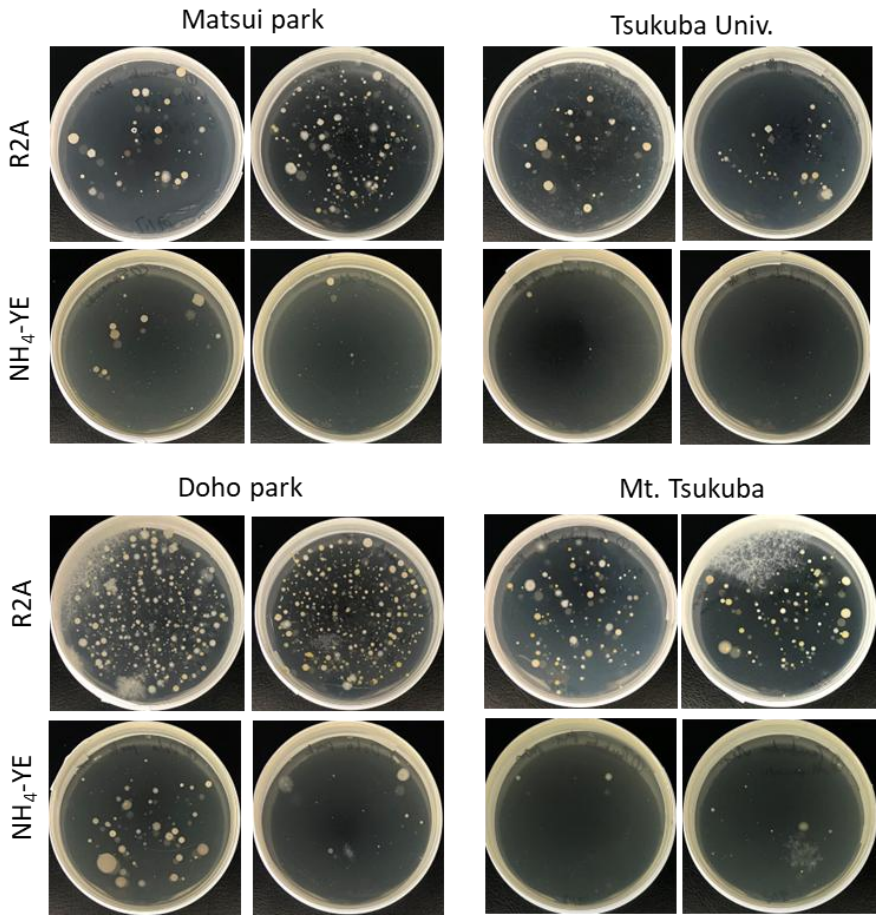

Figure 3. 11 Representative samples collected from Tsukuba sampling

3.3.2 Urease-positive percentage by sampling sites

a) Sapporo sampling

Removing those samples governed by fungal colonies, twenty-two samples were obtained, with 8, 6, 4, 4 isolates from Hokkaido University, Maruyama, Maeda, and Satoland, respectively. Among 577 tested colonies, fifty-seven were identified as urease positive by simple urease tests. The urease-positive percentage by sites is summarized in Table 3.5. In general, more ureolytic strains were found in sites with more dense vegetation. Totally, fifty-seven isolates (10 from HU, 30 from MY, 13 from MD, and 4 from SL) were selected and examined in the calcium carbonate precipitation tests.

Table 3. 5 Summary of isolates in each site of Sapporo sampling

Site ID	HU	MY	MD	SL	Beach	SUM
Tested colonies	125	253	103	96	256	833
Positive	10	30	13	4	10	67
Urease +, %	8	11.86	12.62	4.17	4	8

b) Okinawa sampling

In total, thirty-one samples were collected during the first sampling conducted in Naha and Nago. Unfortunately, most agar plates were completely occupied by fungal mycelium instead of bacterial colonies. However, there are some samples from Kakazu Park still growing a certain number of bacterial colonies. Table 3.6 below shows the number of tested colonies and urease-positive percentage by site. Among 8 sampling spots, Kakazu samples had grown colonies many times higher than the other spots did and about 30 % of them are urease positive. On average, 22% of tested colonies of this sampling showed urease activity in the simple urease test, which is higher than what we expected, since this number obtained from the last sampling in Sapporo was 12%. The reason is that the diversity of culture medium was increased for this sampling, and general culture medium R2A for heterotrophic bacteria grew a high percentage of bacteria producing urease. Similar to Sapporo sampling, less than 10% of bacteria colonies growing on NH_4-YE were identified as urease-positive.

Table 3. 6 Summary of isolates in each site of first Okinawa sampling

Site ID	SR	KU	NK	CR	BS	21C	KK	UR	SUM
Tested colonies	6	12	9	8	4	30	76	17	162
Positive	1	3	1	1	1	4	22	3	36
Urease +, %	16.7	25	11.1	12.5	25	13.3	28.9	17.6	22.2

For the second trial of sampling in Naha city, twenty-four samples (12 samples on NH_4-YE and 12 samples on R2A) were collected from 12 spots. Similar to what we obtained from the first trial; general medium grew more ureolytic bacteria than the NH_4-YE medium did. Bacteria colonies having a chance to grow without competition with the help of fungicide explained this difference in numbers of colonies between two times of samplings. As summarized in Table 3.7, 10-20 % of bacteria colonies were identified as urease positive.

Table 3. 7 Summary of isolates in each site of second Okinawa sampling

Site ID	SR	KK	US	SUM
Tested colonies	107	62	78	247
Positive	20	14	9	43
Urease +, %	18.7	22.6	11.5	17.4

c) Tsukuba sampling

In total, twenty-six samples were collected from thirteen sampling spots in Tsukuba. One sample did not grow any colonies. Table 3.8 presents the urease-positive percentage of 25 samples (12 NH_4-YE samples and 13 R2A samples). Similar to Okinawa sampling, about 20 % of tested isolates were found to be ureolytic bacteria. More urease-positives were found from samples obtained from parks and mountain areas. As mentioned before, these areas usually with dense vegetation, which means there is a large population of bacteria that are ready to be aerosolized on leaves' surfaces. On the way return to Sapporo, several samples were obtained from the observation deck in Haneda airport. Probably, influenced by the strong wind, not many bacteria were caught, but many of them are ureolytic bacteria.

Table 3. 8 Summary of isolates in each site of Tsukuba sampling

Site ID	MP	TU	DP	MT	TS	HD	SUM
Tested colonies	87	29	116	111	20	17	380
Positive	15	4	29	25	2	5	80
Urease +, %	17.2	13.8	25	22.5	10	29.4	21.1

3.3.3 Discussion on distribution features of ureolytic airborne bacteria

A comparison of occurrence of ureolytic bacteria from three sampling sites is illustrated in Figure 3.12. In the Sapporo sampling, NH_4-YE is the only medium used. On average, about 12 % of the isolates were found to be ureolytic bacteria. For the following samplings in Okinawa and Tsukuba, some modifications of culture media were made to enhance the efficiency of screening. With the addition of nickel chloride in the culture media, the percentage of urease positives increased. Due to these changes, samples from Tsukuba and Okinawa are showing a higher urease-positive percentage than Sapporo sampling, while samples from Tsukuba and Okinawa did not exhibit a significant difference. It is worth noting that even higher percentage of ureolytic bacteria were found in Tsukuba and Okinawa sampling, these isolates were showing a weaker capacity in calcium carbonate precipitation than isolates found in Sapporo sampling, suggesting a weaker urease activity.

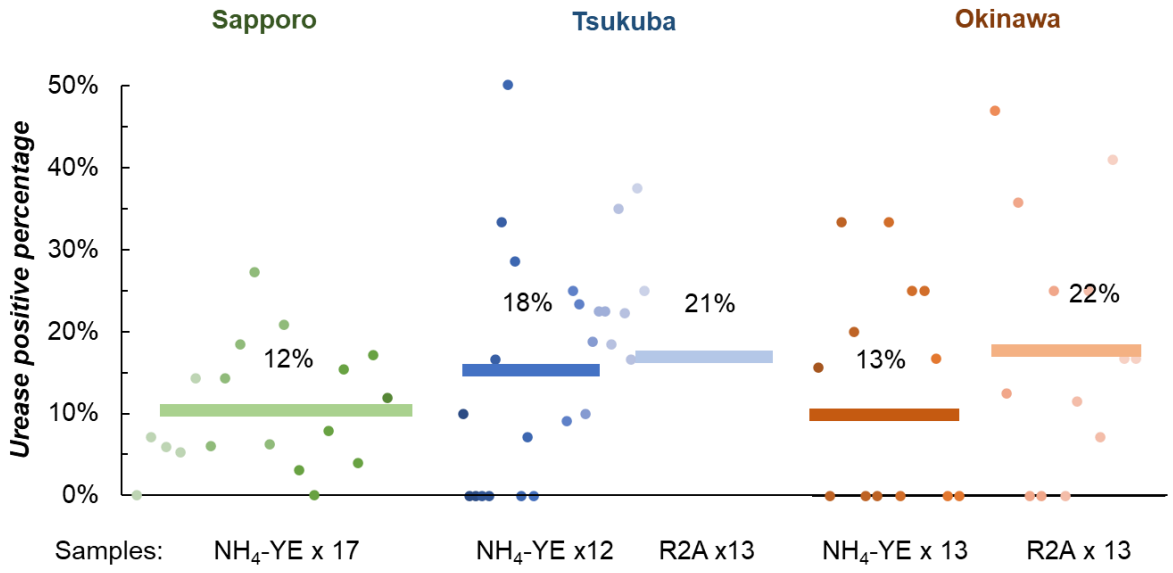

Figure 3. 12 Comparison of the occurrence of ureolytic bacteria from three sampling sites

3.3.4 Monitoring of calcium carbonate precipitation rate

a) Sapporo sampling

Figure 3.13 presents the percentage of calcium ions consumed by fifty-seven isolates from four sites in Sapporo after 48-h and 120-h incubation. On average, isolates from HU were shown to have carbonate precipitation capability in the test, followed by MY, MD, and SL isolates. Isolates that precipitated more than 50% calcium ions after 48-h incubation and that precipitated more than 80% calcium ions after 120-h incubation were selected as potential candidates for MICP application. Five isolates from HU, five isolates from MY, and two isolates from MD were identified by 16S rRNA gene analysis and then characterized in the following tests.

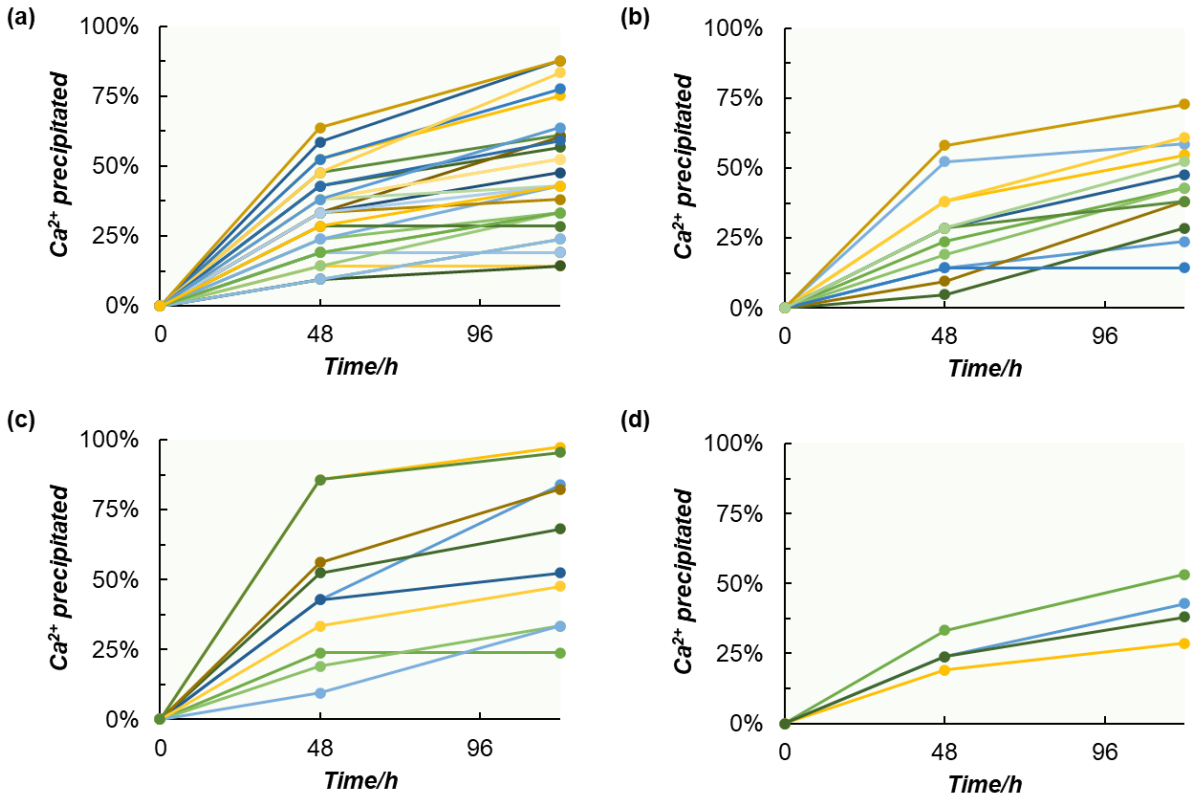

Figure 3. 13 Calcium carbonate precipitation test. The percentage of calcium ions consumed after 48 h and 120 h incubation (a) HU isolates; (b) MY isolates; (c) MD isolates; (d) SL isolates

b) Okinawa & Tsukuba sampling

Figure 3.14 (a) shows the precipitation of calcium carbonate by seventeen isolates from NH_4-YE samples obtained in Okinawa, and Figure 3.14 (b) presents thirteen isolates from NH_4-YE samples obtained from Tsukuba. Figure 3.14 (c) depicts that most isolates from R2A media could only induce a small amount of precipitation even after 144- hour incubation. If the previous screening conditions were applied to these isolates, it would filter out three from thirty isolates. Additionally, we can hardly find potential isolates from R2A samples, since most of them did not show a significant urease activity in the precipitation test. To ensure that we have sufficient isolates to make comparisons between sampling sites, isolates were selected based on the precipitation ranking. In total, six isolates from Okinawa sampling, and twelve isolates from Tsukuba sampling were selected and proceeded to gene analysis.

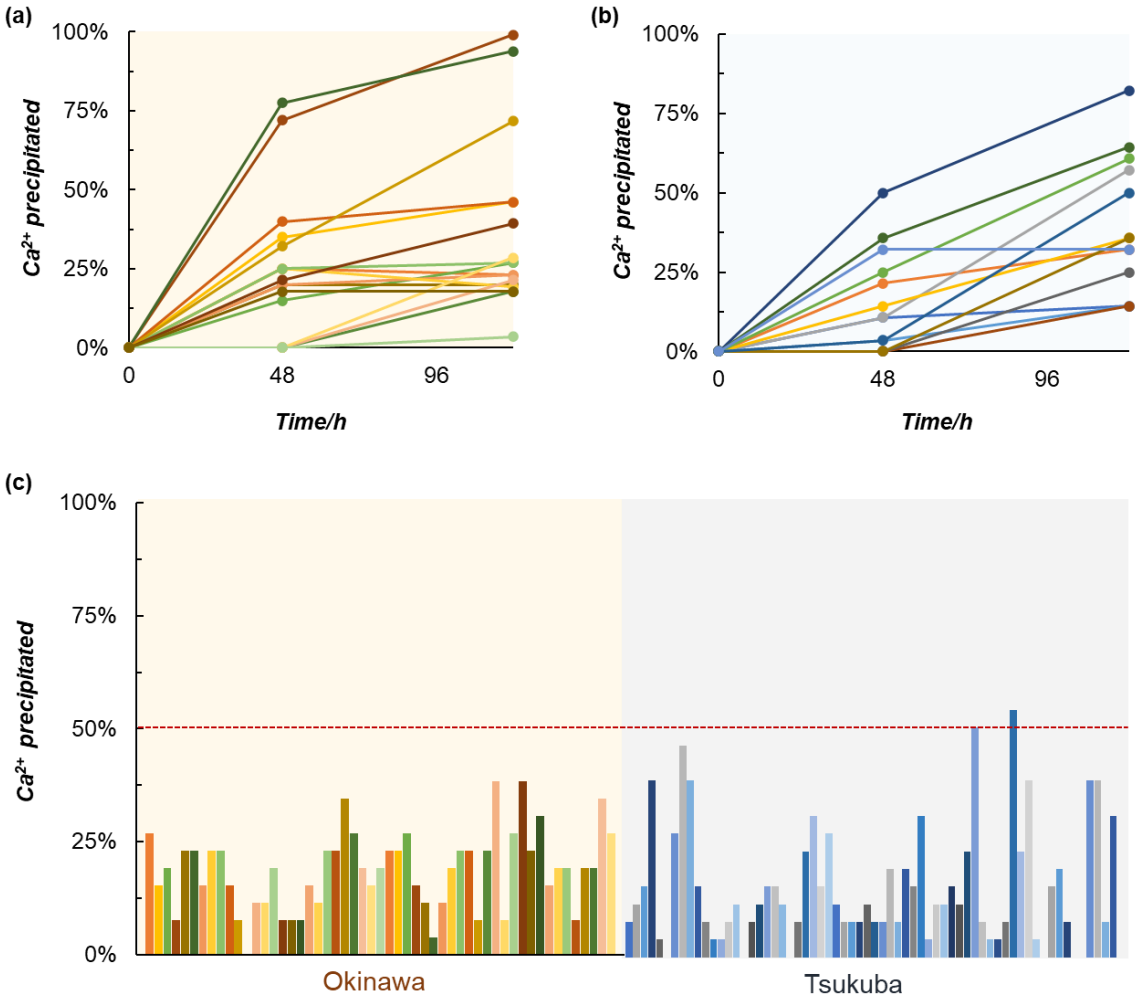

Figure 3. 14 Calcium carbonate precipitation test. The percentage of calcium ions consumed after 48 h and 120 h incubation (a) 17 isolates from Okinawa- NH_4-YE samples; (b) 13 isolates from Tsukuba-NH_4-YE samples; (c) 117 isolates from R2A samples after 144 h incubation

3.3.5 Results of bacterial gene analysis

a) Sapporo sampling

The sampling method based on cultivation finds more Gram-positive strains since Gram-negative bacteria are more likely to lose their cultivability in the air (Després et al., 2012). The findings in this study are no exception. Table 3.9 summarizes basic information of twelve isolates that all belong to Biosafety Level 1, including one opportunistic pathogen. Most of the sequences matched with known species with their identity as high as 99%. According to the information provided by "Micro Atlas Project" (2023), the closest strains were mostly previously from origins such as soil and animals. What

stands out among isolates are RII-2 and FU3 isolated from a rice field and a forest on campus, which were identified as strains from *Lederbergia* genus that was reclassified from *Bacillus* genus by Gupta et al. (2020). It should be mentioned here that the first strain RII-2, *Lederbergia lenta* (synonym: *Bacillus lentus*), was reclassified from *Bacillus* genus that consists of many ureolytic strains. The latter one is a new species in the *Lederbergia* genus since it has a similarity of 96.6% to its closest species. These two isolates were able to precipitate carbonate in a fleeting period, which is much more active than other isolates, therefore, they are proceeded to the characterization tests.

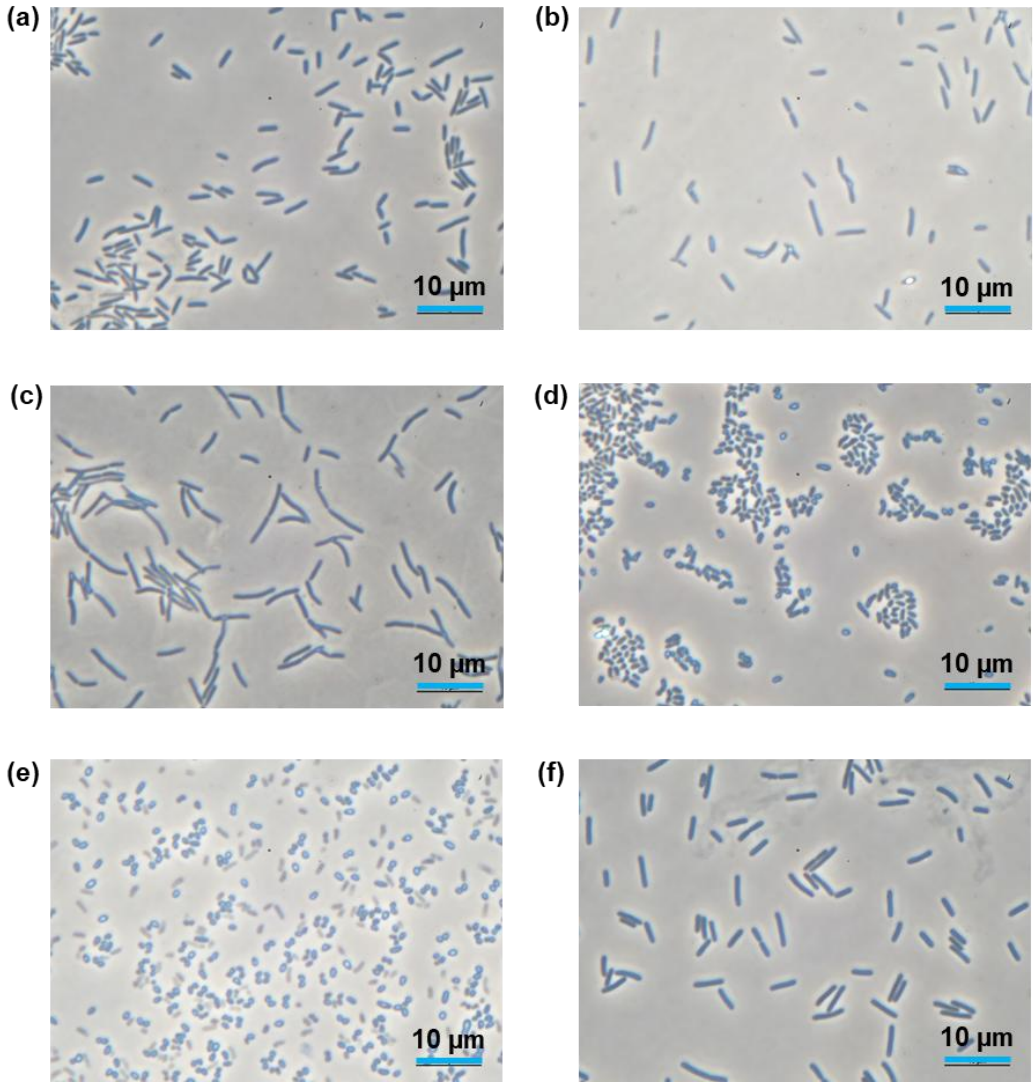

Figure 3. 15 Microscope images of four isolates after certain period of cultivation: (a-b) FU3 after 24 h and 120 h; (c) RII-2 after 24 h; (d-e) MY3-21 after 24 h and 120 h; (f) MY2-9 after 24 h

Twelve isolates are from *Firmicutes* phylum (7 isolates) and *Actinobacteria* phylum (5 isolates) which are two of four major phyla that contain most of the culturable species. In particular, five strains from *Actinobacteria* phylum were found to belong to *Glutamicibacter* genus that was reclassified from *Arthrobacter* genus (Busse 2016). As many bioremediation reports have found that strains from this genus could survive a prolonged period and changing conditions (Alvarez et al. 2017; T. He et al. 2017; Mengping Chen et al. 2022). It would be an advantage if they could be applied to MICP research. Therefore, MY3-21, one *Glutamicibacter* species showing good precipitation capacity was selected and characterized in the following tests. On the other hand, MY2-9 was selected and examined as a representative strain of *Sporosarcina* genus among twelve isolates. Microscope images (Microscope BBX50F4, Olympus, Japan) in Figure 3.15 show the morphology features of selected four isolates. FU3 and RII-2 are two typical rods, while the former (0.9–1.0 × 1.5–5.0 μm) is shorter than the latter (0.9–1.0 × 2.5–7.0 μm). MY2-9 has a similar cell shape and size (0.9–1.0 × 4.0–7.0 μm) as RII-2. MY3-21 shows a pleomorphism like *Arthrobacter* genus, changing their shape from rods to cocci (1.0–1.1 × 1.0–2.5 μm) during cultivation.

b) Tsukuba & Okinawa sampling

Among 147 tested isolates obtained from Tsukuba and Okinawa, eighteen were finally selected and identified by 16S rRNA gene analysis. Table 3.10 below summarizes the taxonomy information. Similar to the Sapporo sampling, about half of them were found to be spore-formers, and most of them are related to *Sporosarcina* and *Bacillus* species. For the non-spore formers, four of them are from *Staphylococcus* genus, one of the most common residents of animal skin, and two of them were from *Micrococcaceae* family. There is an unexpected finding that one plant-derived Gram-negative species (OS5-5) and one found in mining site Gram-negative strain (KK7) were identified. The former was reported as a plant pathogen in some case studies, so it is not an ideal candidate for application. The latter one was reclassified as a novel species in recent years. As a new species, more investigations are needed to understand its performance in MICP process. Since isolates that belong to BSL 2 and opportunistic human pathogens are not suitable for field scale applications, candidates were selected from the rest of the isolates. Of particular interest, one strain was identified as *Sporosarcina ureae* which is known for tolerating high concentration (up to 8%) of urea (Madigan et al. 2021). Besides, one pigmented strain *Micrococcus luteus* has been found in oligotrophic environments for extended periods of time (Greenblatt et al. 2004). With desired features suitable for MICP applications, these strains were selected and proceeded to the characterization process.

c) Discussion on taxonomy

Figure 3.16 compares the composition of isolates from Sapporo sampling with isolates from Okinawa and Tsukuba sampling. As can be seen in Figure 3.16(a-b), these two collections share a great similarity in terms of taxonomy. From Sapporo sampling, about half of isolates were found to be *Sporosarcina* and *Bacillus* related strains, and the other half, five strains from *Actinobacteria* phylum, were found to belong to *Glutamicibacter* genus that was reclassified from *Arthrobacter* genus (Busse, 2016). With versatile features, *Arthrobacter* is frequently reported as potential bacteria for various environmental problems. Until now, only a few studies have researched the application of *Glutamicibacter* bacteria for MICP applications. It is believed that this study could successfully explore the potential of these unique bacteria. Isolates from Okinawa and Tsukuba sampling were put in the same group for comparison. Similarly, this collection consisted of more than half of *Sporosarcina* and *Bacillus* related species, and human commensals like *Staphylococcus* species and strains from *Micrococcaceae* family. These bacteria have a large population across the world since they inhabit most environments. The interesting finding is that two Gram-negative strains were identified from Okinawa samples, which is rare in the air samples.

Figure 3.16(c) analyzed the composition of spore-formers in two collections. About half of them were found to be spore formers, belonging to *Sporosarcina* and *Bacillus* genus. Many airborne bacteria survive in the air in dormant forms and reactivate when the environment becomes favorable for them to grow. Since some of the isolates are not a spore former, they should be resilient against adversity like desiccation and starvation. Although these bacteria usually exhibit a weak urease activity, their long-term performance might be an advantage in the treatment process. From the perspective of urease regulation, these strains are more likely to produce urease when the nitrogen source in the environment is getting limited. It means that their activity may increase after some time if they can survive in the cementation media. Up to now, few studies have been investigated the applicability of bacteria with weak urease activity for MICP treatment. Further investigation in this study tries to provide fresh insight into the role of bacteria with low activity in biocementation treatment.

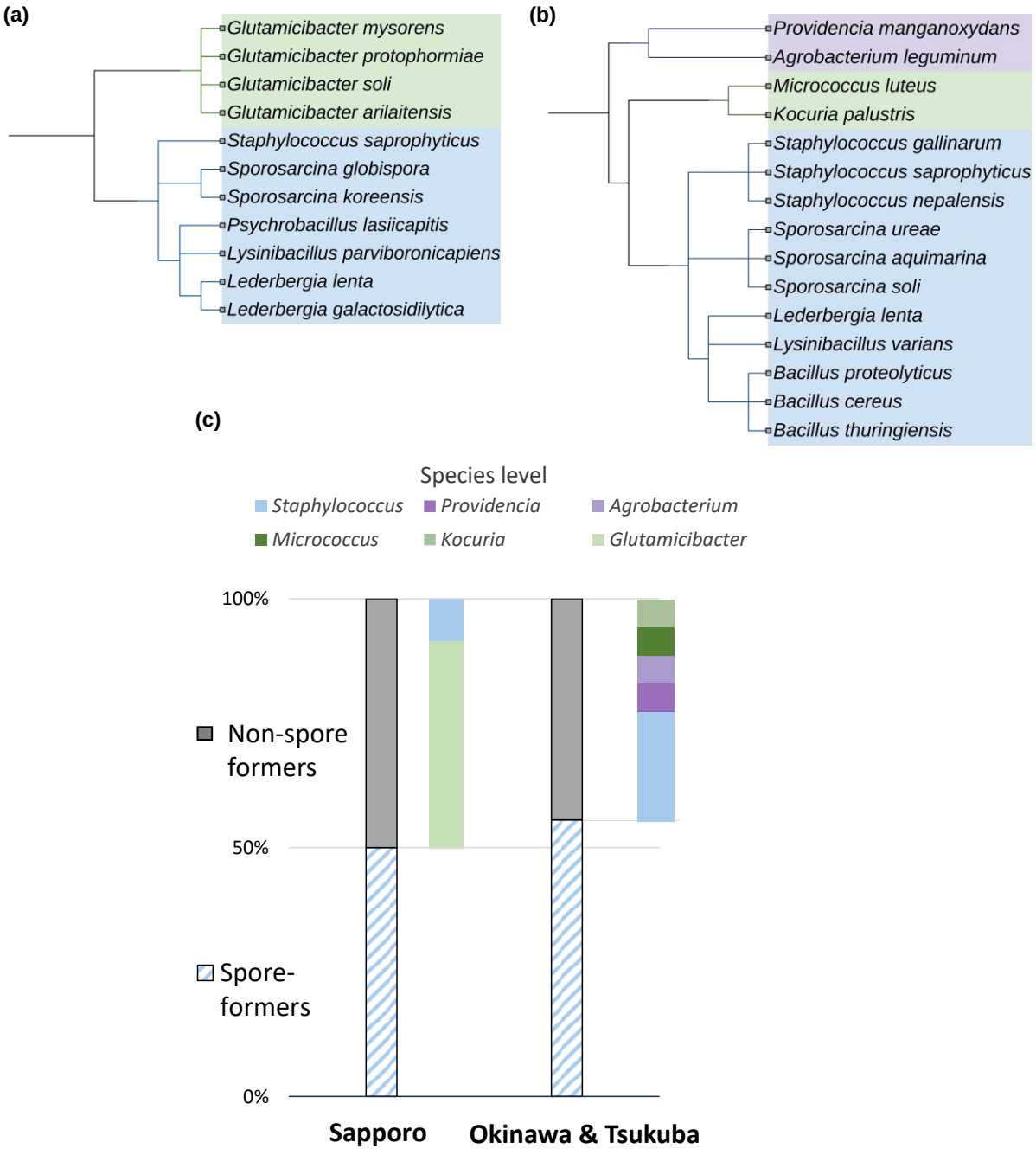

Figure 3. 16 Comparison of (a) Sapporo and (b) Okinawa & Tsukuba bacterial collection in terms of taxonomy; (c) composition of spore-formers in each collection.

Table 3. 9 Summary of the taxonomy of twelve isolates from Sapporo.

Isolate ID	Isolation source	Closest taxonomy				Global distribution (MicrobeAtlas) Samples from different sources			
		Genus_Species	Family	Identity	Accession	Aquatic	Soil	Animal	Plant
RII-2	Rice paddy (HU)	*Lederbergia lenta*	*Bacillaceae*	98.5%	AB021189	15	300	167	12
FU3	Forest (HU)	*Lederbergia galactosidilytica*	*Bacillaceae*	96.6%	AJ535638	93	412	390	82
FU17		*Glutamicibacter soli*	*Micrococcaceae*	99.4%	EF660748	82	74	419	45
FU4*		*Staphylococcus saprophyticus*	*Staphylococcaceae*	99.9%	AP008934	1554	930	23674	763
CH3	Cow house (HU)	*Glutamicibacter protophormiae*	*Micrococcaceae*	99.2%	X80745	1	33	39	4
MY5-21	Summit-220m	*Glutamicibacter mysorens*	*Micrococcaceae*	99.2%	AJ639831	29	26	63	9
MY3-21	Low brush -150 m	*Glutamicibacter arilaitensis*	*Micrococcaceae*	99.1%	AJ609628	149	92	507	69
MY3-15		*Lysinibacillus parviboronicapiens*	*Bacillaceae*	99.1%	AB300598	8	122	9	3
MY2-9	Low brush- 110 m	*Sporosarcina globispora*	*Planococcaceae*	98.6%	X68415	2194	10719	3706	1805
MY1-15	Low brush -70 m	*Glutamicibacter soli*	*Micrococcaceae*	99.4%	EF660748	82	74	419	45
MD4-29	Maple forest	*Sporosarcina koreensis*	*Planococcaceae*	99.6%	DQ073393	-	-	-	-
MD4-5		*Psychrobacillus lasiicapitis*	*Bacillaceae*	99.3%	KP219721	3	15	3	1

Table 3. 10 Summary of the taxonomy of eighteen isolates from Okinawa and Tsukuba.

Isolate ID	Closest taxonomy				Global distribution (MicrobeAtlas) Samples from different sources			
	Genus_Species	Family	Identity	BSL	Aquatic	Soil	Animal	Plant
KK7	*Providencia manganoxydans*	*Morganellaceae*	99.7%	1				
KK11	*Staphylococcus gallinarum*	*Staphylococcaceae*	99.49%	1	1554	930	23674	763
KK18	*Lysinibacillus varians*	*Bacillaceae*	99.6%	1	49	72	210	6
2C10	*Staphylococcus nepalensis*	*Staphylococcaceae*	99.67%	2	20	26	679	34
OS4-1	*Staphylococcus saprophyticus*	*Staphylococcaceae*	98.85%	2	1554	930	23674	763
OS5-5	*Agrobacterium leguminum*	*Rhizobiaceae*	99.28%	1	822	595	3746	731
TF2-2	*Bacillus proteolyticus*	*Bacillaceae*	97.14%	1	446	1920	1524	1079
TF3-6	*Bacillus thuringiensis*	*Bacillaceae*	97.55%	1	446	1920	1524	1079
TF3-9	*Bacillus cereus*	*Bacillaceae*	96.47%	2	446	1920	1524	1079
TF7-5	*Sporosarcina sp.*	*Planococcaceae*	98.28%	1				
TF7-7	*Sporosarcina soli.*	*Planococcaceae*	98.34%	1				
TF7-14	*Sporosarcina sp.*	*Planococcaceae*	98.57%	1				
TF7-15	*Lederbergia lenta*	*Bacillaceae*	96.4%	1	15	300	167	12
TF7-19	*Sporosarcina aquimarina*	*Planococcaceae*	97.24%	1	113	383	478	53
TF7-22	*Staphylococcus saprophyticus*	*Staphylococcaceae*	98.66%	2	1554	930	23674	763
TF7-26	*Sporosarcina ureae*	*Planococcaceae*	99.93%	1	414	999	2247	172
TS2-7	*Kocuria palustris*	*Micrococcaceae*	97.94%	1	8089	5873	47774	2290
TS3-7	*Micrococcus luteus*	*Micrococcaceae*	100%	1	9695	6060	37863	3126

3.4 Conclusions

In this chapter, we collected airborne bacteria from different climate zones in Japan, aiming to obtain resilient candidates for MICP application. Sampling spots in three sites (Sapporo, Tsukuba, Okinawa) include parks, campuses, farms, coastal areas, etc. The percentage of urease-positive isolates from different samples were compared to understanding how climatic characteristics influence the occurrence of ureolytic airborne bacteria. Identified ureolytic bacteria were then examined in the carbonate precipitation tests to confirm their ability to induce precipitation. Selected isolates were then identified in gene analysis. Further investigation is needed to gain a more comprehensive understanding of their survivability and adaptability under changing conditions. Based on the findings from these tests, the following conclusions can be drawn.

I. Terrestrial samples showed about 10-20% population of urease-positive airborne bacteria. General media screened out more ureolytic bacteria than selective media did, while the latter found more bacteria with high urease activity.

II. The first round of screening evaluated approximately 1500 colonies, which found 204 ureolytic isolates. For the second round, thirty of them were selected to proceed to gene analysis based on their ability to produce precipitation.

III. Ureolytic bacteria from different sampling sites showed great similarities in taxonomy. Among selected isolates, half of them were found to be *Bacillus* related spore-forming species, and non-spore-formers are from *Micrococcaceae* family and *Staphylococcus* genus.

Chapter 4 Characterization and applicability evaluation of selected airborne bacteria strains

4.1 Background

As mentioned earlier, it is widely acknowledged that most airborne bacteria belong to generalist groups which make use of various nutrient sources and survive distinct environments. They are usually from *Firmicutes*, *Pseudomonadota* and *Actinobacteria* phyla, which contain the most culturable bacteria. For spore formers, it is difficult to identify if they were caught in spore form or living cell form. For example, well known ureolytic bacteria like *Sporosarcina* species and *Bacillus* species (commonly researched ureolytic bacteria) from *Firmicutes* phylum are probably staying in the air as spore forms. For non-spore forming strains, either they are ubiquitous with a large population that are ready to be aerosolized, or they have other survival strategies to stay viable in the air. Normally, these bacteria were studied less in the field of biocementation because spore-formers are believed to have absolute advantages in applications. However, the well-known ureolytic bacteria like *Sporosarcina* genus have certain limitations in real field application when it encounters untypical problematic soils. For instance, they typically have a strong preference for alkaline conditions and weak tolerance to acidic conditions. Therefore, for non-spore forming strains, there are potentials for biocementation applications waiting to be explored.

Based on the bacterial capabilities of precipitating calcium carbonate, thirty isolates were selected and proceeded to gene analysis in the previous chapter. Among the identified isolates, strains with unique features were then characterized in a series of tests, including urease activity test, temperature dependency test, pH dependence test, survivability test. Isolates standing out from several rounds of screening were then evaluated in the sand column test to confirm their applicability. The results showed that the excellent efficiency of precipitation induced by two candidates confirmed the promising applicability of airborne bacteria toward biocementation. With the development of biotechnology, more studies adopt metagenome analysis to investigate the airborne microorganism communities, which reveals the taxonomic features of both living and dead microorganisms. There is a research gap in associating the gene information obtained in these studies with the expression of these microbes in distinct environments. This study adopted a conventional method to evaluate the airborne bacteria which culture the bacteria in the laboratory conditions and examine bacteria. Although some of the results obtained in laboratories cannot reflect the behavior of airborne bacteria when they are floating in the air, the results provide us with insight into how these bacteria could survive in a harsh environment like air, what the mechanisms could be, and how they can be applied in microbial induced carbonate precipitation.

4.2 Materials and methodology

4.2.1 Selection of strains

As discussed in the previous chapter, isolates from three sampling sites were selected based on their capacity of precipitating calcium carbonate. Isolates from Sapporo sampling had shown a superior performance, therefore, strains from three species, *Lederbergia* sp. (RII-2, FU3), *Sporosarcina* sp. (MY2-9), *Glutamicibacter* sp. (MY3-21, FU17) were characterized by temperature and pH dependence tests. On the other hand, isolates from Okinawa and Tsukuba samplings exhibited a weak urease activity. Based on the calcium carbonate precipitation amount, five strains (KK7, KK18, TF7-15, TF7-26, TS3-7) were characterized. They are from five distinct genera, *Providencia*, *Lysinibacillus*, *Lederbergia*, *Sporosarcina*, and *Micrococcus*.

4.2.2 Temperature dependence of bacterial growth and urease activity

Selected isolates which showed excellent performance at precipitation tests were cultured under varying temperatures, ranging from 15 °C to 35 °C. Bacteria were inoculated from an agar plate to a 5 mL-preculture test tube and cultured for 24 h at 30 °C. For main-culture, 1 mL of pre-culture was inoculated to 100 mL of culture media, then incubated in a shaker at 160 rpm, at varying temperature. Quantitative measurement of bacterial population was conducted using a spectrophotometer, which assesses the turbidity of a solution by measuring its optical density of a specific wavelength of light (600 nm).

A quick determination of urease activity is using a conductivity meter to monitor the conductivity changes with time (Whiffin, van Paassen, and Harkes 2007). This measurement uses a compact EC meter (LAQUAtwin EC-33, HORIBA, Ltd., Kyoto, Japan). With 3-point calibration, the accuracy is ±2% full scale. For testing, 0.5 mL of bacterial culture is added into a beaker with 50 mL of 0.1 mol/L urea solution placed in a stirrer in a water bath (Thermal Robo TR Series, AS ONE Corporation) at set temperature. One sampling of 0.12 mL is taken from the bacteria-urea solution and measured at 5-min intervals (0, 5, 10, 15-min). The calibration curve of this measurement is shown in Figure 4.1.

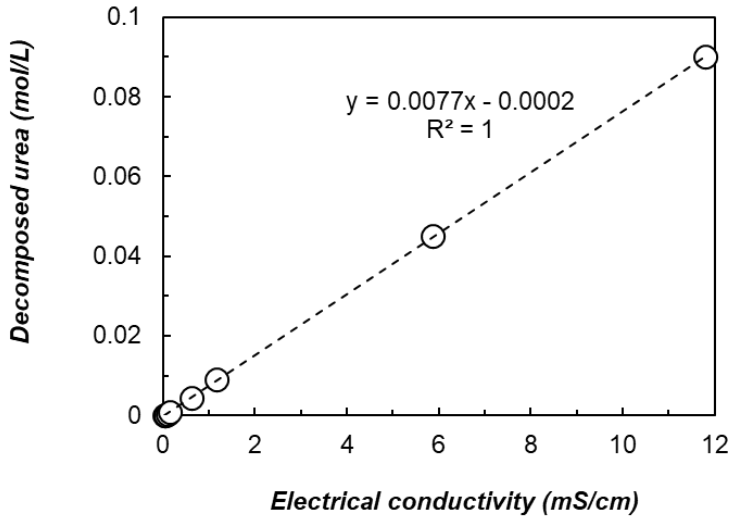

Figure 4. 1 Calibration curve of determination of urease activity by electrical conductivity

4.2.3 pH dependence of urease activity

Quantitative measurement of bacterial urease activity in the temperature dependency test did not buffer the test solution to maintain the pH level. For pH dependency test, the electrical conductivity measurement cannot be monitored and transferred as hydrolyzed urea. Therefore, the urease activity test in this experiment refers to the Berthelot test, which determines the ammonia as Indophenol (Bolleter, Bushman, and Tidwell 1961). Figure 4.2 presents the procedures and reaction mechanisms in the experiment. First, one sample is collected from the bacteria-urea solution every 5 minutes and treated with phenol-nitroprusside and sodium hypochlorite (see Figure 4.2 a). Due to the production of Indophenol, sample color darkens as concentration increases. Finally, the results are quantificationally transferred as the concentration of ammonium ions using the calibration curve (see Figure 4.2 b).

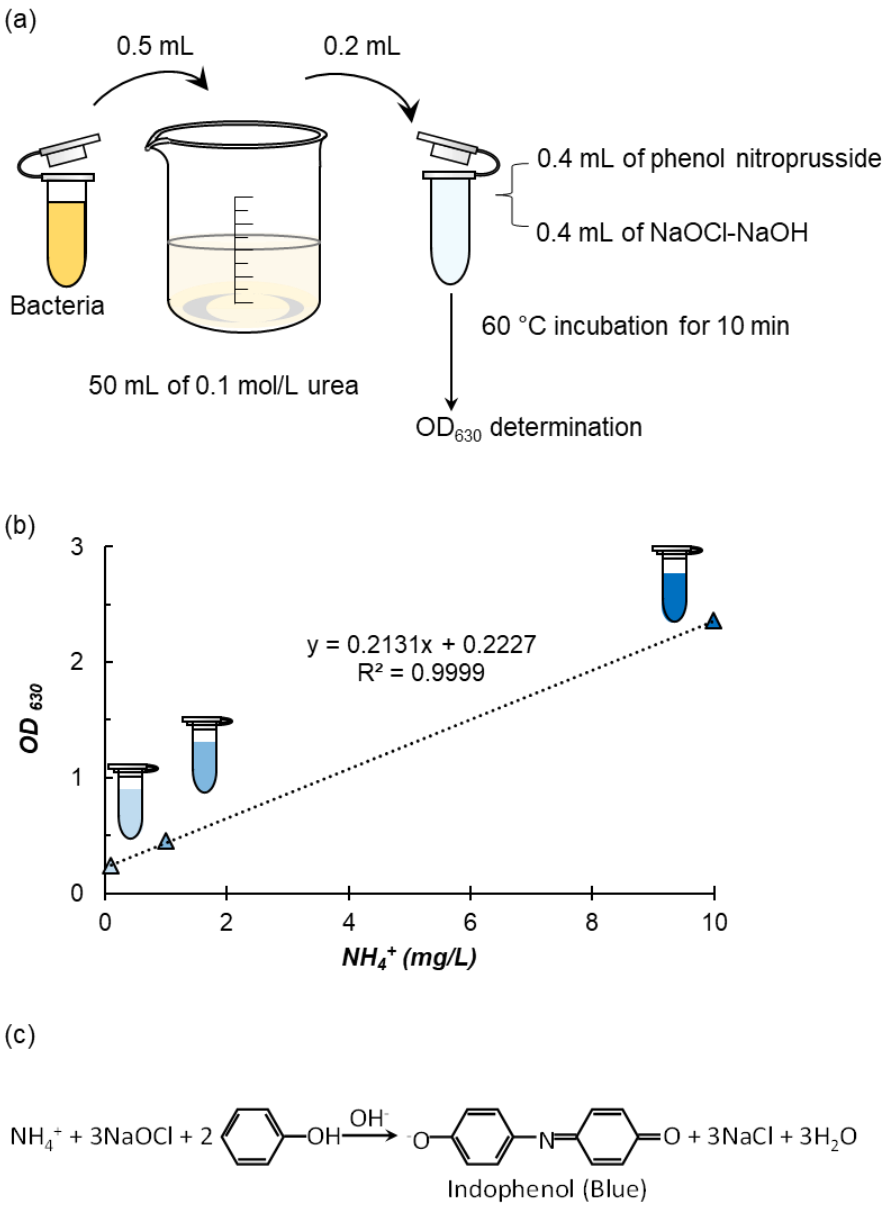

Figure 4. 2 Quantitative measurement of urease activity by Indophenol test (a) experimental flow; (b) calibration curve; (c) chemical reaction

4.2.4 Calcium carbonate precipitation in batch test

For each case, 10 mL of cementation solution (final concentration: 0.5 mol/L) and bacteria (2 mL of the main culture) are included in a 15-mL test tube. The concentrations of calcium ions were monitored during the test. The calcium meter used in this measurement is a compact Ca^{2+} meter B-751 (HORIBA, Ltd., Kyoto, Japan), with a measurement range from 4 to 9900 ppm. For each sampling, 100 μL was taken and diluted before measurement.

a) Precipitation test using various calcium sources

Considering the cost efficiency for field scale applications, it would be a great advantage if the selected bacteria can utilize various calcium sources to induce carbonate precipitation. Unlike chemical grade reagents, alternatives for calcium or urea from other sources might have some compositions that affect the cementation process, which should be considered in future investigations. According to the tolerance test in the last chapter, most tested strains tolerate a concentration of calcium chloride and urea up to 0.5 M. Therefore, the test solution was set as the same concentration range, from 0.25 M to 1.0 M, with three types of calcium salts: i) calcium chloride; ii) calcium nitrate; iii) calcium acetate. Experimental design is described in Table 4.1 and Table 4.2 presents the information of three calcium salts.

Table 4. 1 Precipitation tests with varying concentrations and sources of cementation media

Ca^{2+} source	Tested strains	Ca^{2+} & Urea (mol/L)	Cultivation temp. (°C)	Incubation temp. (°C)	Incubation time (h)	Evaluation
$CaCl_2$ $Ca(NO_3)_2$ $Ca(CH_3COO)_2$	RII-2 FU3 TF7-15 FU17 MY2-9 KK7	0.25 0.5 0.75 1.0	30	25	24 48 96 168	Conc. of Ca^{2+} (ppm) CFU/mL

Table 4. 2 Information on chemical reagents

Product	Maker	Description
Calcium chloride anhydrous	FUJIFILM Wako Chemicals	$CaCl_2$, chemical purity 95.0+%, pH of a 50 g/L solution at 25℃: 8.0-10.0
Calcium nitrate tetrahydrate	FUJIFILM Wako Chemicals	$Ca(NO_3)_2 \cdot 4H_2O$, chemical purity 98.5+%, pH of a 50 g/L solution at 25℃: 4.0-6.0
Calcium acetate monohydrate	NACALAI TESQUE, INC	$Ca(CH_3COO)_2 \cdot H_2O$, chemical purity, 99.0+%, pH of a 50 g/L solution at 25℃: 6.5-8.5

b) Precipitation test with humic acid

The humic acid (HA) used in this study is a commercial product purchased from FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan. Our previous studies found that bacteria response to humic acid differs across species. To make sure that the selected species are applicable to a wide range of soils, it is necessary to understand how the bacterial performance in biocementation is affected by humic acid. For this reason, a series of precipitation tests were conducted to determine the effect of humic acid on different tested strains. Table 4.3 presents experimental conditions and cases in this test.

Table 4. 3 Precipitation test with the presence of HA

HA (%)	Tested strains	Ca^{2+} & Urea (mol/L)	Cultivation temp. (°C)	Test temp. (°C)	Shaking incubation time (h)	Evaluation
0					24	Ca^{2+} (ppm)
0.5					48	Ca^{2+} (ppm)
	RII-2					
1	FU17	0.5	30	25		
2	KK7					
4	MY2-9				96	Ca^{2+} (ppm)
5					168	Ca^{2+} (ppm)

4.2.5 Sand column solidification test

a) Bacterial cultivation

Bacteria from 20 % glycerol stock store at -80 °C were inoculated onto an NH_4-YE agar plate using a cell spreader. After 48-72 hours incubation at 30℃, colonies on the plate were inoculated into 5 mL of culture media in a test tube. The pre-culture was ready after 24 hours cultivation. Then 1 mL of pre-culture was inoculated into 100 mL of culture media in a flask. The bacteria were cultivated in main culture for 48 hours to reach the stationary stage of growth with a stable production of urease.

b) Basic properties of tested sand

Figure 4.3(a) illustrates the grain size distribution of Mikawa sand. Composition analysis was conducted using a dispersive X-Ray Fluorescence (XRF) spectrometer (JSX-3100R II JOEL, Tokyo, Japan), revealing the main contents of 97.65% of SiO_2, 0.89% of Al_2O_3, 0.43% of Fe_2O_3, 0.18% of K_2O, 0.11% of MgO, and 0.11% of Na_2O.

c) Experimental setup

The barrel part of a 50 mL disposable syringe (30 mm in diameter) was used as the mold to shape and compact coarse silica sand into columns. For each specimen, 60 g of Mikawa sand was added. Mikawa sand, one of the Japan standard sands, is used in this study. To prevent the sand from being washed out during treatment, the barrel's bottom was lined with a filter paper piece before filling the mold with sand. Similarly, a filter was used to reduce the adverse effect of large organic particles in the nutrient broth on carbonate precipitation. The two-phase injection strategy adopted in this study is in accordance with previous research (Meiqi Chen, Gowthaman, Nakashima, Komatsu, et al. 2021). The injection of each treatment is presented in Table 4.4. Each step of the treatment is explained pictorially in Figure 4.3(b).

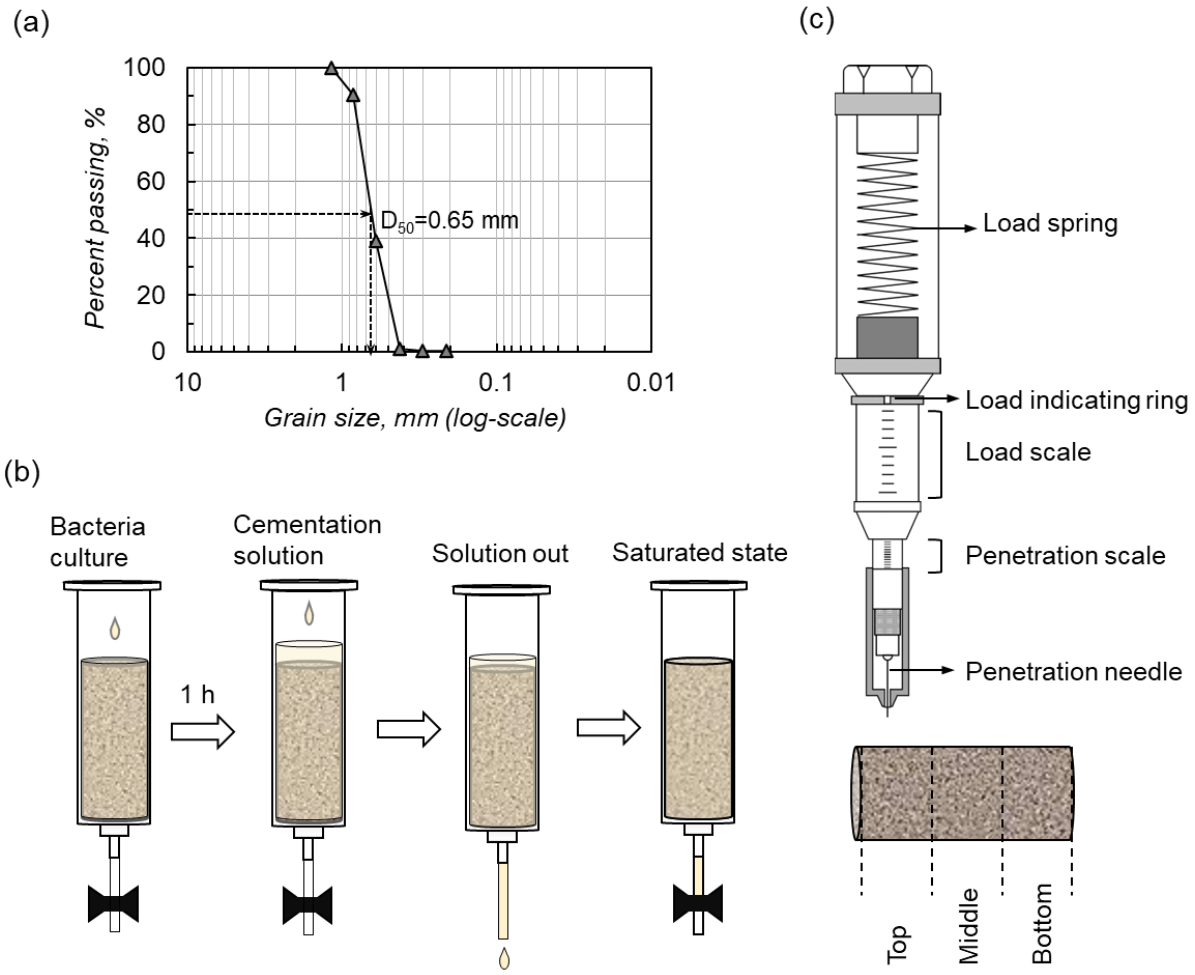

Figure 4. 3 (a) Grain size distribution of test sand; (b) procedures of treatment; (c) needle penetrator and three points measurement of penetration test

Table 4. 4 Injection of each treatment

Injection	Content
N=1,8	12 mL of bacterial culture, 12 mL of 0.5 M of $CaCl_2$, urea and 1.5 g/L nutrient broth.
N=2-7,9-14	12 mL of 0.5 M of $CaCl_2$, urea and 1.5 g/L nutrient broth.

4.2.6 Needle penetration test

Before measurement of unconfined compressive strength (UCS), MICP-treated samples were rinsed with distilled water to remove soluble salts and then oven-dried at 60°C for 48 h (Ahenkorah et al.

2021). The needle penetration test adopted to estimate the UCS is recommended by International Society for Rock Mechanics (ISRM) for testing soft rocks and cemented soil. There is a regression relationship (Eq. 4.1) between the penetration depth and the penetration resistance, which was developed by testing 50 cemented soil samples and 114 natural soft rock samples (Danjo and Kawasaki 2016). A schematic diagram of needle penetrometer is illustrated in Figure 4.3(c).

$$log\ (y) = 0.978\ log\ (x) + 2.621 \quad \text{(Eq.4.1)}$$

where y is the UCS; x is the penetration gradient (N/mm) determined by the penetration depth and penetration resistance.

4.2.7 Determination of calcium carbonate content

The carbonate content of treated samples was measured as the total gas volume generated after a complete reaction with hydrochloride in a closed chamber system. It is a simplified method developed by Fukue, Nakamura, and Kato (1999), and has been frequently used by researchers these days to estimate the carbonate content. First, 2 g of dry samples and 20 mL of HCl solution (2 mol/L) are placed into a chamber separately. Then, the chamber is sealed and equipped with a pressure gauge, which indicates the chamber's pressure change. By shaking the device, samples are allowed to mix and react with the acid solution. Then, the final stable pressure value is used as a parameter to estimate the samples' carbonate content. After transferring the gas volume to the mass of the carbonate through a developed calibration curve (see in Figure 4.4), the carbonate content is obtained using Equation 4.2.

$$Carbonate\ content\ (\%) = m_c / (m_s - m_c) \times 100\% \quad \text{(Eq.4.2)}$$

where m_c represents the weight of calcium carbonate in the tested sample, and m_s is the tested soil sample's total weight.

4.2.8 SEM and XRD analysis

The instrument used for microscale observation in this study is Miniscope TM 3000 (Hitachi, Tokyo, Japan). Samples were oven-dried at 60 ° before being observed. and the X-ray diffraction pattern was analyzed using MultiFlex (Rigaku Co., Ltd., Tokyo, Japan) to assess the polymorphs of calcium carbonate. The measurements were conducted using Cu-Kα radiation at 40 kV, 40 mA, 6.5°/min.

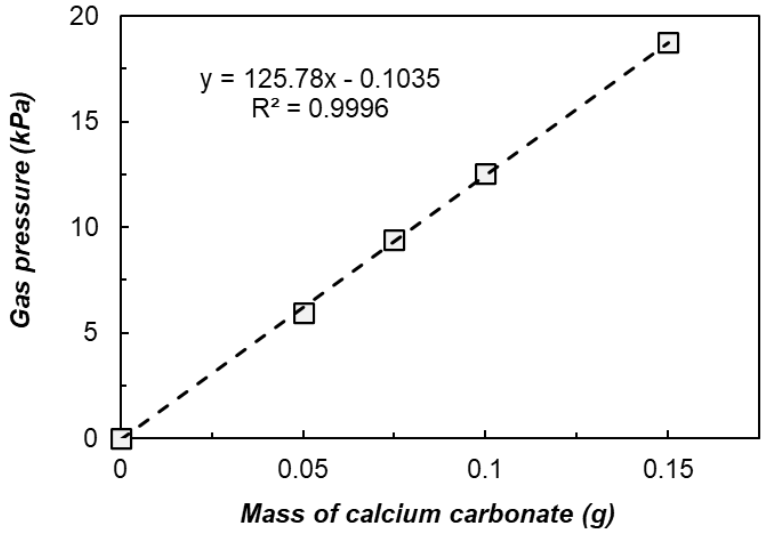

Figure 4. 4 Calibration curve of calcium carbonate content determination

4.3 Results and discussion

4.3.1 Temperature dependence of bacteria

a) Selected strains from Sapporo sampling

Four isolates that showed excellent performance at precipitation tests were characterized by urease activity tests under varying temperatures, ranging from 15°C to 35°C. At the same time, the growth curve of each strain was monitored every 24 h. As seen in Figure 4.5(a-b), there is a rapid increase of growth rate and urease activity with increasing temperature both in the case of RII-2 and FU3. And the activity of the former is likely to continue increasing, whereas that of the latter will decline when the temperature exceeds 35°C. The growth curve and urease activity of two strains under 20°C and 30°C, showed a peak activity after 24-h or 48-h cultivation and a steady decline after their stationary phase. In general, RII-2 and FU3 might be two potential candidates for MICP in a tropical temperate region. The temperature dependence of two strains from Maruyama Mountain is illustrated in Figure 4.5(c-d) in which MY3-21 and MY2-9 show notable differences compared to the previous two strains in several respects, such as the growth pattern and activity under varying temperatures. Although both tend to have a higher growth rate under low temperatures, the activities have shown optima at 25°C and 20°C. In reported studies on the closest strain of MY2-9 *Sporosarcina globispora*, it is often classified as a psychrophile (J. P. Wang et al. 2015). It is unique among twelve isolates that are mostly reported as common mesophiles. Ideally, this strain could be a good precipitator for cold regions. On the other hand,

what is unexpected in this figure is that MY3-21 showed limited activity although it was a good carbonate precipitator in previous tests. Similar behaviors were also shown in the cases of FU17 and CH3 (Figure 4.5e-f). Possible explanations are that the cell membrane composition of *Glutamicibacter* sp. (glutamic acid in the peptidoglycan interpeptide bridge) might contain certain groups that facilitate the precipitation process and species of this genus might have a good tolerance toward high concentrations of calcium source and urea. It has been reported that some amino acid groups influence precipitation (Polat, Özalp, and Sayan 2021; Nawarathna et al. 2021) . In this case, this strain could be a potential candidate for MICP application. In this baseline study, the feasibility of applying airborne bacteria was confirmed by small-scale solidification tests under room temperature using two strains with the highest activity among isolates, RII-2 and FU3.

b) Selected strains from Okinawa and Tsukuba sampling

Five isolates were characterized in this collection, including one from *Sporosarcina* sp. (TF7-26), one from *Lederbergia* sp. (TF7-15), one *Lysinibacillus* sp. (KK18), one *Micrococcus* sp. (TS3-7) and the Gram-negative strain, *Providencia* sp. (KK7). As can be seen in Figure 4.6, all of them have a typical growth pattern under varying culture temperatures. These isolates were cultured at temperatures that range from 15 to 35 °C. The results show that two isolates from Tsukuba city, TF7-15 and TF7-26, could grow at a wide range of temperatures, and the former showed significant activity in a moderate temperature range. However, the other isolate, TS3-7, only showed good growth at temperatures higher than 25 °C, and its urease activity is lower than the detectable value. All of them have a common pattern in terms of their urease activity, showing a better performance with the increasing temperature. In the case of two isolates from Okinawa, the growth curve of KK18 showed significant growth at temperatures ranging from 25-35 °C but no growth occurred at 15 and 20 °C. KK7 has a rapid growth from 20-35 °C, and a good urease activity lasting for one week.

The findings in this experiment showed that some of the isolates could precipitate a significant amount of carbonate in carbonate precipitation tests but show negligible activity in the urease test. This phenomenon was also found in other strains of *Glutamicibacter* sp., same from *Micrococcaceae* family. Based on the previous results on the survivability of bacteria, these bacteria tend to survive for a longer time than other ureolytic bacteria from *Sporosarcina* and *Bacillus* genus. Based on the capability of precipitating calcium carbonate, four candidates (TF7-15, TF7-26, KK7, KK18) were selected and proceeded to further characterization tests.

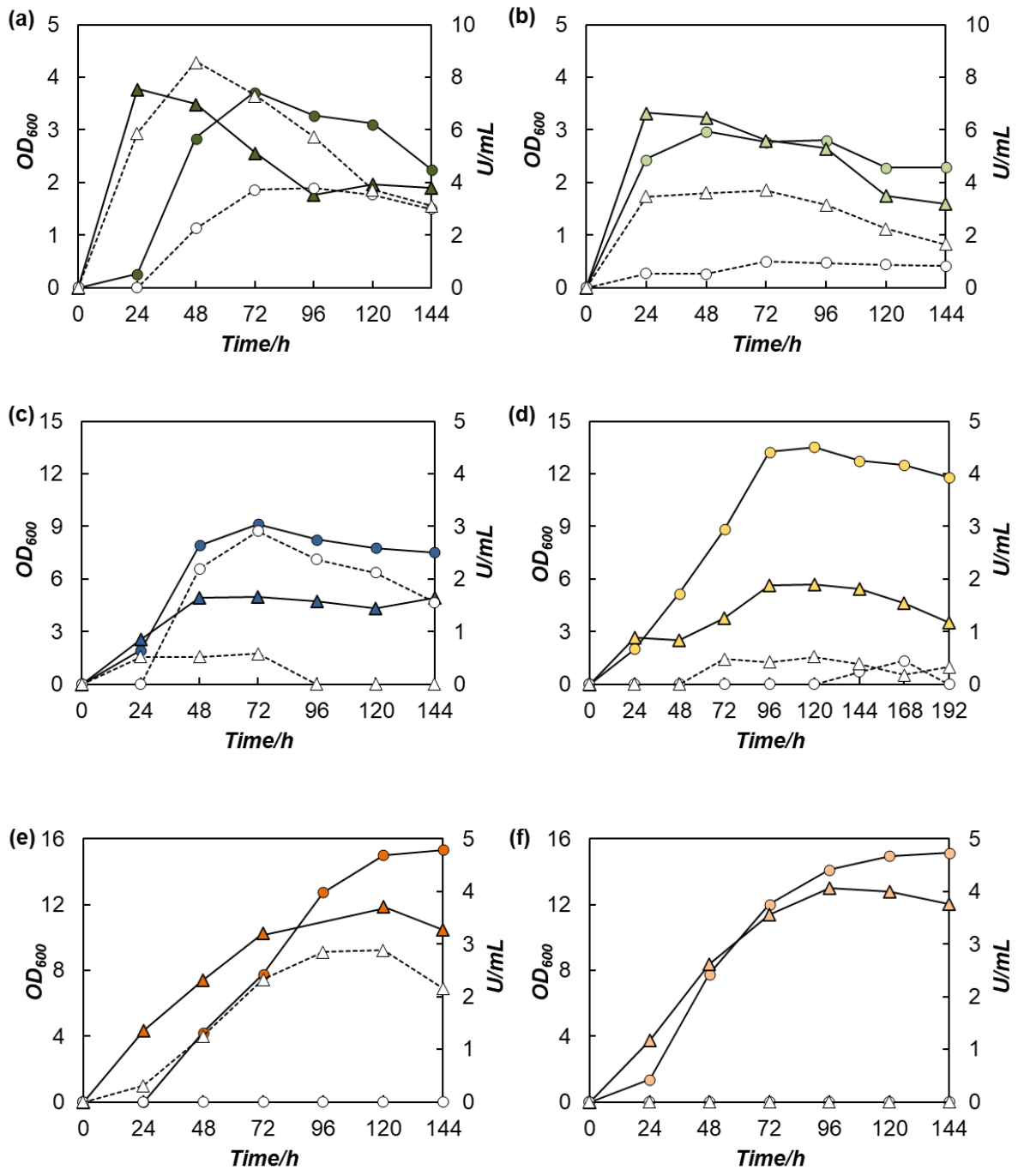

Figure 4. 5 Temperature dependence of four selected isolates from Sapporo samplings. Growth and activity changes with time under 20 ℃ (OD600-●; U/mL-○) and 30 ℃ (OD600-▲; U/mL-Δ) of (a) RII-2; (b) FU3; (c) MY2-9; (d) MY3-21; (e) FU17; (f) CH3

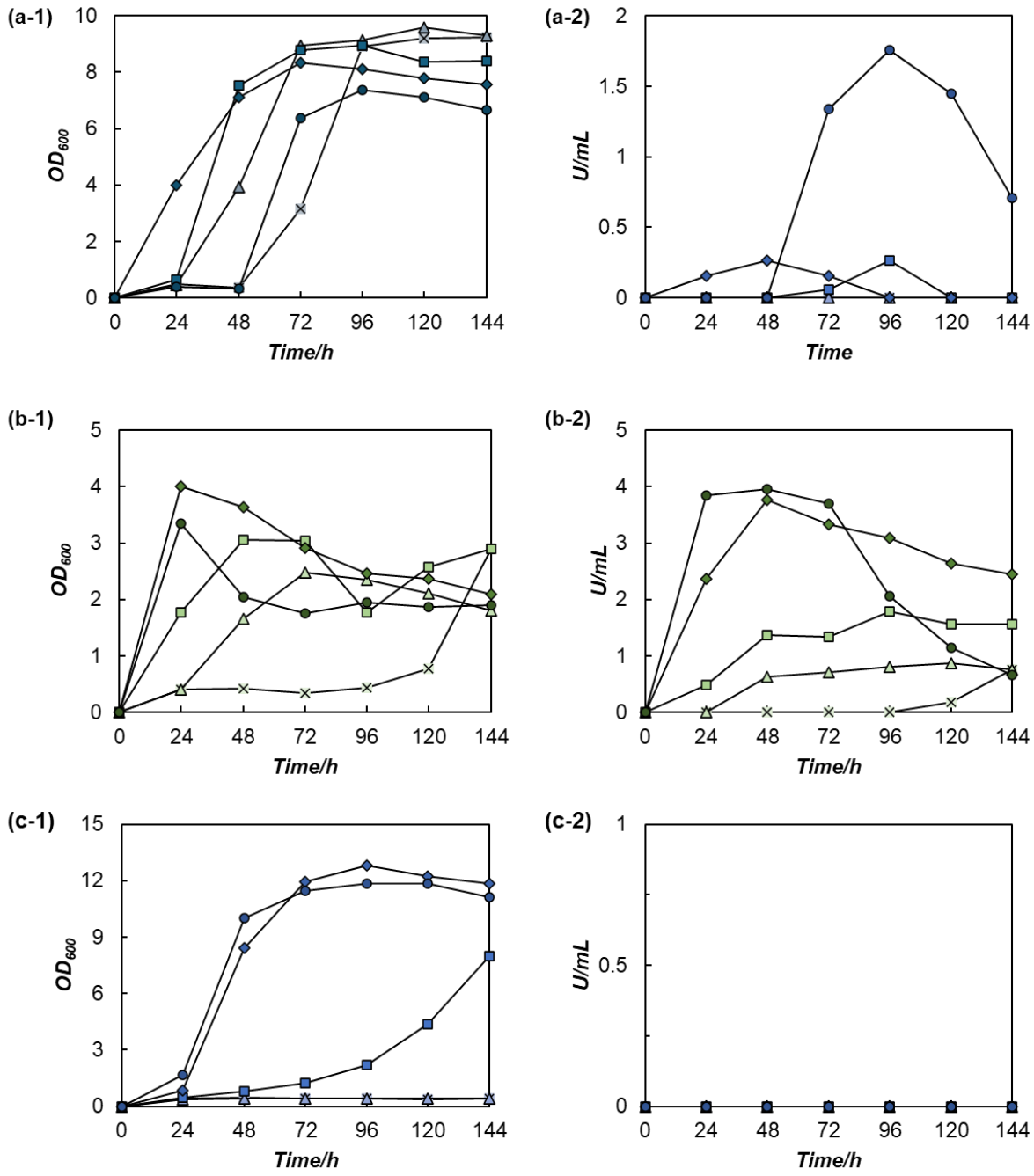

Figure 4. 6 Temperature dependence of five isolates from Okinawa and Tsukuba samplings. Growth and activity changes with time under ×-15 °C , ▲- 20 °C , ■- 25 °C , ♦-30 °C, ●-35 °C of (a) TF7-26; (b) TF7-15; (c) TS3-7; (d) KK7; (e) KK18

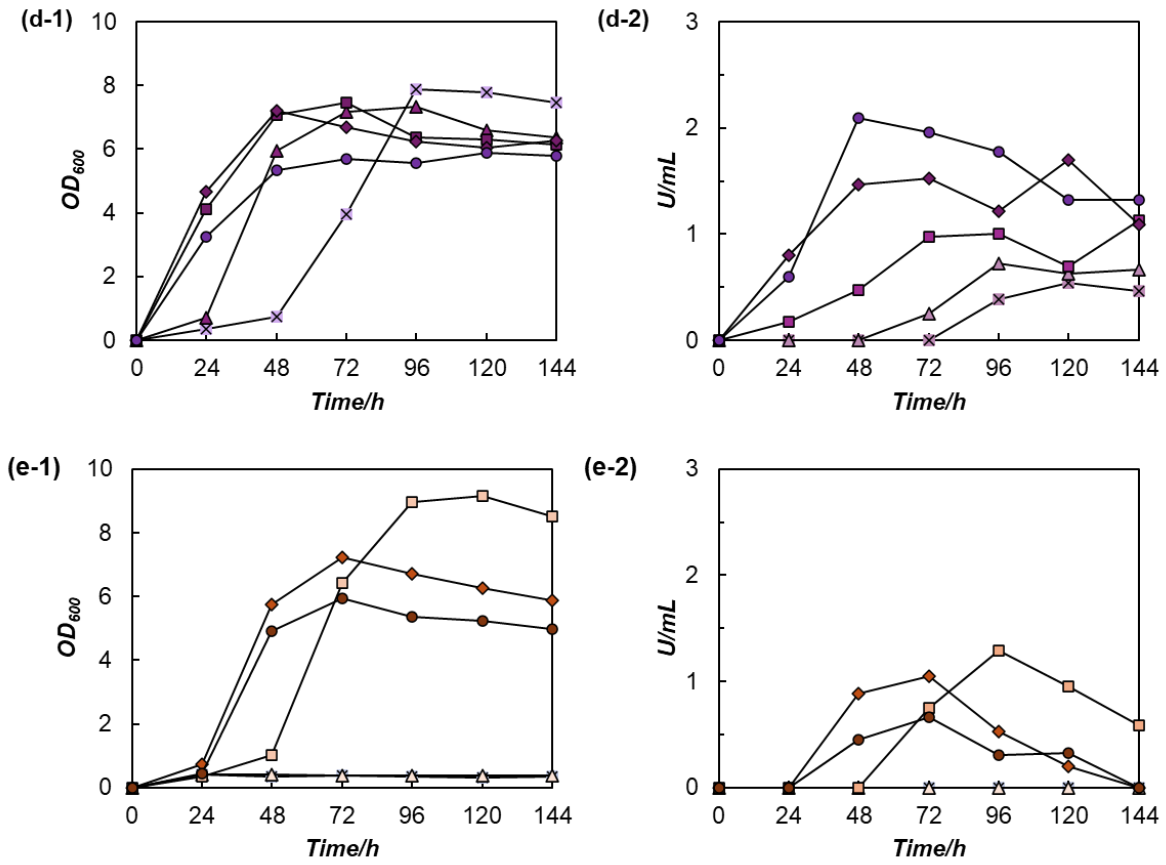

Figure 4. 6 (continued) Temperature dependence of five isolates from Okinawa and Tsukuba samplings. Growth and activity changes with time under ×-15 °C , ▲- 20 °C , ■- 25 °C , ♦-30 °C, ●-35 °C of (a) TF7-26; (b) TF7-15; (c) TS3-7; (d) KK7; (e) KK18

c) Discussion

A comparison between Sapporo (Green) isolates and Okinawa (Orange) & Tsukuba (Blue) isolates were shown in Figure 4.7. As expected, some isolates were featured by the climate zone where they inhabit. For instance, one isolate (MY2-9) from Sapporo was found to have cold tolerance which enables it to grow in a 4 °C refrigerator, and one isolate (KK18) from Okinawa only grows at temperatures higher than 20 °C. However, there are two exceptions, two *Lederbergia* species, showing mesophilic growth patterns but not growing at 15 °C.

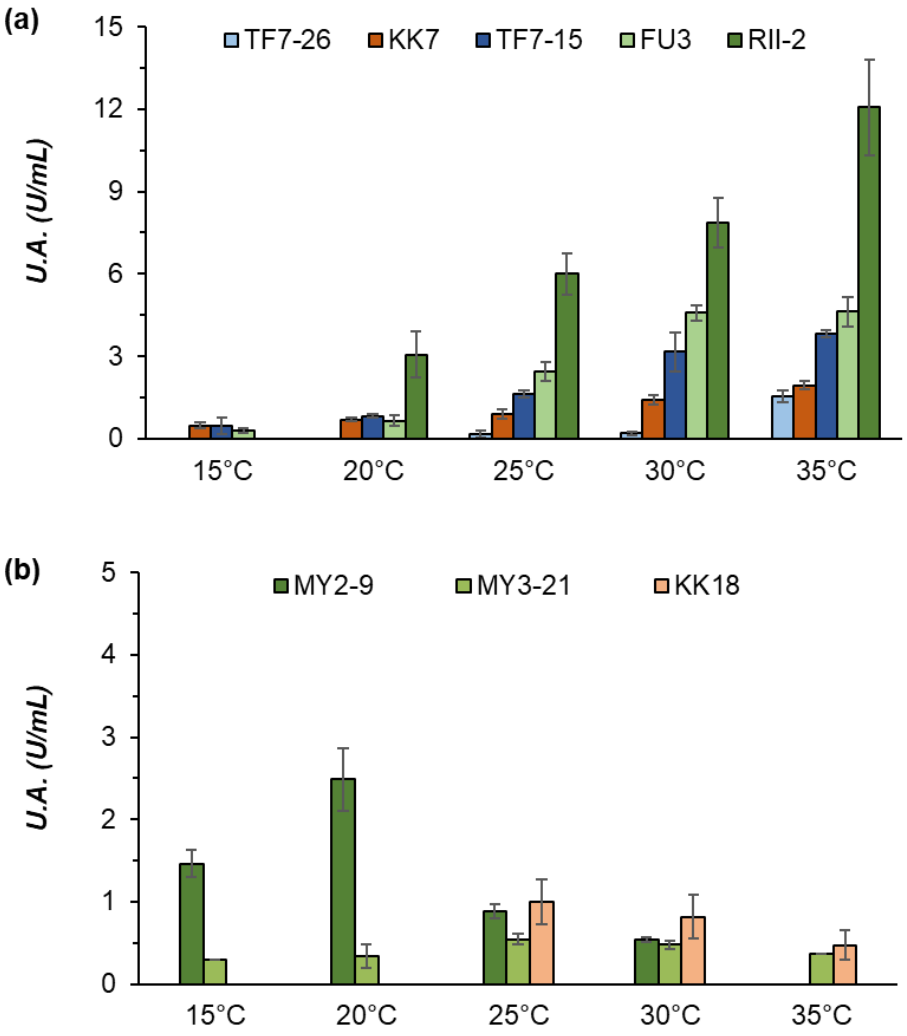

Figure 4. 7 Peak urease activity under 15-35 °C of eight selected isolates from three sampling sites. (a) isolates show preference to elevated temperature; (b) isolates show preference to relatively low temperature

4.3.2 pH dependence of bacteria

The pH dependence of selected isolates from temperature dependence tests were characterized at room temperature. Five strains from different genera (*Providencia*, *Sporosarcina*, *Lederbergia* and *Glutamicibacter*) were examined. Since the urea solution is buffered by EDTA phosphate buffer, the results showed a lower urease activity than the electronic conductivity showed. As can be seen in Figure 4.8, most strains exhibited higher urease activity at alkaline conditions, showing no activity at pH lower than six. Two exceptions are KK7 and RII2. The former prefers acidic conditions in terms of urease production. A possible explanation is that this strain has a urease regulation which expresses the related genes to hydrolyze urea in order to increase the pH level of the environment to survive. A typical species

is the human pathogen, Helicopter pylori, which survives human stomach by urease activity. The latter one might have some resilient characteristics to overcome the acidic condition. As a result, the bacteria can produce urease in a constrained amount. Both have an enormous potential in the biocementation application towards some acidic problematic soils or the treatment of polluted acidic materials. After obtaining a better understanding of the selected MICP candidates, the next experimental stage is to develop a mixed culture of high MICP efficiency. The target is to enhance tolerance under harsh environments and keep a high performance of applied bacteria during the MICP process.

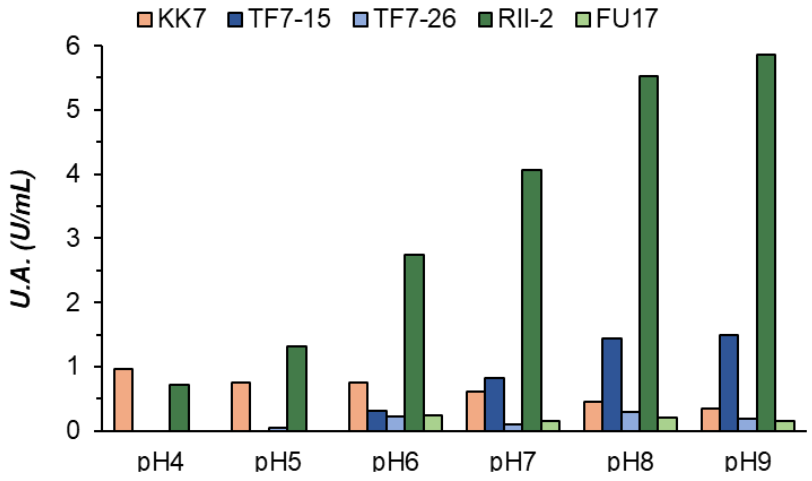

Figure 4. 8 Urease activity under pH 4-9 of 5 selected isolates from three sampling sites

4.3.3 Evaluation of tolerance in precipitation tests

To understand how the selected strains tolerate the high concentration of cementation solution, a series of precipitation tests were performed. Bacteria cultivated in main culture for 72 hours were applied to precipitation tests with varying concentrations of cementation media (0.25 M, 0.5 M, 0.75 M, 1 M) using three types of calcium sources (calcium chloride, calcium nitrate, and calcium acetate). At the same time, the survivability after one-week incubation was examined by plate counting method. After mixing, the test solution was diluted and inoculated on agar plates. Colonies in a countable number on plates were counted after 48 hours of incubation.

a) Effect of concentration of cementation media and types of calcium sources

As can be seen in Figure 4.9, three *Lederbergia* strains (RII-2, FU3, TF7-15) and stood out from six tested strains in terms of tolerance to various concentrations and types of calcium sources in this test.

The results demonstrated that TF7-15, the *Lederbergia* species, had a similar performance with FU3, since they are close in terms of taxonomy. The *Sporosarcina* strain, MY2-9, also exhibited a good performance in cases of calcium chloride and calcium nitrate as calcium sources, while a relatively weak performance was observed in cases of calcium acetate. Another *Sporosarcina* strain, TF7-26, was also evaluated in the precipitation test with changing concentrations of cementation media, while the *Sporosarcina ureae* strain seems to be less tolerant to concentrated solution (see Appendix A). As for the other two strains, KK7 and FU17, both were able to induce precipitation steadily when the concentration was lower than 0.5 M. As the concentration increases up to 1.0 M, the precipitation was mostly inhibited. Regarding the influence of concentrations of cementation media, it is generally acknowledged that concentrated the calcium salts can greatly inhibited the urease activity (Whiffin 2004). Generally speaking, tested bacteria have relatively higher tolerance to chloride and acetate, especially acetate. Calcium acetate can serve as an energy and carbon substrate for diverse microorganism (Zhuang et al. 2019). Therefore, high concentration of calcium acetate might be less unfavorable to these bacteria compared to the other two calcium salts.

Many studies have investigated the influence of various calcium sources on MICP. Achal and Pan (2014) first tried to figure out the tremendous role of calcium sources on MICP using a *Bacillus* strain. The authors examined the effect of four calcium sources as an amendment with a low concentration (25-50 mM) into culture media, revealing that calcium chloride is the preferred media followed by calcium nitrate. Testing by a similar method, Otlewska and Gutarowska (2016) studied the abiotic factors using three strains (two *Bacillus* sp., one *Arthrobacter* sp.) and three types of calcium sources, while the results found calcium acetate is the most preferred calcium source. The calcium carbonate morphology varies from species to species. The preference of calcium sources in precipitation test might also be strain-specific. A recent EICP study evaluated the cementation effectiveness using three calcium sources of 1.0 M concentration, which revealed that calcium chloride is the best calcium source, followed by calcium acetate, calcium nitrate (Weng et al. 2024). The results revealed a complete inhibition of urease activity by 1.0 M of calcium nitrate, which can also partially explain the findings in this study.

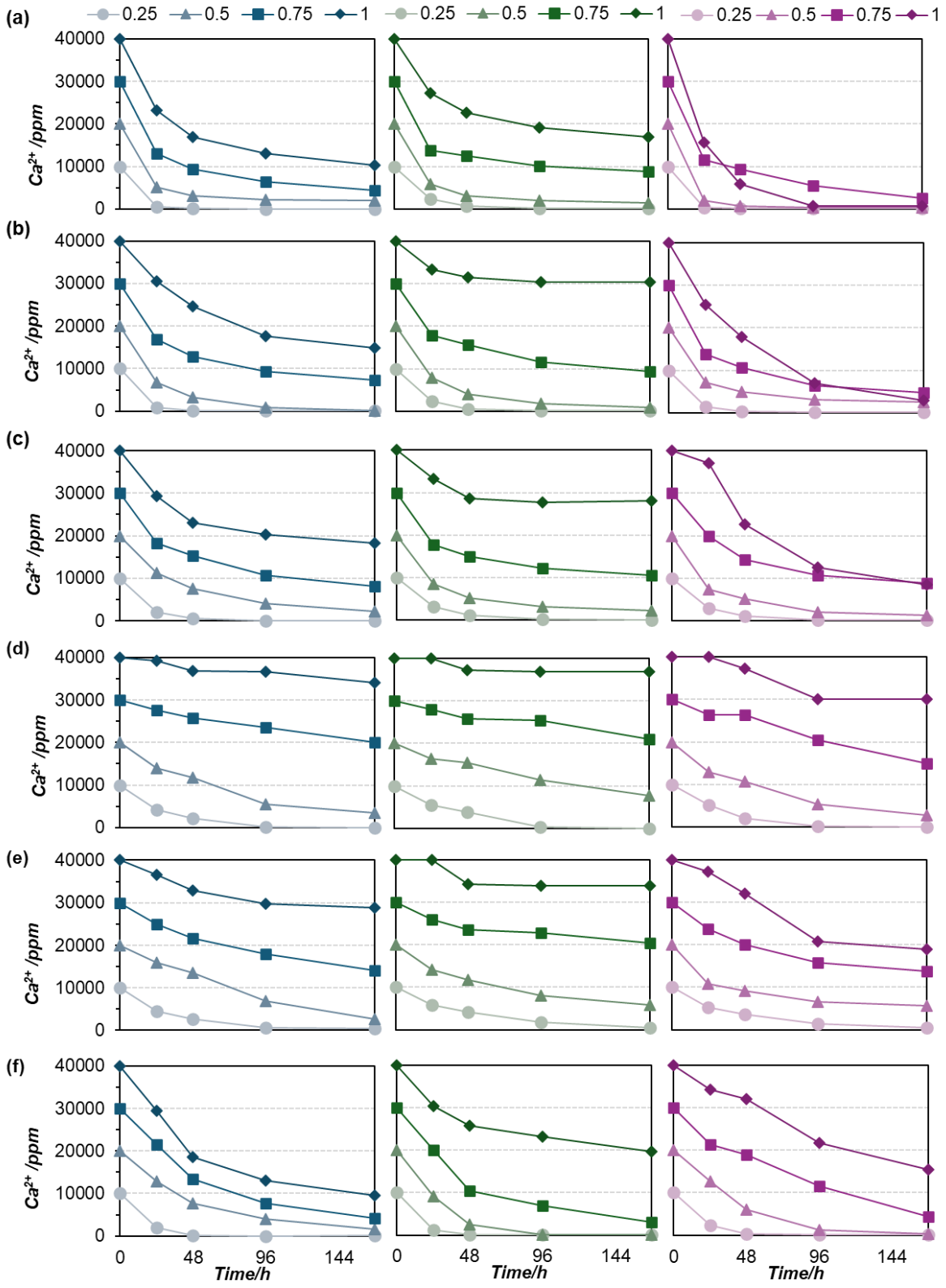

Figure 4. 9 Monitoring of calcium ion concentration changes in precipitation batch test with varying concentrations and sources of cementation media (calcium chloride in blue, calcium nitrate in green, and calcium acetate in purple). (a) RII-2; (b) FU3; (c) TF7-15; (d) KK7; (e) FU17; (f) MY2-9

b) Survival of bacteria in various cementation media

As seen in Figure 4.10, the bacterial survival in cementation media of different calcium sources and concentrations differs. Briefly, tested strains can be divided into two groups based on their survival condition. The first group (RII-2, FU3, TF7-15, MY2-9) has weak survivability when the concentration is more than 0.5 M, at the same time, showing high selectivity to cementation media. Strains in this group showed a relatively high adaptability to calcium chloride, as they survive up to 0.5 M of calcium chloride. The second group (KK7, FU17) has relatively high survivability in all tested conditions. In particular, FU17 has considerable viable cells in all calcium salts with concentrations up to 1.0 M.

From the perspective of their initial population, the second group has a larger population (CFU/mL: $10^{\wedge 11\text{-}13}$) than the first group (CFU/mL: $10^{\wedge 7\text{-}9}$) in the beginning of the test due to their high utilization efficiency of energy. And changes in the initial population can result in significant differences in the final survival rate. Based on the estimation, the first group has a survival rate in the range of 0.1-10%, the second group is up to 20% in the concentration of 0.5 M. Assuming that they have similar survival rate, the latter have certain advantages in a long-term performance. Taken together, the former group exhibited a better performance in precipitation than the latter did, while the latter exhibited a higher survival rate in test solutions. Considering long-term effectiveness of biocementation, the latter could also be a suitable candidate for stable cementation in a long period of time.

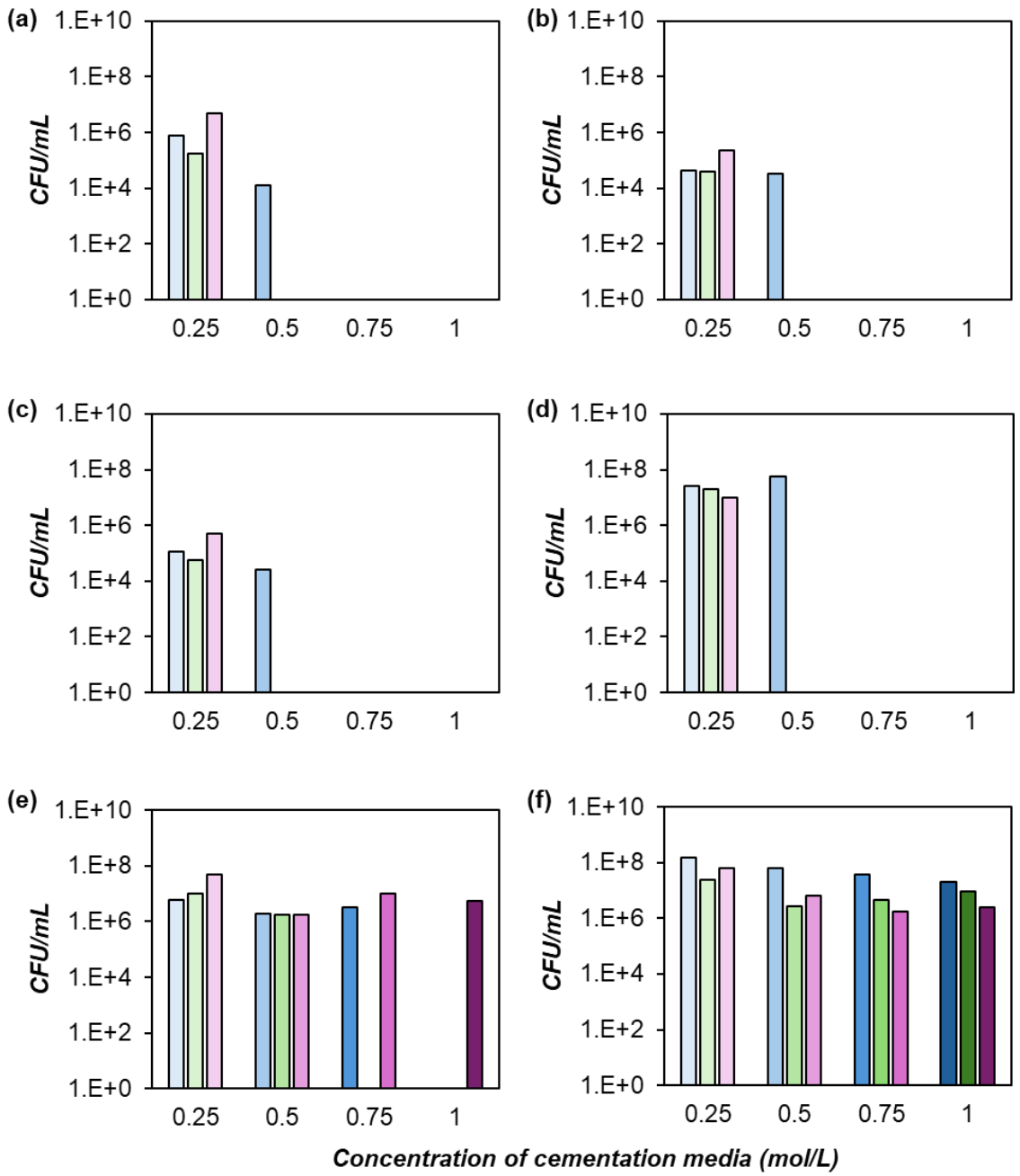

Figure 4. 10 Survival of bacteria in three types of cementation media (calcium chloride in blue, calcium nitrate in green, and calcium acetate in purple) after 168 h. (a) RII-2; (b) FU3; (c) TF7-15; (d) MY2-9; (e) KK7; (f) FU17

c) Precipitation with the presence of HA

As seen in Figure 4.11, with a small addition of HA in the cementation media, the precipitation rate of KK7 and FU17 was significantly enhanced after 168 hours incubation. For the other two strains, the

effect of HA seems to be negligible. Remarkably, the precipitation of KK7 was doubled with the HA addition, and the effect increases with the addition amount increases. In the cases of FU17, the precipitation was retarded at the beginning stage, which was almost completely inhibited at the first day incubation. But the effect gradually turned to be positive by the end of the test. For the other two strains, the effects are much smaller. In particular, RII-2 was slightly affected by the presence of HA. MY2-9 has a similar performance to FU17, but the fluctuation is much smaller than the latter. General mature soil profile usually has organic matter content from 1 to 5 % of the dry weight in the surface soil and decreases with depth. These findings confirmed that they are able to precipitate carbonate with the presence of small amount of HA. For soils that are slightly organic, four strains can be potential candidates for MICP applications.

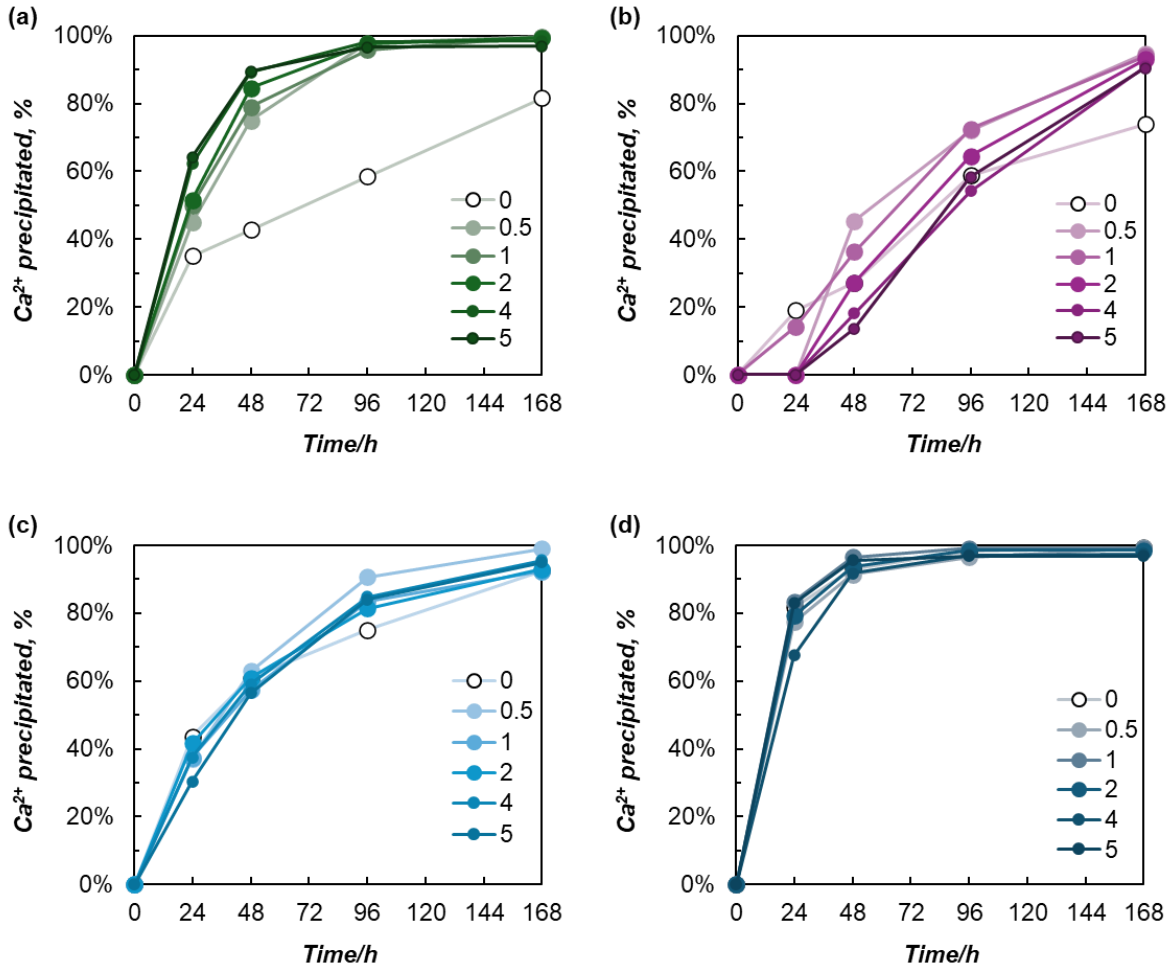

Figure 4. 11 Monitoring of calcium ion concentration changes in precipitation batch tests with 0, 0.5, 1, 2, 4, 5 % of HA addition. (a) KK7; (b) FU17; (c) MY2-9; (d) RII-2

4.3.4 Calcium carbonate morphology induced in different conditions

a) Calcium carbonate induced by different bacteria

The morphology of calcium carbonate induced by bacteria is governed by many factors, some of them are closely related to bacterial metabolites and activity in the cementation media (Azulay et al. 2018; J. Zhang et al. 2021). In terms of urease activity, for example, when the bacteria have relatively low urease activity, the slow formation of calcium carbonate usually leads to a large size of precipitates. Contrarily, the high-activity bacteria induce precipitates of much smaller size than low-activity bacteria. On the other hand, the presence of extracellular polymeric substances produced by bacteria was found to influence the formation and morphology of calcium carbonate. Figure 4.12 presents various calcium carbonate morphology precipitated by different bacteria strains.

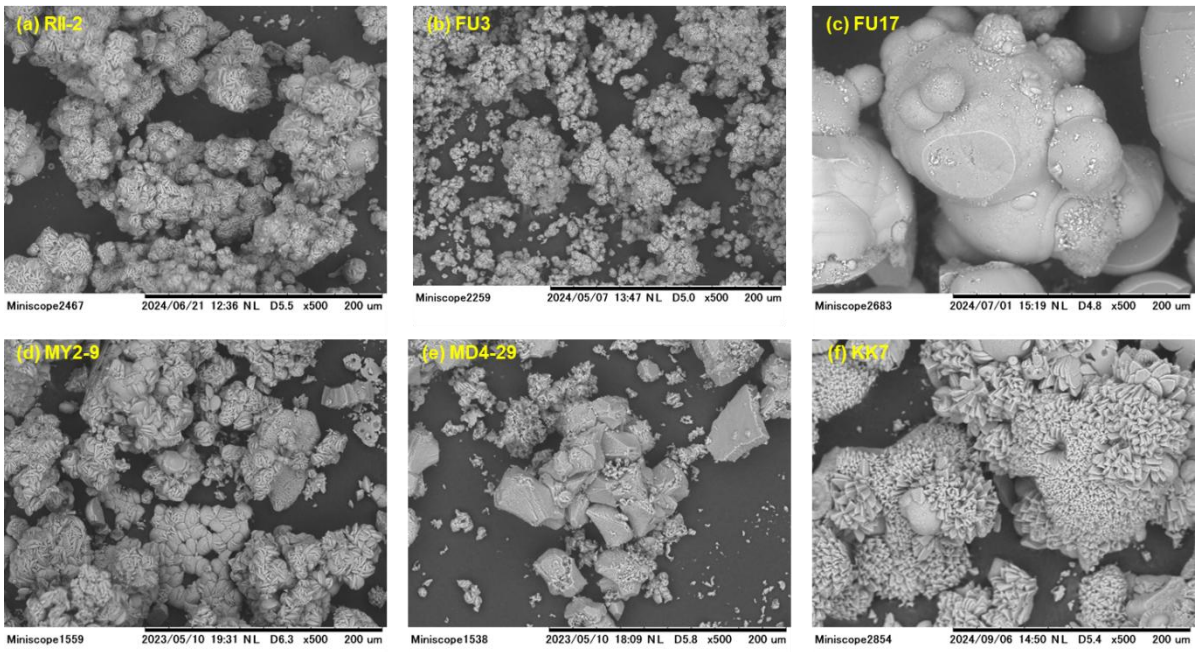

Figure 4. 12 Variety of calcium carbonate morphology induced by six representative strains (Magnification of images: × 500)

Among the precipitates, calcium carbonate produced by FU17, *Glutamicibacter* sp., showed an extra-large spherical crystal (~200 µm in diameter) that is identifiable by naked eyes, which is very likely to be vaterite formation. *Glutamicibacter* species have a typical feature with the presence of glutamic acid functional group in the peptidoglycan interpeptide bridge, which might lead to the precipitation of

calcium carbonate in a vaterite structure (Polat, Özalp, and Sayan 2021). The observation in this study is consistent with these findings. On the other hand, a previous study has revealed that extracellular polymeric substances play an essential role in the formation of agglomerated globules which are similar to the observation in this study. It was found that microbes produce nanobacteria-like structures as nucleation sites for biomineralization, protecting cells from entombment (Bontognali et al. 2008). Possibly, the FU17 has a similar self-preservation strategy, leading to an enhanced survival rate in the precipitation tests. The calcium carbonate formation in cases of KK7, the *Providencia* sp., consists of small crystals aggregating into a large "cauliflower". For other fast precipitators from *Lederbergia* genus or *Sporosarcina* genus, the crystal seems like a tetrahedral growth form of calcite. Other calcium carbonates formed by different strains can be found in Appendix B. Generally speaking, crystals formed by strains from the same genus share certain similarity in the calcium carbonate morphology.

b) Calcium carbonate formed in different cementation media

As mentioned earlier, high concentrations of cementation media inhibit the urease activity of bacteria, thus, leading to a reduction in precipitation rate in the long term. Theoretically, the formation of calcium carbonate is fast when the concentration of substrate is high. However, either the bacteria will lose viability, or the urease enzyme will be deactivated with the precipitation process going-on. Previous studies have shown that the size of calcium carbonate tend to decrease with the increasing concentration of cementation media. Dubey et al. (2022) compares the calcium carbonate formation induced by a bacterial consortium and different strains from *Sporosarcina* species. The size analysis of the study revealed that a significant reduction of the crystal size was observed with increasing concentration of cementation media (0.25- 2.0 M) in the cases using standard strain, *S. pasteurii* (ATCC 11859), while the size difference of calcium carbonate induced by other cases was much less. It means that bacteria from the same species can induce quite different carbonate formation under changing conditions, suggesting that the calcium carbonate morphology produced in varying concentrations of cementation media can be strain specific. Figure 4.13 shows the calcium carbonate produced by RII-2, the strain with best MICP performance among tested strains, under varying conditions. From the images, it can be seen that the changes in crystal size of various cases are not noticeable in most cases. One exception is the case of calcium acetate. The portion of vaterite and amorphous calcium carbonate increases with increasing concentration of cementation media. Using *S. pasteurii* (KCTC 3558), Gorospe et al. (2013) tested the effect of seven calcium salts on the calcium carbonate morphological features. The results revealed that organic salts lead to more formation of vaterite than calcite, which is consistent with the findings in this study. More calcium carbonate crystals formed by different strains can be seen in Appendix B.

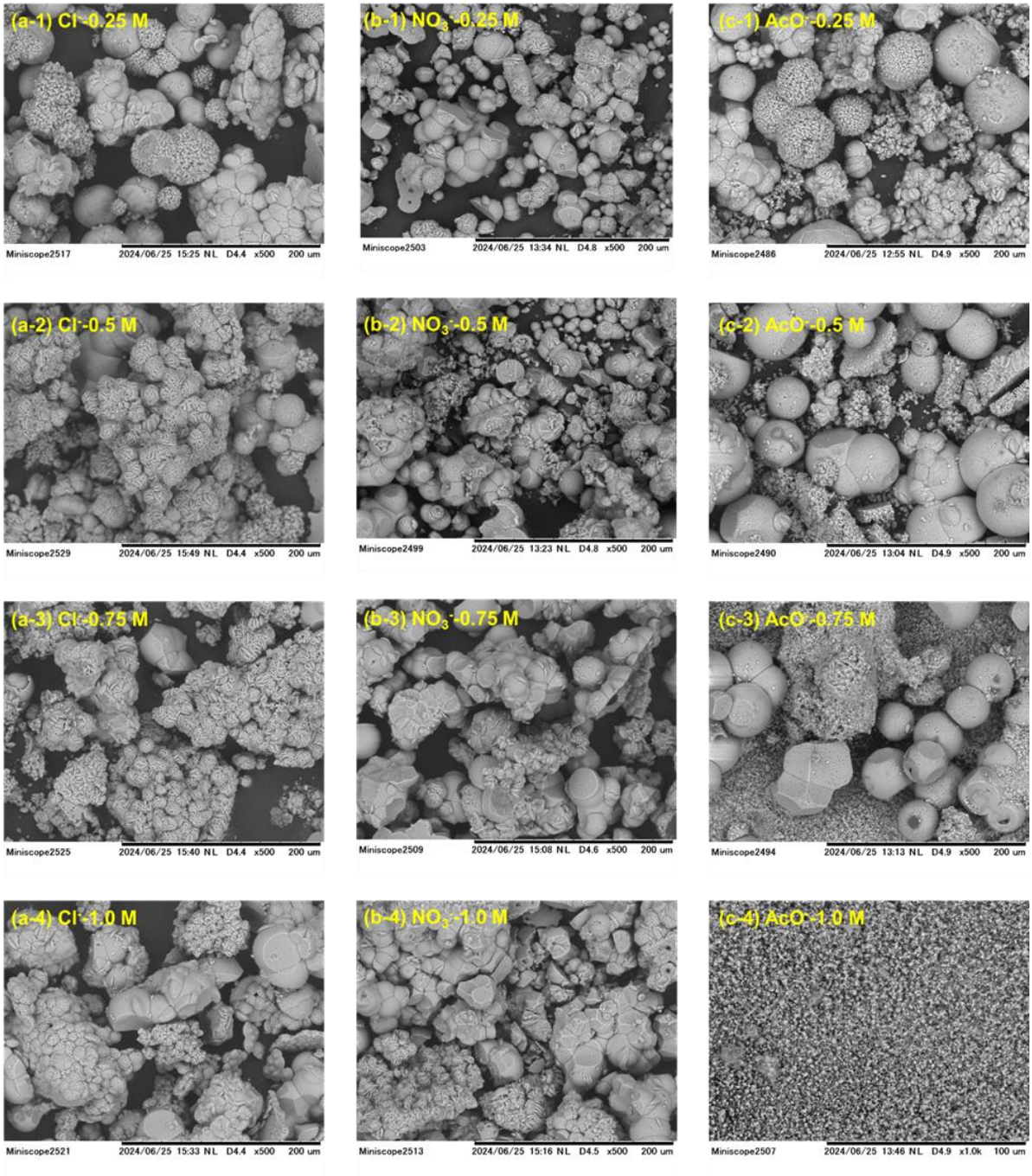

Figure 4. 13 Calcium carbonate induced by RII-2 strain with varying calcium salts and concentrations of cementation media. (a) calcium chloride; (b) calcium nitrate; (c) calcium acetate

4.3.5 Evaluation of solidified sand columns

a) Estimated UCS

To evaluate the applicability of selected candidates for MICP treatment. Two *Lederbergia* strains (RII-2&FU3) were examined in a small-scale sand solidification test. Treated samples were evaluated both mechanically and chemically. The bulk density changes in samples after treatment were shown in Figure 4.14(a), revealing a density increment of about 12% in two cases. The results of the estimated UCS obtained from needle penetration tests are presented in Figure 4.14(b). What can be seen in this figure is the increase of strength with depth, which results from a general pattern of calcium carbonate distribution in coarse sand columns. As the bacteria permeate through the coarse sand column from the sample surface to the bottom, some cells are trapped along the way down while others go down with effluent.

b) Calcium carbonate content

The calcium carbonate content results seen in Figure 4.14(c) were consistent with the UCS tendency, increasing the carbonate content led to an improvement in strength. It is worth noting that approximately 90% of the calcium ions provided were precipitated in the case of RII-2 and FU3 precipitated about 80% of that, indicating a highly efficient utilization of reagents for MICP. From the distribution of the calcium carbonate precipitation and the corresponding strength improvement, FU3 seems to be contributing to a more uniform cementation than RII-2, although it has a lower activity compared to RII-2. From the microscope images in the previous chapter, FU3 is smaller in cell size, however, more clusters are forming compared to the RII-2 arrangement. Extracellular polymeric substances of bacteria have been found to affect the formation of calcium carbonate in terms of morphology and structure (Azulay et al. 2018). Differences in biopolymers at the bacterial surface may contribute to a higher retention rate at the upper layer of sand columns. Since FU3 is a new species in *the Lederbergia* genus, its characteristics are still unknown. Therefore, more investigation is needed to understand the mechanism behind this finding.

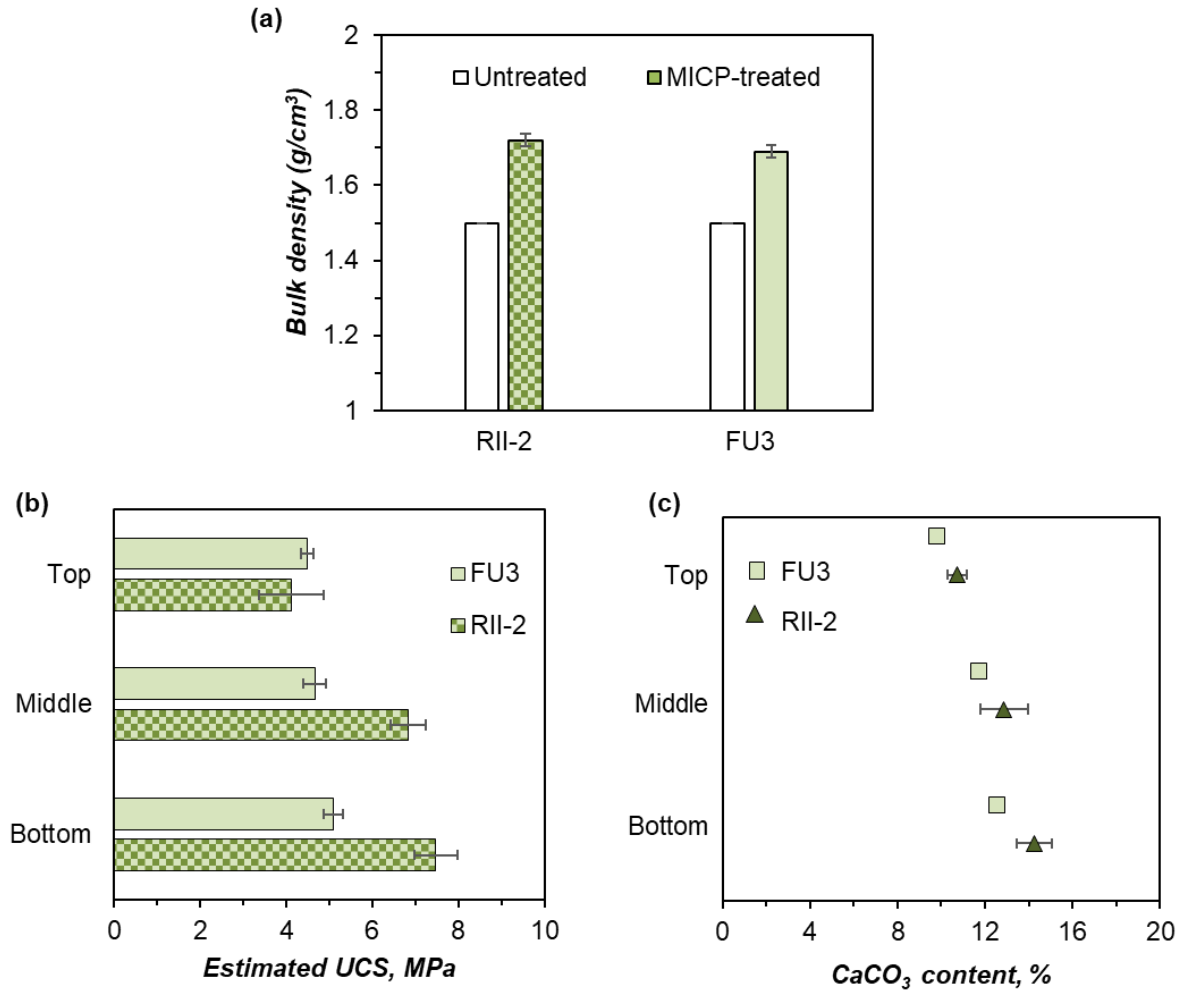

Figure 4. 14 Mechanical evaluation of MICP-treated samples. (a) before-after comparison of bulk density; (b) estimated unconfined compressive strength; (c) estimated calcium carbonate content of top, middle, bottom of treated samples

c) SEM and XRD analysis

Figure 4.15 illustrates the MICP-treated samples under scanning electron microscope observation and analysis of XRD. From the SEM images, there is no significant difference found between the precipitates induced by the two strains in terms of morphology, and mostly the size of precipitates fell into the range of 10–20 μm. It can be inferred from the tetrahedral form that the precipitates are calcite, which was further confirmed by the peaks in XRD patterns. Khanjani et al. (2021) investigated the controlling factors of microbial-induced $CaCO_3$ precipitation using *S. pasteurii*, from which the results found that the morphology of $CaCO_3$ changes with increasing Ca^{2+} and urea concentrations, and the vaterite formation dominates at initial stages and transforms to calcite after a certain period under

aqueous conditions. In this study, the transformation of calcium carbonate phases might have been done during the treatment, which explains why calcite is the only calcium carbonate form identified.

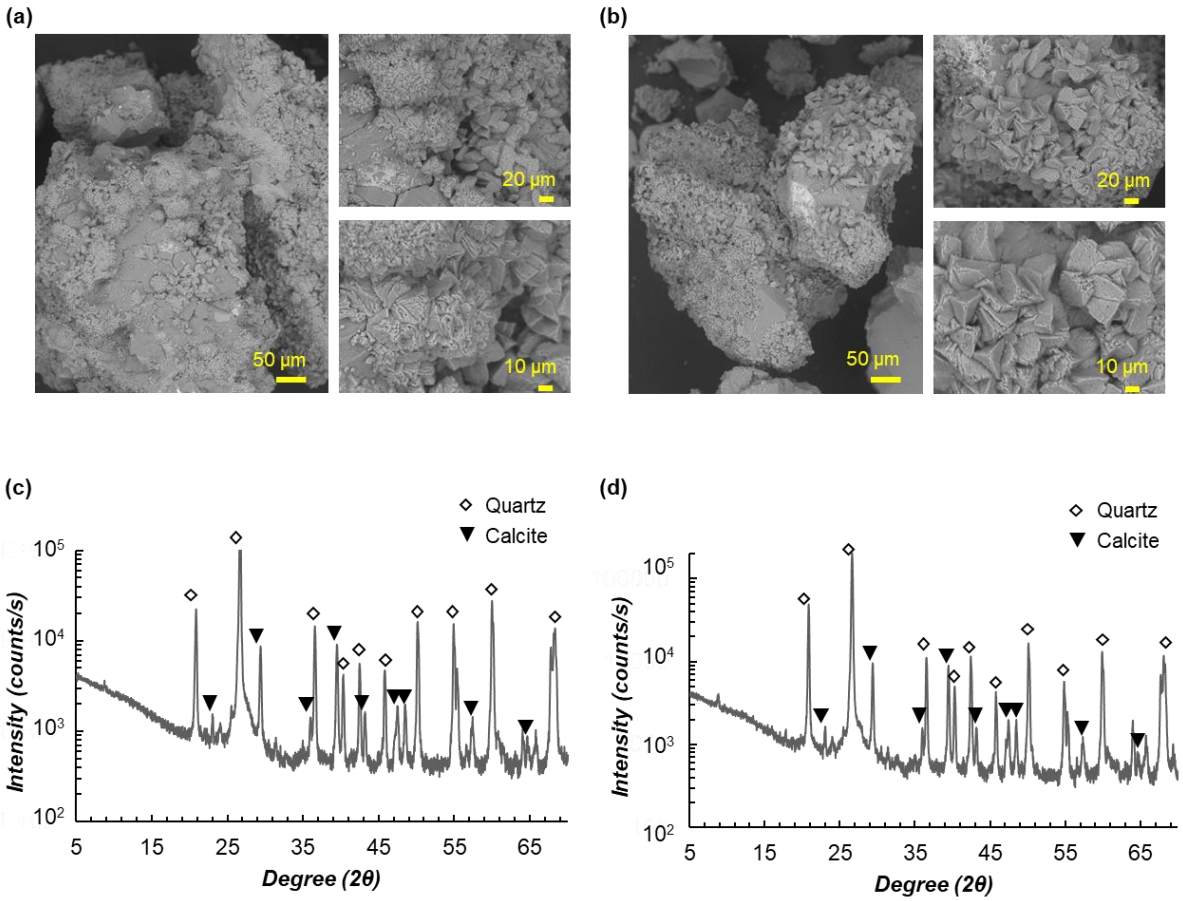

Figure 4. 15 Scanning electron microscope images of precipitates for (a) RII-2; (b) FU3. X-ray diffraction analysis results for (c) RII-2; (d) FU3

d) Discussion

The correlation between unconfined compressive strength with calcium carbonate content is usually discussed as important evidence of MICP efficiency, which could vary to some degree depending on the grain size distribution of tested sands, applied bacterial species, and treatment design. In this study, a comparison seen in Figure 4.16 was made to show the difference between studies on coarse sand with different species applied (Liang Cheng, Cord-Ruwisch, and Shahin 2013; Danjo and Kawasaki 2016; Mahawish, Bouazza, and Gates 2018; Terzis and Laloui 2019; Hoang et al. 2020). As a, namely, gold standard of ureolytic bacteria, *S. pasteurii* has been the preferred strain for most MICP researchers due

to its excellent performance. What can be seen in this figure is that the *Lederbergia* species in this study is comparable in bonding efficiency to that of *S. pasteurii*, which is a remarkable outcome that confirms the applicability of these two strains. What was found in our previous sand column solidification tests under the same experimental conditions (bacterial urease activity 3.5 U/mL) is that the bacterial performance began to decrease significantly after 5 days, leading to a relatively low calcium carbonate content and weak cementation effect (Meiqi Chen, Gowthaman, Nakashima, Komatsu, et al. 2021). Although the bacteria showed similar urease activity under urease test conditions, their performances in sand column solidification tests differ. Possibly, two *Lederbergia* strains have a better tolerance towards high concentration of calcium ions and ammonium and a higher survival rate under nutrient-deficiency environment. To confirm this postulation, further investigation is necessary for the subsequent stage of this research. Overall, these two strains could be good precipitators for MICP application in moderate climates.

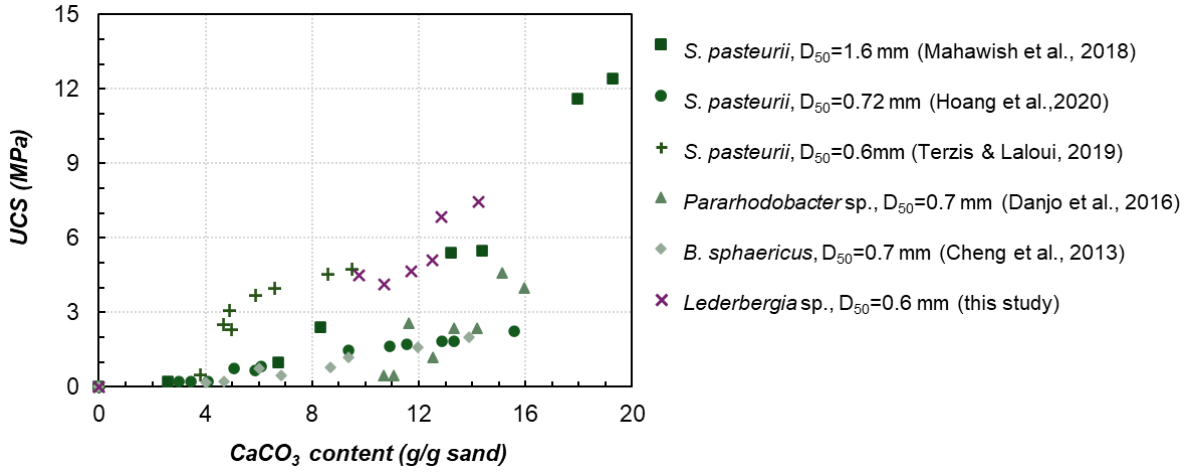

Figure 4. 16 Relationship between calcium carbonate content with UCS-a comparison with published studies on coarse sand treated by MICP

Compared with soilborne bacteria, airborne bacteria are more likely to be resilient strains. It has been reported that they can survive desiccation, nutrient deficiency, temperature shifts, harmful UV radiation, etc., which is possibly associated with DNA repair mechanisms, pigmentation, aggregation behavior, and adaptation to nutrient deficiency (Smets et al., 2016). Since the airborne bacteria usually withstand desiccation, it is highly possible that they could also survive in the process of freeze drying for obtaining a culture starter (Gratia et al. 2009). This would be an essential advantage over other bacteria with less resistance to desiccation when considering the production and application of ureolytic bacteria for MICP. Potential performance under nutrient deficiency conditions indicates the daily nutrient supply

may not be necessary for the treatment, which might also contribute to a less cost of bacterial cultivation. On the other hand, when they are applied to regions with significant temperature differences between daytime and nighttime, they are more likely to survive and induce continuous precipitation. For example, MY2-9, which thrives at a wide range of temperatures and produces cold adaptive enzymes, might be able to perform stable precipitation under changeable temperatures. Overall, with many characteristics of great bio-technological significance, ureolytic airborne bacteria are supposed to be able to apply to MICP for various purposes.

4.4 Conclusions

Airborne bacteria selected from different sampling sites were assessed by a series of tests to evaluate their applicability for biocementation. The findings provide new insights into isolation sources for ureolytic bacteria that are applicable to MICP. The following conclusions can be drawn.

I. Bacterial growth pattern and urease activity of selected isolates under 15°C–35°C cultivation showed that these isolates have climatic features. Two high activity *Lederbergia* strains thrive at relatively high temperatures, while two representatives of *Sporosarcina* and *Glutamicibacter* genera grow better at low temperatures. Besides, the pH dependence of their urease activity also showed great diversity. Interestingly, one *Providencia* sp. was found to exhibit relatively higher activity in acidic conditions.

II. The tolerance test with various calcium sources of varying concentration revealed that bacterial survival in the cementation media greatly differs from species to species. Some bacteria with excellent MICP performance have a surprisingly low survival rate compared to those strains with low activity. Further investigation is needed to gain a more comprehensive understanding of their survivability and adaptability under changing conditions.

III. Sand column solidification tests using two *Lederbergia* strains confirmed the applicability of airborne bacteria for MICP, showing estimated UCS ranging from 4 to 8 MPa and precipitation of 80%–90% total calcium source. Overall, this study provides new insight into ubiquitous airborne bacteria for geotechnical engineering applications.

Chapter 5 Development of high adaptability biocementation by mixed cultures of airborne bacteria

5.1 Background

Airborne bacteria are believed to have some unique features to survive in the air. Compared to the soilborne bacteria that we isolated from various soils, a greater diversity in taxonomy was found in the airborne isolates. For example, some strains from the *Micrococcaceae* family that are less known to researchers in this field may have potential in biocementation applications, which need to be explored further. On the other hand, it should be noted that bacteria growing in laboratory conditions can behave quite differently from the way they behave in their natural habitat, which means that airborne bacteria may show some resilient features in the air but do not express them in laboratory culture (Barberán et al. 2017). Many previous studies have found that bacteria have more complex responses to different inhabits than we could imagine. In the field of bioengineering, there are many attempts to construct "microbial community" to reach a high efficiency and stable performance for different purposes. Either this community is artificial or synthetic, the combination or association of microorganisms usually results in more powerful metabolic capabilities than those of single strains (Massot et al. 2022). However, due to the constraint of technology, some mechanisms in their functioning process remained unrevealed. Currently, two of the most challenging parts are the difficulty in monitoring the complex interactions in the community and the storage of a performance stable microbe consortium (Stewart 2012; Massot et al. 2022). Few studies have tried using multiple bacteria for MICP treatment. Gat et al. (2014) cocultured *S. pasteurii* with non ureolytic bacteria *B. subtills* for carbonate precipitation. The authors concluded that the non-ureolytic bacteria led to a decrease in pH level, while the precipitation rate was facilitated by these bacteria as nucleation sites. A recent study investigated synergistic biocementation by two ureolytic bacteria from *Comamonas* and *Bacillus* genus, revealing a significant increase in carbonate precipitation in comparison to the average precipitation amount of mono strains (Rajasekar et al. 2024).

Considering the limitations of bacterial consortium, this study tried to cultivate single strains separately and apply multiple strains for biocementation instead of coculturing multiple strains to construct a consortium. In this chapter, strains selected from previous chapters were examined in various conditions after being mixed into pairs with different ratios. The results showed that bacteria tend to have more positive interactions than negative interactions in the precipitation process. One of the interesting findings is that with a small portion of low-activity bacteria mixed into high-activity bacterial culture, the precipitation efficiency significantly increased. Even in an environment that is unfavorable for precipitation, mixed strains exhibited a higher precipitation rate than mono strains did. Taken together,

introducing competition or other interactions from other strains makes it possible for us to gain more understanding about the potential of these strains.

5.2 Materials and methodology

5.2.1 Selected strains

As described in the previous chapters, ureolytic bacteria of great diversity were isolated from the air. Figure 5.1(a) compares the global occurrence of ten bacterial genera identified in this study based on the "Micro Atlas Project" (2023) database. Many of them are frequently identified in soil, animals, water, and plants, with more than hundred thousand of samples. Based on the previous findings, strains from four species *Lederbergia*, *Sporosarcina*, *Providencia*, and *Glutamicibacter*, were selected to proceed to further investigation. The former two are more often found in soil samples, the latter two are frequently isolated from animal samples.

In this study, three *Lederbergia* strains (RII2, FU3, and TF7-15) are closely related with *Bacillus* species. Among these isolates, RII-2 and FU3 have relatively high urease activity, and their applicability for biocementation has been proved in the sand column solidification test. One *Sporosarcina* species MY2-9 has cold tolerance which makes it possible to grow at low temperatures as low as 4 °C, therefore, it has a great potential for application in cold regions. *Glutamicibacter* strains were found in several sites in Sapporo sampling. Existing research found that bacteria from this genus probably have a large population across the world (see in Figure 5.1b). Based on the Microbe Atlas database, *Glutamicibacter* bacteria were identified globally with a relatively large population compared to *Sporosarcina* species. Moreover, it has been reported that these bacteria are versatile in making use of various nutrients, thus surviving harsh environments (Santos et al. 2020). Currently, to our knowledge, only a few studies have investigated the applicability of *Glutamicibacter* strains for MICP application. Based on a comparison of five *Glutamicibacter* strains on the results of characterization tests, FU17 was selected as the representative strain. *Providencia* species are frequently found in animal samples, and some species are notorious pathogens that cause urinary tract infections and diarrhea. The strain (KK7) isolated from Okinawa is close to a recently proposed novel species, *Providencia manganoxydans*, which was originally isolated from heavy metal-contaminated soils. According to the report, it can oxidize Mn (II) and survive in a harsh environment (Z. Li et al. 2022). An interesting finding of this strain is that it tends to show relatively higher urease activity in acidic conditions than in alkaline conditions, which could be a great advantage when considering the application of MICP to stabilize acidic problematic soils. Basic characteristics of four strains are presented in Table 5.1.

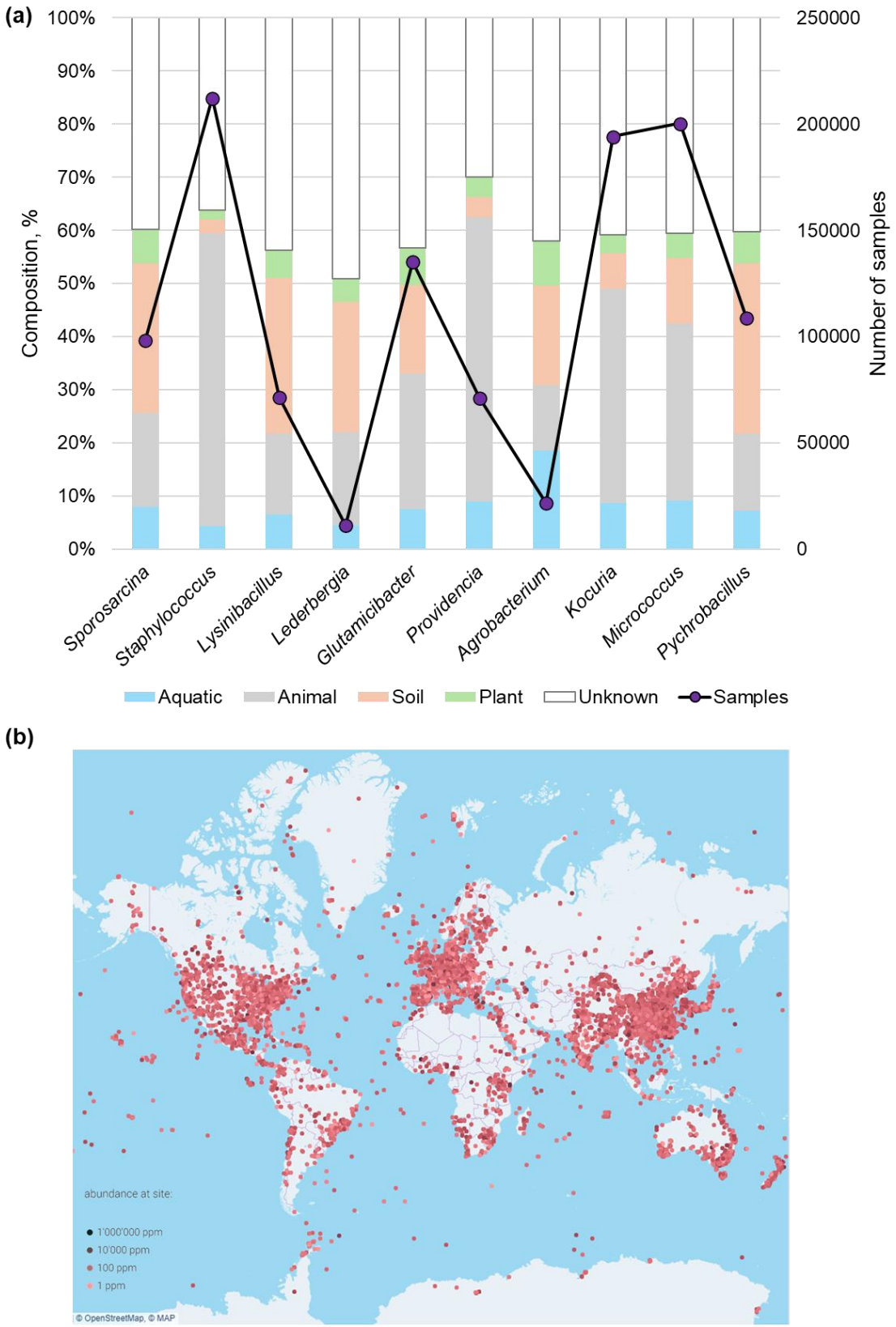

Figure 5. 1 (a) Prevalence of ten identified genera in different environments; (b) abundance of *Glutamicibacter* genus (Data from Microbe atlas Database)

Table 5. 1 Basic characteristics of selected strains

Genus	Strain ID	Gram	Morphology	Temp. range	Optimal pH range
Lederbergia	RII-2 FU3	+	Rods 0.9–1.0×2.5–7.0 μm	mesophile	8-9
Glutamicibacter	FU17 MY3-21 MY5-21 MY1-15	+	Rods/cocci 1.0–1.1×1.0–2.5 μm	mesophile	6-7
Sporosarcina	MY2-9 MD4-29	+	Rods 0.9–1.0×4.0–7.0 μm	psychrotroph mesophile	8-9
Providencia	KK7	-	Rods -0.5×1.5 μm	mesophile	4

5.2.2 Precipitation tests using mixed strains

In the previous chapters, a series of precipitation tests were conducted with mono strains. After examination, strains were selected from different genera to make a combination that typically consists of a fast precipitator and a slow precipitator, or a typical precipitator and a non-typical precipitator. For example, strains from *Lederbergia* genus are typical ureolytic bacteria with high urease activity and a common mesophilic growth pattern. *Glutamicibacter* species are usually weak in activity but show a stable performance since they survive the test solution for the long term. This type of combination of different precipitators is evaluated in precipitation tests. Other non-typical strains such as *Providencia* species had a relatively high urease activity at acidic conditions compared to other tested strains. Therefore, they could be coupled with common strains that perform better at alkaline conditions to achieve high-efficiency precipitation across a wide pH range. This experiment started with mixing two strains with different ratios and mixing methods. After selecting pairs with high precipitation efficiency, these pairs were tested in tests with less favorable conditions, such as low temperature, high concentrations of cementation media and low initial pH.

a) Mixing of strains from different genera with changing ratios

The first trial was the mixing of two *Sporosarcina* sp. with four *Glutamicibacter* sp. Table 5.2 presents the cases of precipitation tests. In this test, the bacteria population was not adjusted, and two strains were mixed in a volume ratio of 1:1. To understand the effect of varying bacteria populations, the second

series of mixing test was conducted with adjustment of bacteria culture before the precipitation test. Table 5.3 presents the cases of mixing two *Lederbergia* sp. with four *Glutamicibacter* sp. The mixing ratio refers to the turbidity ratio. The third series of precipitation tests mixed four strains (*Lederbergia* sp., *Glutamicibacter* sp., *Sporosarcina* sp., and *Providencia* sp.) in pairs. The mixing ratio refers to the volume ratio. Based on the findings of the previous two series, the volume ratio was adjusted into a wider range (see Table 5.4).

Table 5. 2 Precipitation tests mixing *Sporosarcina* sp. with *Glutamicibacter* sp. in pairs

Strain A	Strain B	Volume ratio A: B	Conditions
Sporosarcina sp. MY2-9 MD4-29	*Glutamicibacter* sp. FU17 MY3-21 MY5-21 MY1-15	1:1	0.5 M cementation media 25 °C, 160 rpm

Table 5. 3 Precipitation tests mixing *Lederbergia* sp. with *Glutamicibacter* sp. in pairs

Strain A	Strain B	Turbidity ratio A: B	OD_{600} (A)	Conditions
Lederbergia sp. RII-2 FU3	*Glutamicibacter* sp. FU17 MY3-21 MY5-21 MY1-15	1:1 1:0.5 1:0.25	3.3-3.6	0.5 M cementation media 25 °C, 160 rpm
Lederbergia sp. RII-2	*Glutamicibacter* sp. FU17	1:1 1:0.1 1:0.01	3.5	

Table 5. 4 Precipitation tests mixing four strains in pairs

Strain	Combination			Volume ratio	Conditions
	Strain A	Strain B	Group ID	A/B	
Lederbergia sp. RII-2 *Glutamicibacter* sp. FU17 *Sporosarcina* sp. MY2-9 *Providencia* sp. KK7	RII-2	FU17	RF	1/0 1000/1	0.5 M cementation media 25 °C 160 rpm
	RII-2	KK7	RK	100/1	
	RII-2	MY2-9	RM	10/1	
	MY2-9	FU17	MF	1/1	
	MY2-9	KK7	MK	1/10	
	FU17	KK7	KF	1/100 1/1000 0/1	

b) Precipitation test at low-temperature conditions

From culture media to target soils, applied bacteria usually go through dramatic environmental changes during the treatment period. For example, the temperature can drop from 30 °C to 15 °C on summer nights in Hokkaido. To confirm the influence of temperature shifts, one series of precipitation tests were conducted using selected pairs from previous cases. The blank was set at 25 °C. One set of trial tests was conducted with incubation temperature changing every 12 hours from 35 °C to 15 °C. The results did not show a significant difference (see Appendix C). This set of experiments aimed to evaluate the performance of mixed strains at low temperature conditions. Table 5.5 presents the tested cases.

Table 5. 5 Precipitation tests at low temperatures-a comparison of the performance of mono strains with mixed strains.

Group	ID	Mixing ratio	Concentration of CM	Incubation temp.
Mono strains	RII-2 (R) MY2-9 (M) KK7 (K) FU17 (F)	N/A	0.5 M	15 °C 25 °C (Blank)
Mixed strains	RM	100/1, 10/1, 1/1		
	RK	100/1, 10/1, 1/1		
	RF	100/1, 10/1, 1/1		

c) Precipitation test at high concentrations of cementation media

The concentration of cementation media is one of the important factors that govern the efficiency of cementation, since it directly affects the function of urease enzymes and survival of microbes. Existing studies found that a moderate concentration of cementation media contributed to a relatively more uniform cementation of soil samples than a high concentration did (Erdmann and Strieth 2023). Little attention was paid to evaluating the survival rate of bacteria during the cementation process. Based on the characterization test in the previous chapter, most tested bacteria were not able to survive a high concentration of cementation media. In this series of experiments, it aims to investigate whether the performance of mixed strains could surpass mono strains in a high concentration of cementation media. Table 5.6 presents the tested cases.

Table 5. 6 Precipitation tests with high concentrations of cementation media-a comparison of the performance of mono strains with mixed strains

Group	ID	Mixing ratio	Incubation temp.	Concentration of CM
Mono strains	RII-2 (R)	N/A	25 °C	1 M
	MY2-9 (M)			
	KK7 (K)			
	FU17 (F)			
Mixed strains	RM	100/1, 10/1, 1/1		
	RK	100/1, 10/1, 1/1		
	RF	100/1, 10/1, 1/1		

Table 5. 7 Precipitation tests with high concentrations of HA-a comparison of the performance of mono strains with mixed strains

Group	ID	Mixing ratio	HA addition, %	Conditions
Mono strains	RII-2 (R)	N/A	0 5 10 15	CM: 0.5 M Temp.: 25 °C
	MY2-9 (M)			
	KK7 (K)			
	FU17 (F)			
Mixed strains	RK	100/1		
		10/1		
		1/1		

d) Precipitation test at high HA content

In Chapter 4, isolates were tested with a small addition (0.5, 1, 2, 4, 5 %) of HA in precipitation test. The results revealed that all of them can induce precipitation without much effect from HA. In a mature soil profile, the top layer usually consists of a thin layer of humus, the organic matter in soils. Usually, organic matter is less than 5% of the soil matrix. However, in organic soils, the organic content can be more than 50% of the soil. HA as a fraction of humic substances, its portion in organic matter varies. In this series of experiments, the HA addition was added up to 15%. All tested cases are presented in Table 5.7.

5.2.3 Calculation of mixing effect index

To make a quantitative comparison of mixing effect on the precipitation ratio in each case, an index is defined and calculated based on the equations below (Eq.5.1 & Eq. 5.2). Theoretical precipitation ratio of mixed strains is calculated based on a hypothesis that mono strains induce carbonate precipitation independently without interaction with other strains.

$$Theoritical\ ppt.\ ratio\ of\ mixed\ strains\ (A+B) = \frac{V_{strain\ A} \times ppt.\ ratio\ of\ strain\ A + V_{strain\ B} \times ppt.\ ratio\ of\ strain\ B}{V_{total}} \quad \text{(Eq. 5.1)}$$

$$Mixing\ effect\ index = \frac{Theoritical\ ppt.ratio\ of\ mixed\ strains - ppt.ratio\ of\ mixed\ strains}{Theoritical\ ppt.ratio\ of\ mixed\ strains} \quad \text{(Eq. 5.2)}$$

5.3 Results and discussion

5.3.1 Performance of mono strains and mixed strains in precipitation test

a) Eight pairs of two Sporosarcina strains with 4 Glutamicibacter strains

Figure 5.2 presents the results of calcium carbonate precipitation tests using combination of two Sporosarcina species (MY2-9 and MD4-29) with four *Glutamicibacter* species (FU17, MY1-15, MY3-21, and MY5-21). Mixing ratio in this series (1:1) of tests refers to the volume ratio without any adjustment of bacterial populations. Generally, all cases showed a positive effect after 24-hour incubation. In the beginning stage of the test, most mixed cases showed a retarded precipitation rate. Normalized effect of mixing is compared in Figure 5.3. As can be seen in the figure, the mixing effect index was mostly between 0.2-0.4 for MY2-9 cases, and 0-0.4 for MD4-29 cases. For the former, the negative effect in the beginning stage was less than the latter, and the positive effect was more significant than the latter.

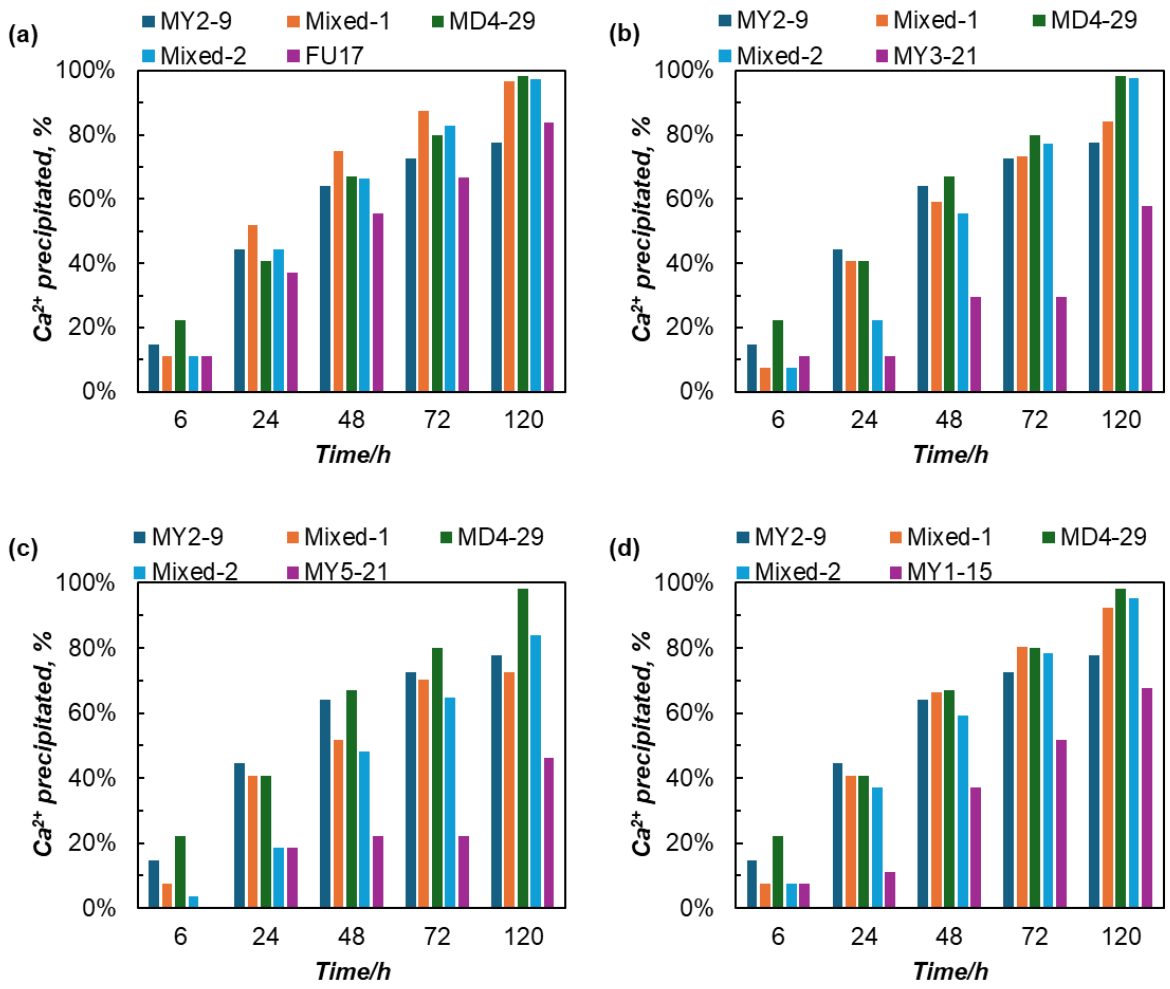

Figure 5. 2 Monitoring of calcium ion concentration changes in precipitation tests mixing *Sporosarcina* sp. with *Glutamicibacter* sp.

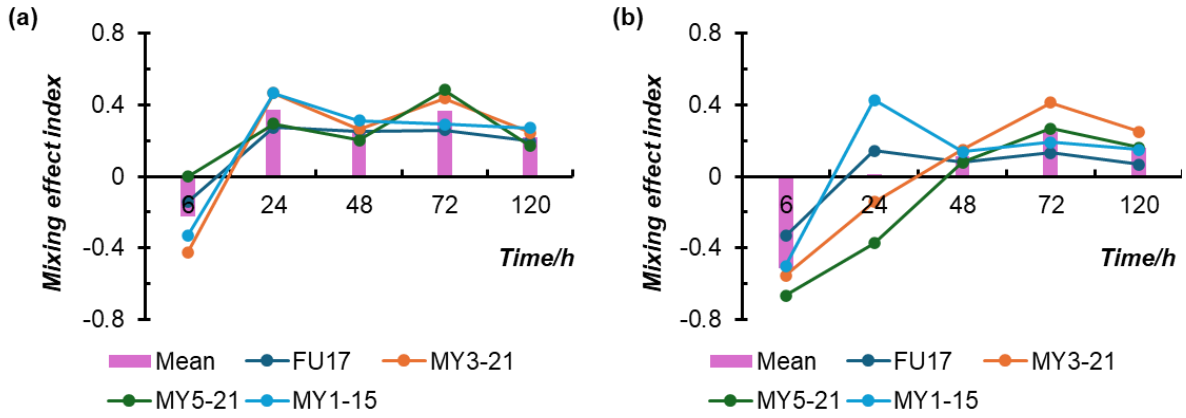

Figure 5. 3 Normalized effect of mixing. (a) pairs with MY2-9; (b) pairs with MD4-29

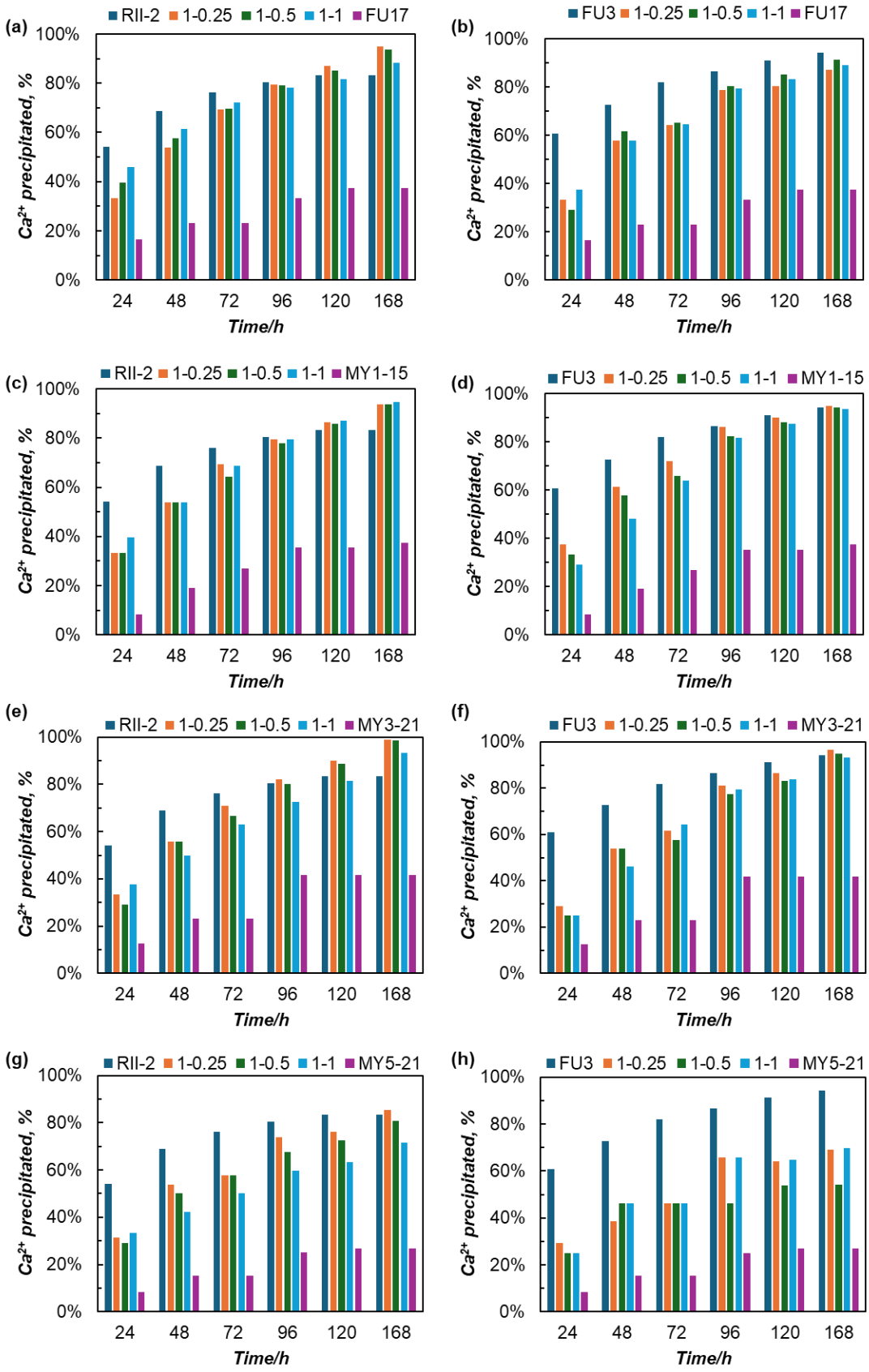

Figure 5. 4 Monitoring of calcium ion concentration changes in precipitation tests mixing *Lederbergia* sp. with *Glutamicibacter* sp.

b) Eight pairs of two Lederbergia strains with 4 Glutamicibacter strains

To better understand the interaction of two strains in the mixed culture, bacterial cultures were adjusted to reach the same turbidity before changing the mixing ratios. In this set of experiments, two *Lederbergia* strains were mixed with four *Glutamicibacter* strains with a ratio of 1:0.25, 1:0.5, and 1:1. As can be seen in Figure 5.4, monoculture of *Lederbergia* strains showed excellent activity at the beginning of the precipitation test, while the *Glutamicibacter* had a significantly decreased performance after population adjustment. As a result, all cases using mixed cultures exhibited a retarded precipitation rate at the beginning of incubation period (~72-hour). Nevertheless, the mixed cultures had a stable precipitation rate till 168-hour incubation which is a significant improvement compared to the cases of monocultures. On the one hand, mixed cultures can contribute to a higher efficiency of source utilization by the end of the treatment, which is a great advantage in reducing the cost of treatment. On the other hand, monocultures of bacteria with high urease activity usually produce a fast precipitation of calcium carbonate in a short period while the rate is decreasing afterwards. This can lead to a dramatic increase in the heterogeneity of cemented samples. In the case of mixed culture, carbonate precipitation occurs at a relatively stable rate which can probably contribute to an increase in homogeneity of cemented samples. Normalized effect of mixing is compared in Figure 5.5. Similar to the previous combination, when bacteria with high performance was mixed with another strain with a weak performance, the mixing effect was relatively small and there was a retarded period at the beginning. An interesting finding is that the mixing effect increases with the decreasing portion of "weak strains". In this set of precipitation tests, volume ratio was adjusted in a small range. Adjustment in a wider range might contribute to a precipitation of higher efficiency.

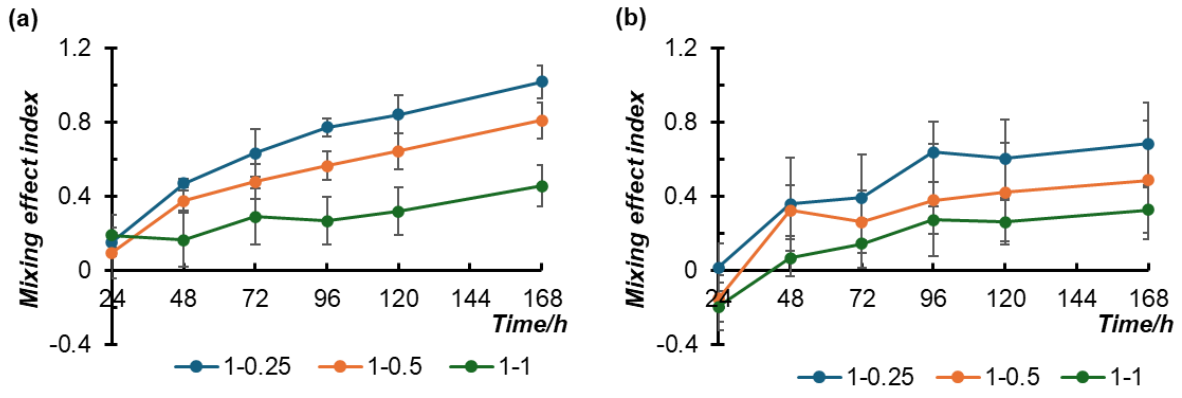

Figure 5. 5 Normalized effect of mixing. (a) pairs with RII-2; (b) pairs with FU3

One more set of precipitation tests was conducted to figure out how the mixing ratio affects precipitation efficiency. Three volume ratios of 1:0.01, 1:0.1, and 1:1 was adopted. Two representative strains (RII-2 from *Lederbergia* sp. and FU17 from *Glutamicibacter* sp.) were selected. Figure 5.6 shows the estimated precipitation amount and mixing effect index in 168-hour incubation. As can be seen in Figure 5.6(a), cases of mixed strains induced more precipitation than cases of mono strains did. The mixing effect demonstrated that the efficiency was highest when the mixing ratio was 1:0.01.

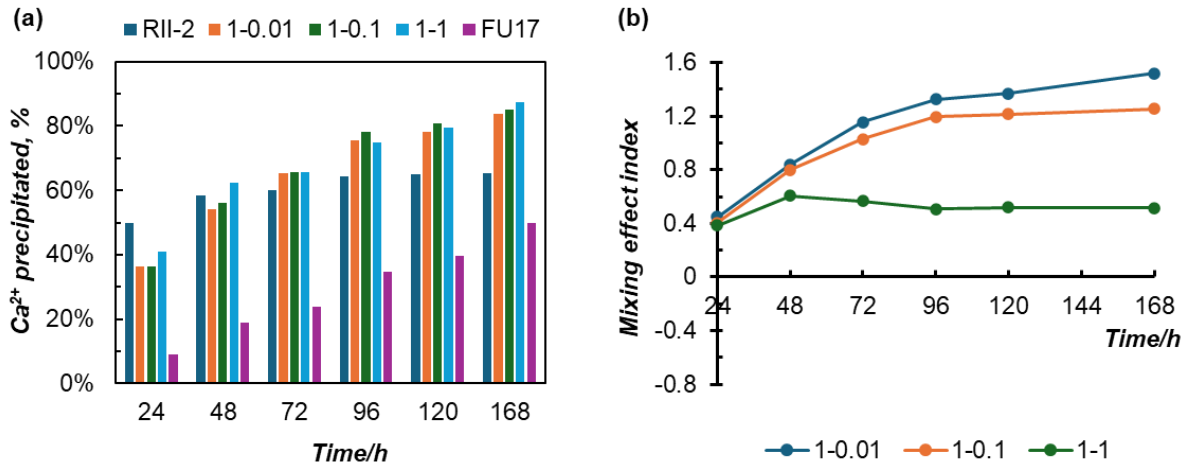

Figure 5. 6 Precipitation tests mixing *Lederbergia* sp. with *Glutamicibacter* sp. in a wide range (a) Monitoring of calcium ions; (b) normalized effect of mixing

Studies focused on the interaction between different bacteria in bacteria consortium found that bacteria tend to cooperate with each other to survive in an environment with less nutrients by making full use of metabolites produced by other bacteria (Goldford et al. 2018). However, when they were placed in a nutrient-rich environment, their interaction with each other leads to a fast extinction (Ratzke, Barrere, and Gore 2020). In the precipitation test, the initial nutrient in the culture media was diluted by the adjustment of volume ratio. Therefore, there is a possibility that nutrient availability might affect the performance of mixed strains. On the other hand, the mixing trials in the two sets of precipitation tests were limited to the mixing of small portion of weak strains with high- performance strains. In an ideal scenario, mixing a small portion of high-performance strains into weak strains should improve the precipitation rate, if both of two strains can work independently without any interference with each other.

c) Six pairs of one Lederbergia strain, one Sporosarcina strain, one Glutamicibacter strain, and one Providencia strain

To figure out the mechanism of mixing effect, the volume ratio in this set of precipitation tests was fixed without dilution of bacterial culture. Surprisingly, most mixed cases with a high portion of high-performance strains showed an increase in precipitation rate at the beginning of the test, without significant retarded phase (see in Figure 5.7). In particular, the cases with *Lederbergia* sp. RII-2 dominated exhibited a 10-20% increase in the precipitation amount in 48-hour incubation. Contrary to expectations, the mixing effect was not as significant as the cases with RII-2 dominated when high portions of weak strains were mixed with small portion of high-performance strains.

The mixing effect index of six pairs of mixed strains is summarized in Figure 5.8. The best performance was found in cases with RII-2 dominated, which exhibited a stimulated precipitation throughout the 168-hour incubation period. Other cases showed a relatively small mixing effect. Therefore, further investigation of mixed strains was focused on three pairs (RF, RK, RM) with RII-2 dominated (100/1, 10/1, 1/1). In terms of the possible interactions between these strains, it is difficult to elucidate the mechanisms from the gene expression level, but there are some hypotheses that could explain the results obtained in precipitation tests. For example, weak strains like KK7 and FU17 are much smaller in cell size than RII-2 with a typical rod shape. It means that these small cells have a large specific surface area that can be served as nucleation site for calcium carbonate than large bacteria cells. One previous study of using mixed strains for MICP found that calcium carbonate precipitation efficiency can be improved by mixing ureolytic bacteria with non-ureolytic bacteria, which might result from the nucleation site provided by the non-ureolytic bacteria (Gat et al. 2014). It should be noted that the non-ureolytic bacteria were similar rods to the ureolytic bacteria, while there is a significant difference in cell size between two strains in this study. Possibly, a relatively large specific surface area contributed to more nucleation sites than previous studies.

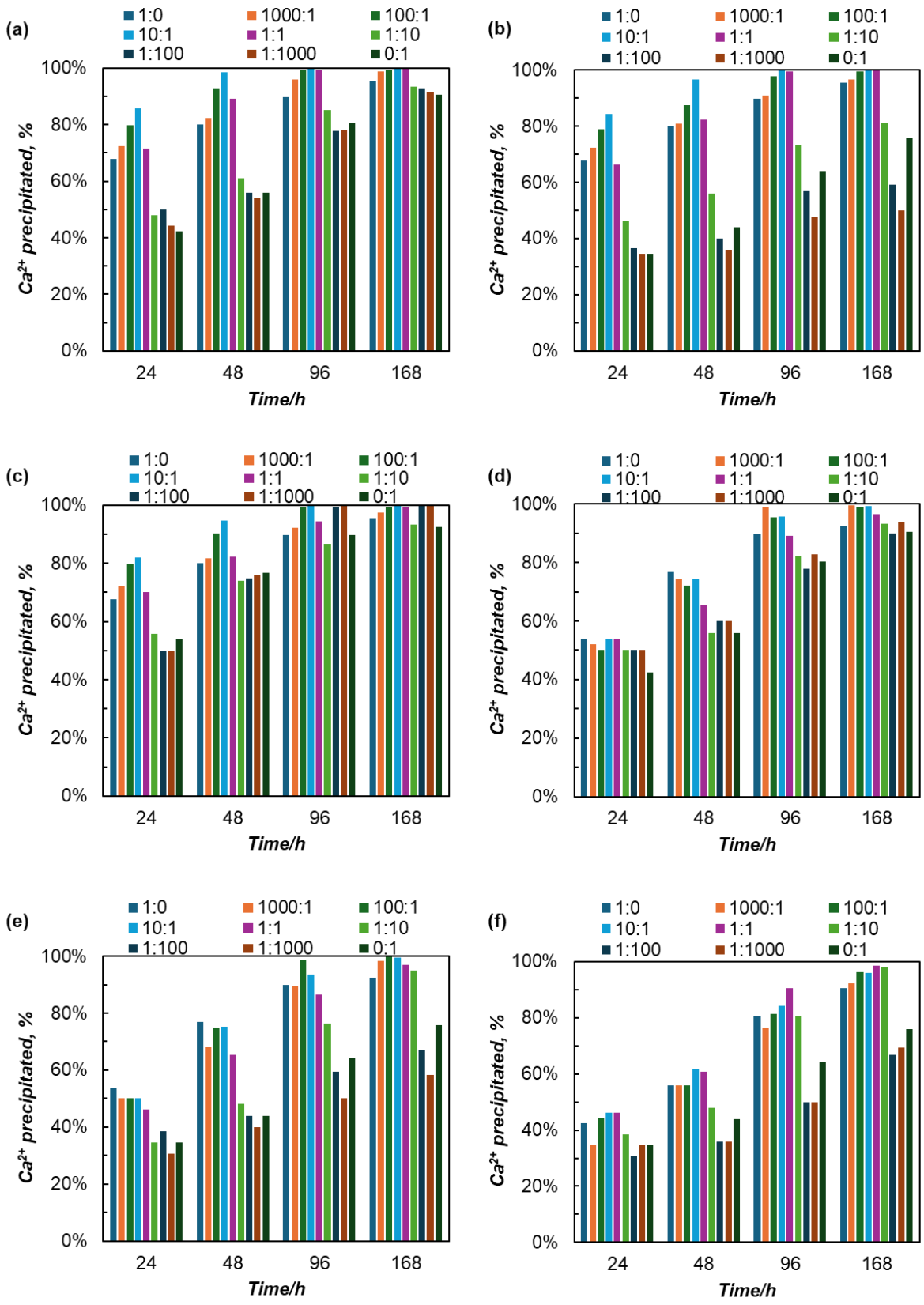

Figure 5. 7 Monitoring of calcium ion concentration changes in precipitation tests mixing four strains in pairs (a) RF; (b) RK; (c) RM; (d) MF; (e) MK; (f) FK

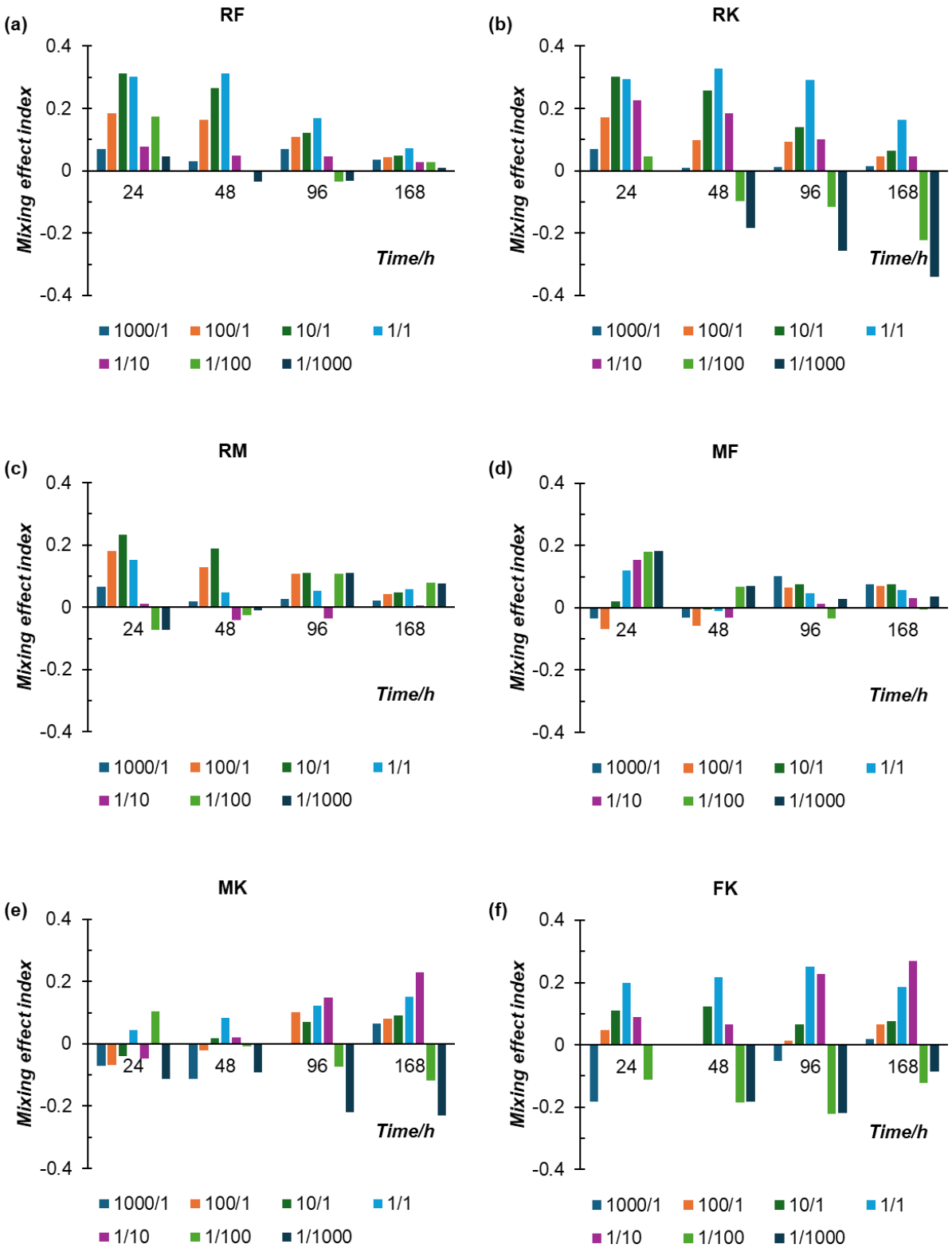

Figure 5. 8 Normalized effect of mixing. (a) RF; (b) RK; (c) RM; (d) MF; (e) MK; (f) FK

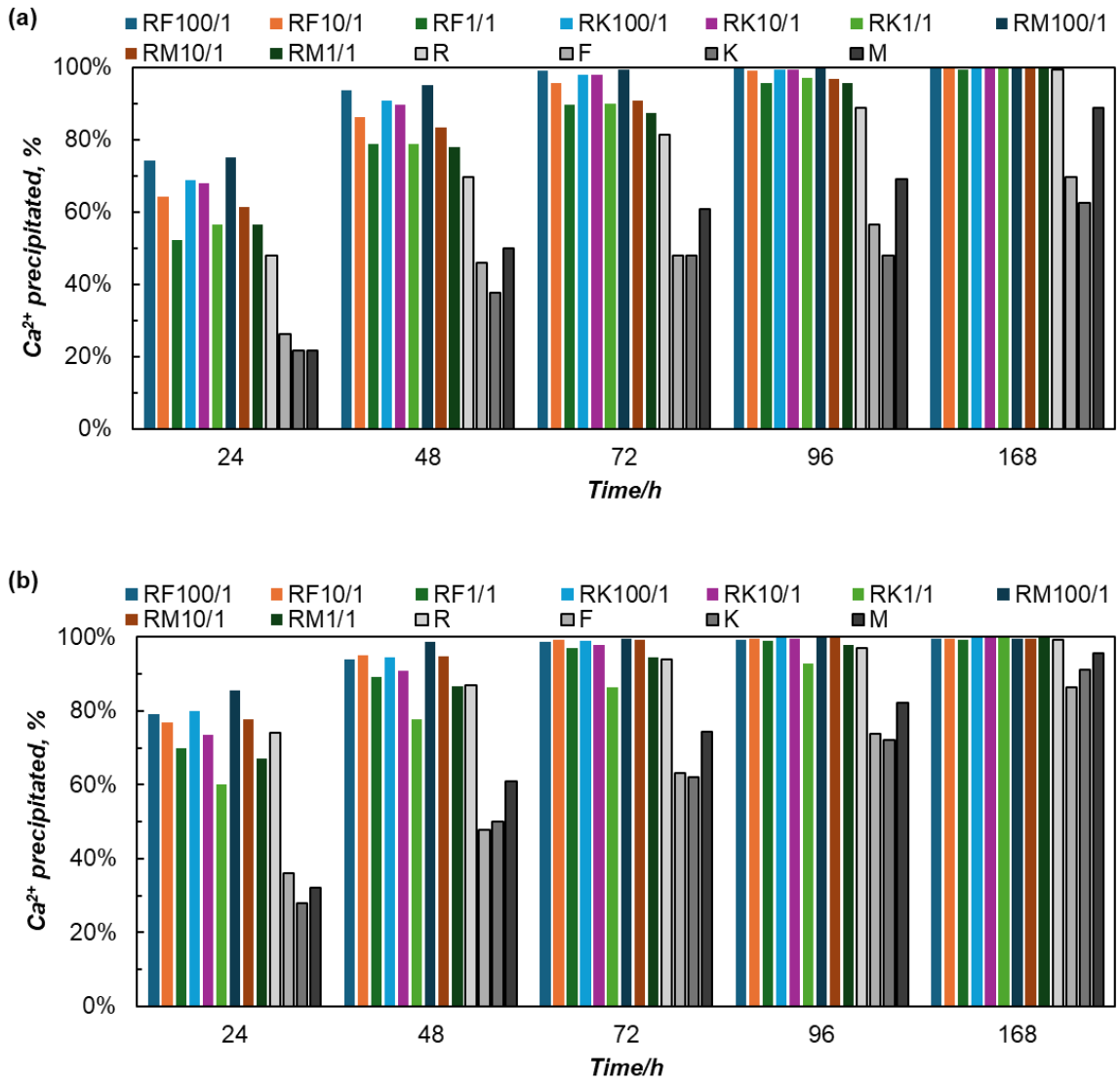

Figure 5. 9 Monitoring of calcium ion concentration changes in precipitation tests- a comparison of mono strains with mixed strains. (a) cases incubated at 15 °C; (b) cases incubated at 25 °C

5.3.2 Comparison of performance at low temperature conditions

Four strains examined in this chapter have been characterized by a series of tests in the previous chapters. Most of them have a typical mesophilic growth pattern. For example, *Lederbergia* strains showed an increasing performance with an increasing temperature from 15 to 35 °C (RII-2 was not able to grow at 15 °C). But one *Sporosarcina* strain (MY2-9) was found growing at a temperature as low as 4 degrees and showing an optimal urease activity at 20 °C. To figure out how mixing of two strains with different temperature ranges can affect the precipitation efficiency at low temperature, one set of precipitation tests was conducted at 15 °C, with a control group at 25 °C. Figure 5.9 presents the estimated

precipitation amount of two sets of tests. Generally, most calcium ions were precipitated by 96 hours, in both of the two sets. A closer observation on the figure can find that cases of mono strains showed a more significant retardation at the beginning of the test than cases of mixed strains did. As can be seen in Figure 5.10(a), the difference between two sets (% precipitated at 25 °C - % precipitated at 15 °C) was quite large after 24-hour incubation. In particular, the difference of RII-2 strain's case is more than 25 %. However, this difference decreased dramatically with time in all cases of mixed strains while most cases of mono strains showed an increase in the difference. From the comparison of mixing effect index between 15 °C cases and 25 °C cases in Figure 5.10(b-c), it can be inferred that bacteria of different strains might be stimulated to cooperate with each other to survive in an environment that is less favorable for them. As a result, the mixed cases with optimal mix ratio achieved almost 100% of calcium carbonate precipitation by 72-hour incubation when the optimal mono case (RII-2) precipitated 80% of the calcium ions. Overall, cases of mixed strains did not show a significant retardation of precipitation compared to cases of mono strains, suggesting an improved utilization rate of cementation media.

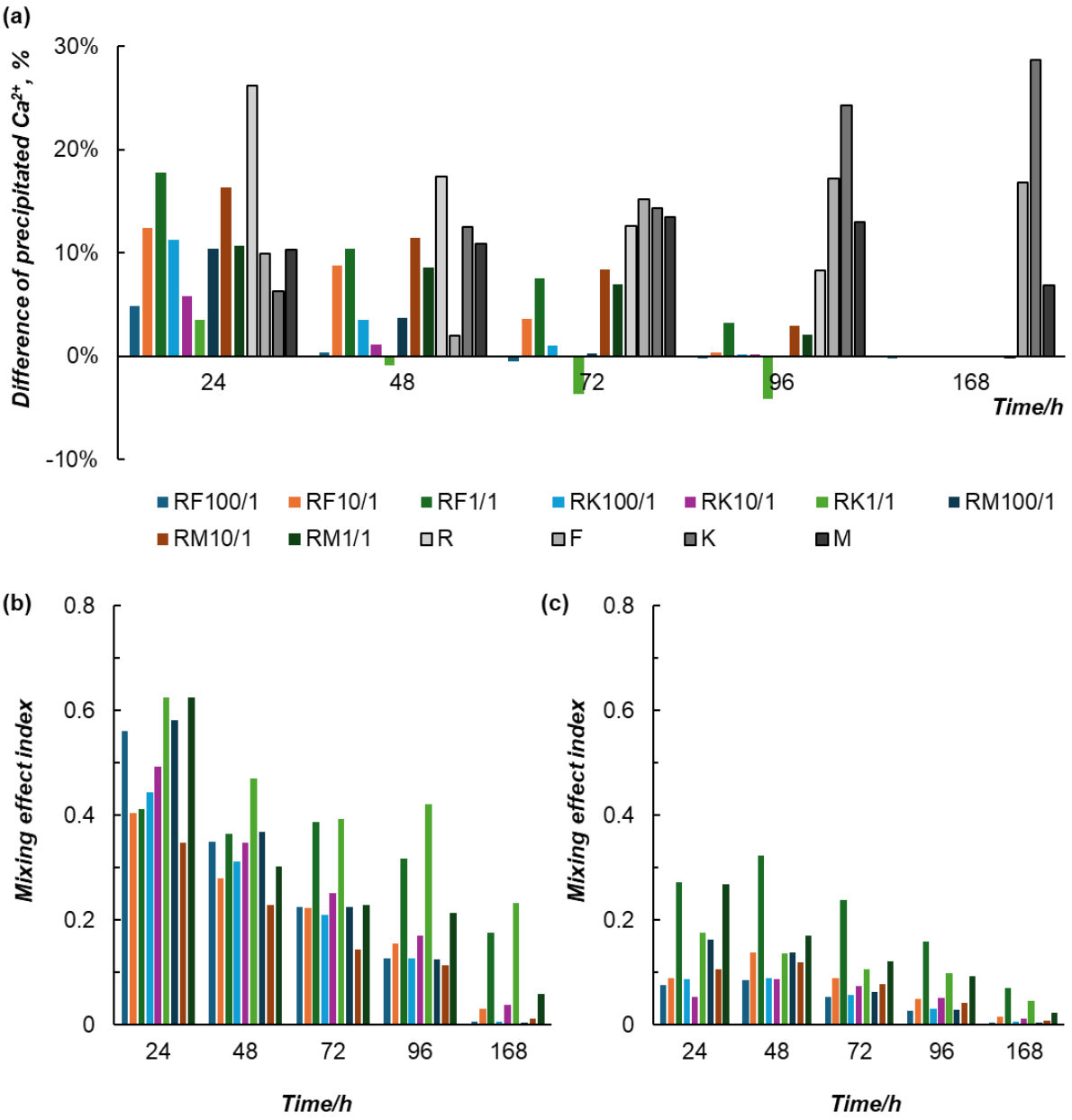

Figure 5. 10 Comparison of two sets of tests at different temperatures (a) The difference of estimated precipitation amount; (b) mixing effect of 15 °C cases; (c) mixing effect of 25 °C cases

5.3.3 Comparison of performance at high concentration of cementation media

In the previous chapter, selected isolates were examined in precipitation tests with various calcium sources and changing concentration of calcium sources and urea, which found that most common ureolytic bacteria from *Sporosarcina* sp. (MY2-9) and *Lederbergia* species (RII-2, FU3 & TF7-15) cannot tolerate a high concentration up to 1.0 M. After 168-hour incubation, two isolates (KK7 & FU17) from *Providencia* sp. and *Glutamicibacter* sp. exhibited a much higher survival rate than the species

mentioned above. These strains did not induce precipitation at a speed as fast as the other strains, but they were able to survive for a longer period than those strains which have a fast precipitation rate only at the beginning stage. Therefore, an ideal situation is that by mixing these strains together, fast precipitators precipitate significant amounts of calcium carbonate at the beginning of the test and slow precipitators can continue the precipitation under a reduced pressure from high concentration cementation media.

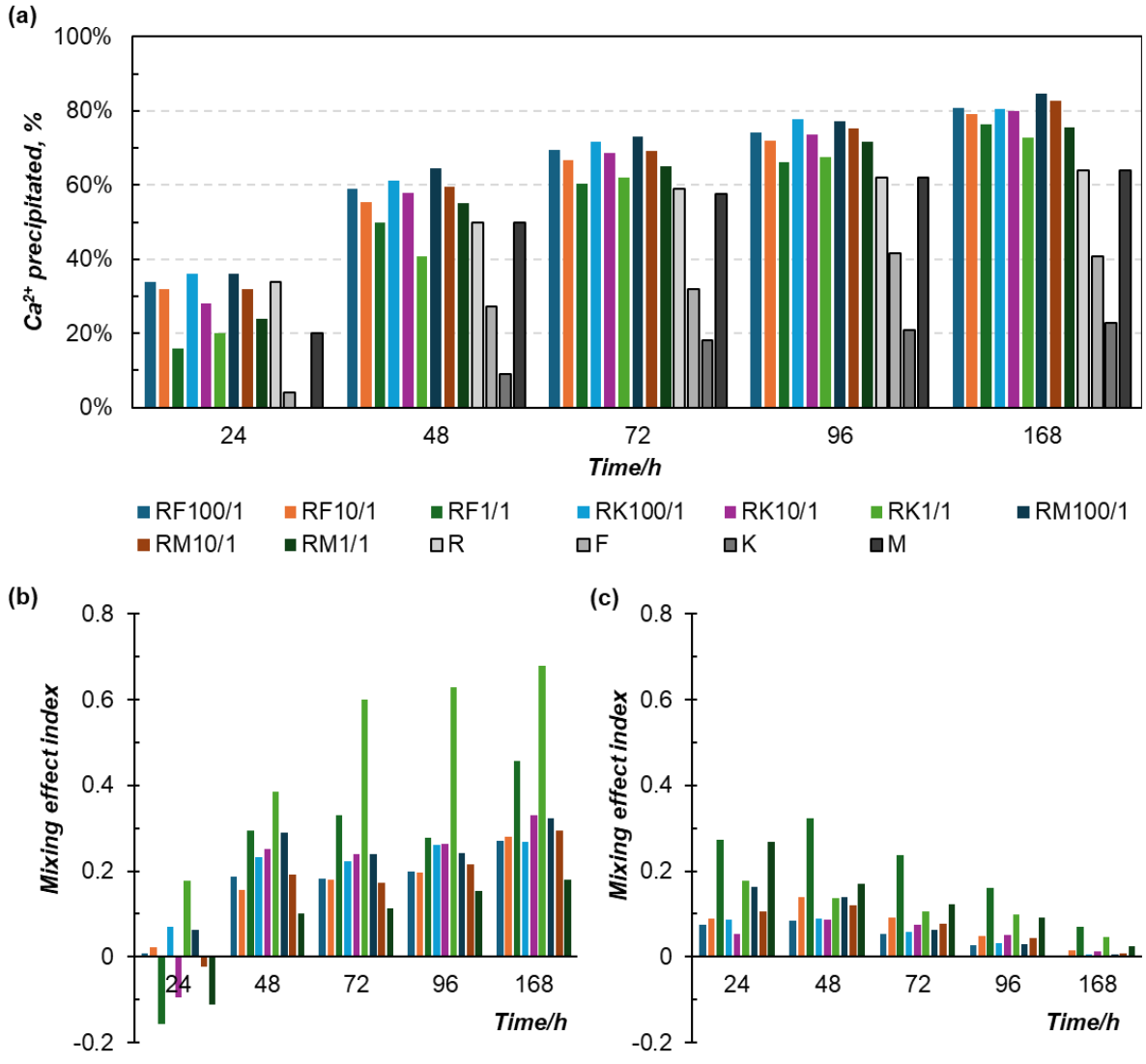

Figure 5. 11 (a) Monitoring of calcium ion concentration changes in precipitation tests with high concentrations of cementation media - a comparison of mono strains with mixed strains; (b) mixing effect of cases of 1 M; (c) mixing effect of cases of 0.5 M

Figure 5.11(a) illustrates the estimated precipitation produced by three pairs of strains mixed with different volume ratios and 4 mono strains in 1.0 M of cementation media. From the results of 24-hour incubation, it can be seen that mixed cases did not exhibit a great difference compared to the optimal mono strain (RII-2). However, most mixed cases surpassed the mono cases from measurement at 48-hour incubation. After 168 hours, most of them reached a precipitation amount more than 80%, which is approximately 20% higher than the best precipitators (RII-2 and MY2-9) from the mono-strain group. Similar to the results in the previous section, the mixing effect index at 1.0 M of cementation media was found to be doubled than the index at 0.5 M of cementation media, as seen in Figure 5.11(b-c). Taken together, the performance of mixed strains in precipitation test at low temperature and high concentrations of cementation media indicates that applying multiple strains can dramatically increase the cementation efficiency, especially when environments are not favorable for bacteria.

5.3.4 Comparison of performance at high HA concentrations

a) Performance of mono strains

Figure 5.12 presents the precipitation curve of four strains. As can be seen, four strains showed a distinct response to the HA addition in precipitation test. Among four strains, *Providencia* sp. (KK7) had a greatly stimulated precipitation rate with a high addition of HA up to 15%. Based on its performance in the pH dependent urease test, it can be inferred that this strain might have a urease regulatory system corresponding to the pH of surroundings. When the pH of the environment is low, it activates the synthesis of urease enzyme to overcome the negative effect of acidity (Cotter and Hill 2003). Although RII-2 has a preference to weak alkaline conditions in previous tests, this strain is able to keep a small urease activity at pH 4. It means that even the acidity might hinder the urease activity, due to the high initial activity, this strain can increase the pH level in a short period and accelerate the precipitation rate after the pH reach to a certain level. On the contrary, if the bacteria fail to overcome the acidity at the initial stage, the bacteria are less likely to precipitate after some time. Take MY2-9 (see Figure 5.12d) as an example, precipitation was completely hindered when the HA addition is up to 10%. How bacteria can cope with acidity determines their survival and metabolic functions in precipitation tests with the presence of HA. It seems that FU17 is a resilient strain that tolerates HA, thus, it can induce carbonate precipitation at a slow but long-lasting precipitation. Studies have shown that bacteria are able to protect themselves from acid using various strategies (Lund, Tramonti, and De Biase 2014). Therefore, the selection of bacterial species is of great importance for the application of MICP on acidic soils. Among the four tested species, RII-2 and KK7 are better choices for stabilization of acidic soils.

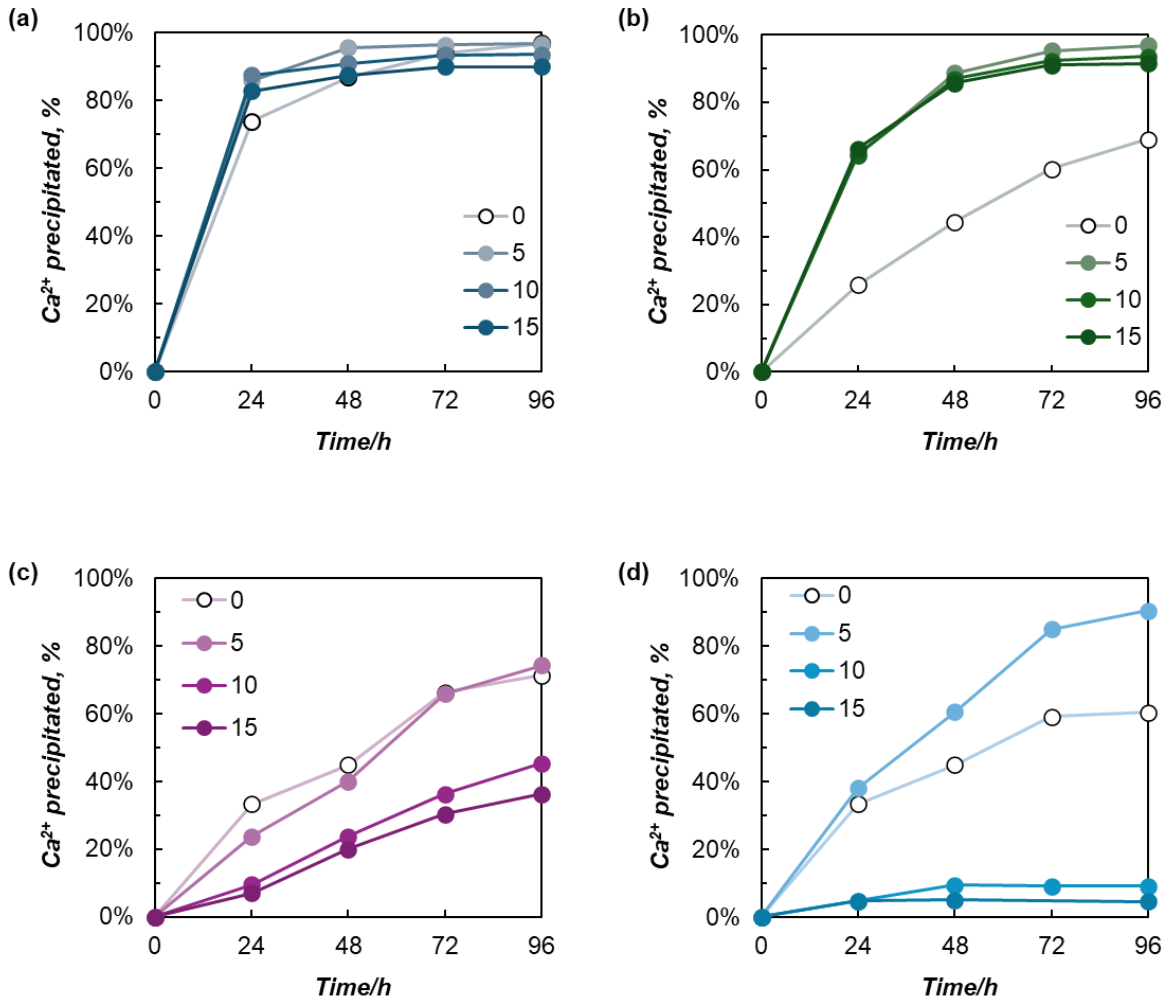

Figure 5. 12 Monitoring of calcium ion concentration changes in precipitation tests with high concentrations of HA-cases of mono strains. (a) RII-2; (b) KK7; (c) FU17; (d) MY2-9

b) Performance of mixed strains

In this experiment, one pair of mixed strains was tested in precipitation test with high concentrations of HA. According to the findings in the previous section, RII-2 and KK7 showed the most tolerance to HA. Therefore, the pairs of two strains with different mixing ratios were examined. Figure 5.13 shows the results of precipitation amount with time. Most mixed strains cases showed a higher precipitation amount than mono strains cases after 48 hours incubation. Taking together, most tested pairs have shown a better adaptation to harsh environments (low temperature, low pH, high conc. of chemicals) than mono strains. Pairs are more likely to overcome the negative effect that hinders the precipitation. Therefore, the application of mixed strains is beneficial to the stabilization of organic soils, or soils in cold regions, contributing to an improved cementation effectiveness.

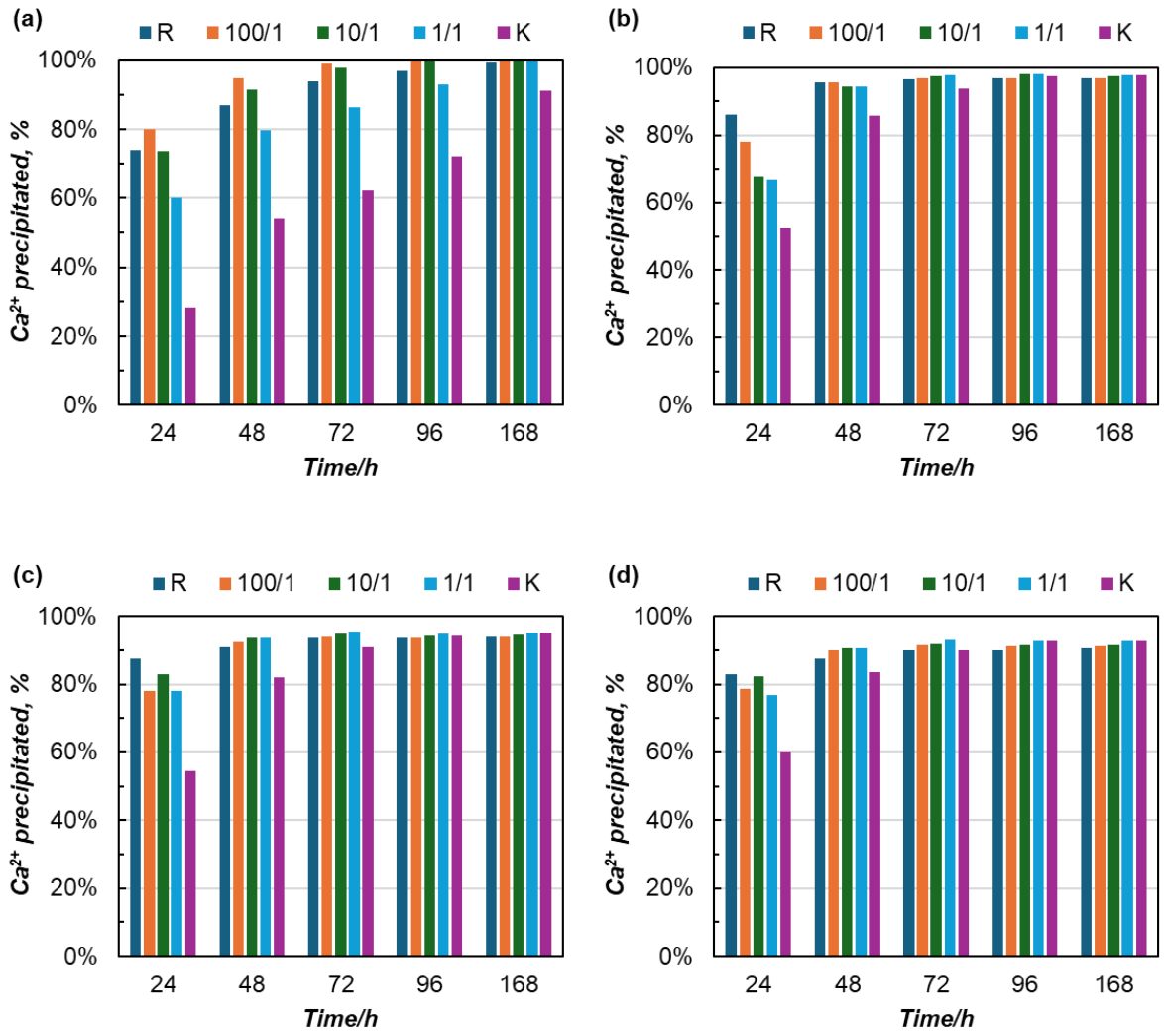

Figure 5. 13 Monitoring of calcium ion concentration changes in precipitation tests with high concentrations of HA-cases of mixed strains RK with (a) 0 %; (b) 5 %; (c) 10 %; (d) 15 % of HA

5.3.5 Calcium carbonate morphology induced by mixed strains

The calcium carbonate morphological features were also discussed in the previous chapters in terms of bacterial difference. As can be seen in Figure 5.14, the carbonate formation was dramatically affected by the mixing of two strains. Most precipitates have both features from two individual strains, and the size usually falls in between. Particular, in all cases with *Glutamicibacter* strain, FU17, the calcium carbonate crystals are apparently larger than other cases. As discussed before, this strain precipitate extra-large size vaterite, probably due to its low activity and some extracellular characteristics. In the observation in this chapter, it seems that the latter factor governs the formation of calcium carbonate, as the precipitation rate was significantly higher than previous mono strain cases. It can be seen that

these large crystals formed an aggregation, which may contribute to a high treatment effectiveness since large crystals generally lead to better cementation. Especially, for coarse sand solidification, the relative size of calcium carbonate precipitates should be large enough to support soil particles (Mujah, Cheng, and Shahin 2019). Further examination of the performance of mixed strains should be conducted to confirm the improvement of cementation effectiveness through mixed strains.

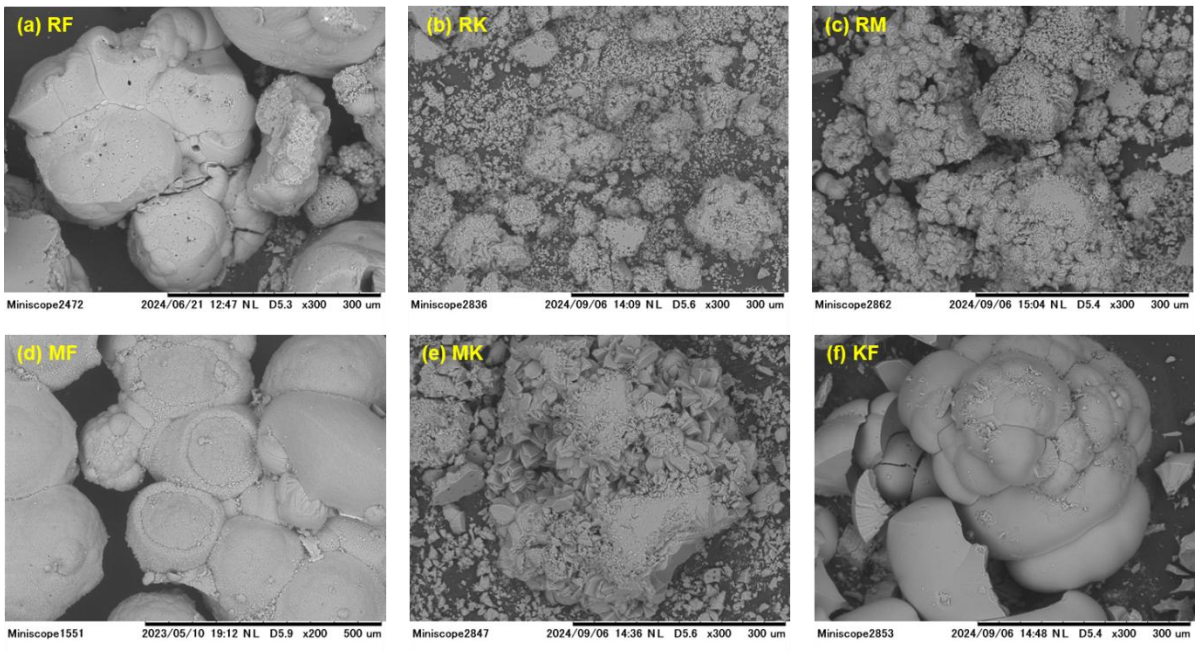

Figure 5. 14 Calcium carbonate induced by mixed strains (a) RII-2 & FU17; (b) RII-2 & KK7; (c) RII-2 & MY2-9; (d) MY2-9& FU17; (e) MY2-9 & KK7; (f) KK7& FU17

5.3.6 Discussion on improvement mechanisms by mixed strains

a) Bacterial roles in different stages of cementation process

Fast precipitators from *Lederbergia* sp. and *Sporosarcina* sp. tend to induce more than 50% of provided calcium source in 24-hour incubation due to their high urease activity, while the precipitation rate of slow precipitators is greatly inhibited by high concentrations of cementation media. These findings have been discussed in the characterization test in the previous chapter. possible explanation is associated with the regulation of bacterial urease. In most cases, urease expression is controlled by urea, nitrogen availability and pH changes (Castro-Alonso et al. 2019). Therefore, mixing bacteria may increase precipitation ratio by sharing the workload in different stages of precipitation process. For example,

triggered by urea, fast precipitators could produce urease in the beginning stage and die in the later stage. With decreasing environmental stress from media and an increasing demand for nutrient, resilient slow precipitators start to produce urease. To confirm this hypothesis, the genetic factors should be studied. Figure 5.15 below depicts a predicted performance of mono strains and mixed strains.

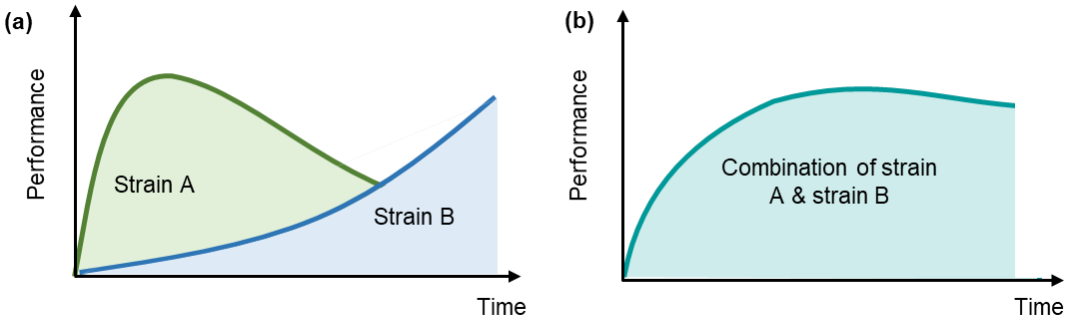

Figure 5. 15 Schematic illustration of performance of (a) mono strains and (b) mixed strains

b) Increase in nucleation sites

Typical ureolytic bacteria with high urease activity are from *Sporosarcina* sp. or *Bacillus* related species, sharing a great similarity in cell morphology. They are usually rod-shaped, with a large cell size ranging from 0.5 × 1.2 μm to 2.5 × 10 μm (Shelef 2003). *Glutamicibacter* sp. is a group closely related to *Arthrobacter* sp. which typically has a rod-coccus growth cycle. The diameter of its cocci can be anywhere between 0.6 and 1.2 μm (Comi and Cantoni 2011). The closest taxonomy of KK7 (*Providencia manganoxydans*) is a novel strain isolated from heavy metal contaminated soils. Its cells are rod-shaped with the dimensions of ~0.5×1.5 μm (Yongchao Li et al. 2019). Generally speaking, bacteria with smaller cell size can make use of nutrients in a more efficient way since they have a relatively large specific surface area than other bacteria with large cells (Madigan et al. 2021). As a result, they usually grow into a large population after reaching stationary phase. In this chapter, selected fast precipitators are relatively large rods, while slow precipitators are small rods or cocci. Based on our previous data of plate counting of bacterial culture prepared in the same condition, their population can be hundreds or thousands of times larger than the other rod-shaped strains. It means that the population of the two strains become equal when the mixing volume of rods is 100 or 1000 times larger than that of the cocci or small rods. As illustrated in Figure 5.16, when the portion of the cocci increases, the overall population also increases.

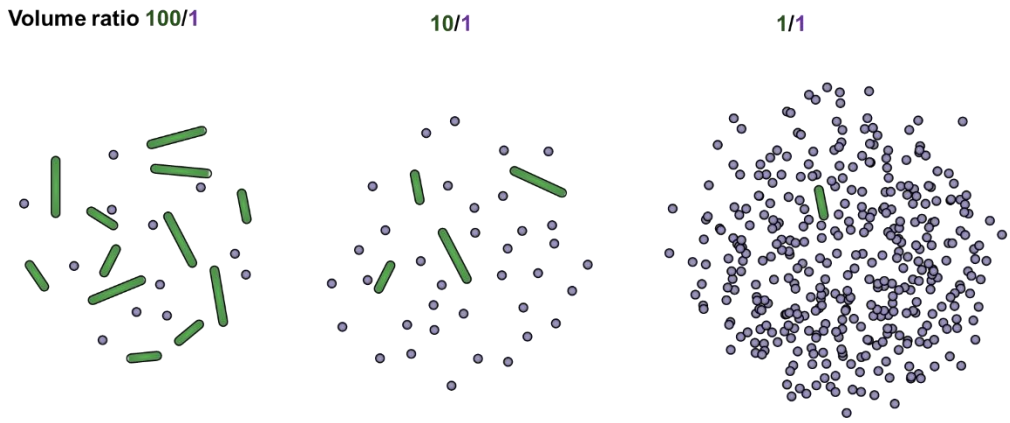

Figure 5. 16 Population differences of rods and cocci in varying mixing ratio

c) Increase of survival rate

Research on microbe consortia has shown that the application of consortium can establish a stable bacteria community leading to long-lasting performance in different fields. Currently, there are still many mysteries regarding the interaction mechanisms of bacteria in a consortium (Goldford et al. 2018). What's more, the storage of a microbe consortium faces certain difficulties since community composition tends to change over time (Massot et al. 2022). All of these problems hinder its broader application. Separate cultivation of bacteria can solve the storage problem, at the same time, forming a consortium like community when mixing together in batch test of calcium carbonate precipitation. In this study, some findings suggest that mixed strains cooperate with each other and contribute to a higher efficiency of precipitation, which probably formed a relatively stable community in the cementation media (Gat et al. 2014; Rajasekar et al. 2024). Existing studies on bacterial consortium revealed that positive interactions occur more frequently when different bacteria were in a hostile environment (Ratzke, Barrere, and Gore 2020). With less nutrient available in the environment, bacteria can make use of metabolites produced by other strains to achieve a "win-win" situation. In a batch test, bacteria provided an environment with pressure from high concentrations of cementation media and great scarcity of nutrients. Figure 5.17 illustrates three basic nutritional interactions: competition, syntrophy, and cross-feeding. It is highly possible that the survival of mixed strains is enhanced by such kinds of cross-feeding behaviors.

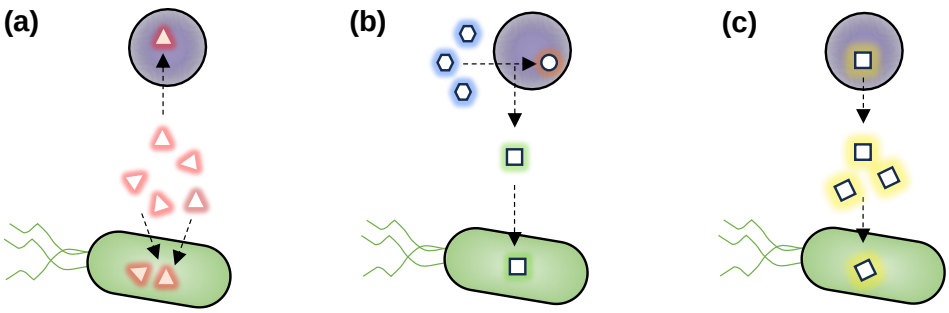

Figure 5. 17 Three basic nutritional interactions: (a) competition; (b) syntrophy; (c) cross-feeding. Modified from Seth et al. (2014).

5.4 Conclusions

In this chapter, selected isolates were mixed with different ratios before examining in the precipitation tests. Several combinations of strains from four genera were evaluated in precipitation tests of normal conditions. Selected pairs with good performance were later tested under less favorable conditions. The findings of this chapter can be summarized as follows.

I. Generally, mixed strains have a better performance than mono strains in the long term. The mixing effect is influenced by the bacterial species, populations, and mixing ratios. With a small portion of slow precipitators added into high activity bacteria, strains with relatively low urease activity, the precipitation rate was significantly enhanced. However, adding high activity strains into slow precipitators did not contribute to the increase in precipitation rate.

II. Pairing of *Lederbergia* sp. with *Glutamicibacter* sp. leads to the most improvement of precipitation efficiency. Selected pairs are able to adapt to less favorable conditions for calcium carbonate precipitation (low temperature, low initial pH, high concentrations of cementation media), inducing more precipitation amounts than mono strains.

III. In the cases of mixed strains, bacteria from different species might play different roles in precipitation process. The precipitation might be improved through increasing effective nucleation sites provided by slow precipitators and enhancing bacterial survival by cross-feeding.

Chapter 6 Influence of humic acid on bacterial performance and effectiveness of biocementation

6.1 Background

Current biocementation research mainly focuses on the utilization of well-known ureolytic bacteria, however, there are some limitations on the application of these bacteria in some unique problematic soils like organic soils because these bacteria generally have a strong preference for alkaline conditions to survive and induce precipitation. Moreover, the temperature dependence of them typically shows a mesophilic pattern which exhibits high activity at high temperatures. Other obstacles in real field applications include the oxygen content, compositions, and local microorganisms' communities of soils. All of these could lead to great differences in the effectiveness of biocementation. On the other side, the role of bacteria in MICP process is frequently investigated in terms of their urease activity, growth patterns, and bacterial population for optimizing the efficiency of treatment. To date, less attention has been paid to the diversity of bacterial characteristics. For example, the functional group on their cell membrane and their metabolites can influence the morphology of calcium carbonate. Additionally, their metabolic activities are actually modifying the environment around them, at same time, their behavior is also shaped by the environment. Many factors related to employed bacteria and soil properties indirectly affect the effectiveness of biocementation, therefore, biocementation still needs to be improved to make the technique more compatible with various soil properties.

The previous stage of this study found that HA disrupts the crystallization of calcium carbonate, and it interacts with different ureolytic strains in a complicated manner (Meiqi Chen et al. 2022). So far, very little is known about how HA affects ureolytic bacteria and what factors should be taken into consideration when selecting strains for MICP application toward organic soils. The purpose of this study is to figure out how HA affects the process of MICP from the perspective of microbe-HA interactions, with a focus on how bacteria growth and bacterial urease activity are influenced by a small HA addition. Results suggested that all three tested strains were stimulated in terms of growth and activity, with two strains showing optimum in a certain amount of addition. For the effect of HA on cementation process, it was revealed that the HA particles can be skeleton to support soil matrix and serve as nucleation sites for carbonate precipitation owing to its great cation exchange capacity. Therefore, HA was proposed as an amendment for improving the effectiveness of biocementation, with many benefits in the treatment process. Overall, some findings in this work help explain what had been observed in the previous stage, and it is hoped that this research will contribute to a better understanding of MICP application in organic soils.

6.2 Materials and methodology

6.2.1 Bacteria cultivation

The HA was added into NH_4-YE media for main culture before autoclave. See other preparations in section 4.2.5 (a) bacterial cultivation.

6.2.2 Tested strains

This experiment tested three strains with different characteristics in the aspect of urease activity. e-4 and PS-1 were isolated from high water content organic soils obtained from Iwamizawa, Hokkaido. Ly. xy was identified in slope sandy soils and its feasibility of MICP application has been investigated before (Gowthaman et al. 2019). The table below presents some basic information about three strains.

Table 6. 1 Basic characteristics of three strains

Strain ID	Source	Closest taxon	Cells	Temp. range	Peak U.A.
e-4	organic soil[a]	*Sporosarcina* sp.	Rods	Mesophile	5 U/mL
PS-1		*Staphylococcus edaphicus*	Cocci	Mesophile	0.8 U/mL
Ly. xy	sandy soil[b]	*Lysinibacillus xylanilyticus*	Rods	Mesophile	1.5 U/mL

[a] Organic soils with 800% water content from Iwamizawa, Hokkaido; [b] Sandy soils from highway slope in Onuma, Hokkaido.

6.2.3 Quantitative measurements

a) Plate counting

Plate count or viable count is a growth-based method to quantify bacteria population. Compared to other common measurements such as total cell count and turbidimetric measures that count both living and dead cells, this method could produce a more accurate result when it aims to investigate the viability of bacteria since only cells that are alive and able to grow are counted. In this method, bacteria culture is spread on an agar plate after serial dilutions, and after incubation, single colonies on plates are counted.

Plates with too many or too few colonies are removed and only plates with 30-300 colonies are considered valid statistics.

b) Electrical conductivity measurement for determination of urease activity

See procedures in section 4.2.2.

6.2.4 SEM analysis

See procedures in section 4.2.8.

6.3 Results and discussion

6.3.1 Effects of HA on bacterial growth

In general, it is believed that the presence of humic substances has a double side effect on bacterial growth and activity, both stimulation and inhibition (Yang, Tang, and Antonietti 2021). In this experiment, with a small addition (0.1, 0.5, 1.0, 1.5%) of HA added into the culture medium, the growth of three species was stimulated to different degrees. Figure 6.1 shows the results of CFU/mL of cases with 0-1.5% HA addition. Tikhonov et al. (2010) reported that HA might affect bacterial reproduction at early stages. Therefore, the bacterial population was confirmed by plate counting after 24-h or 48-h incubation (depending on bacterial growth). It can be seen that two isolates from organic soils exhibit a significant increase in bacteria population. In the case of e-4, the population of 1.5% addition case was 5 times higher than that of control case, and now optimum HA addition was found in added range. An optimum was observed in the case of PS-1 which showed a highest population (approximately 20 times higher) with 1% HA addition. For the strain from sandy soil, Ly.xy, was also stimulated to some degree, while it is much less affected compared to other two strains. To test strains in this study, the HA might be an indirect external energy and carbon source for them (Lipczynska-Kochany 2018).

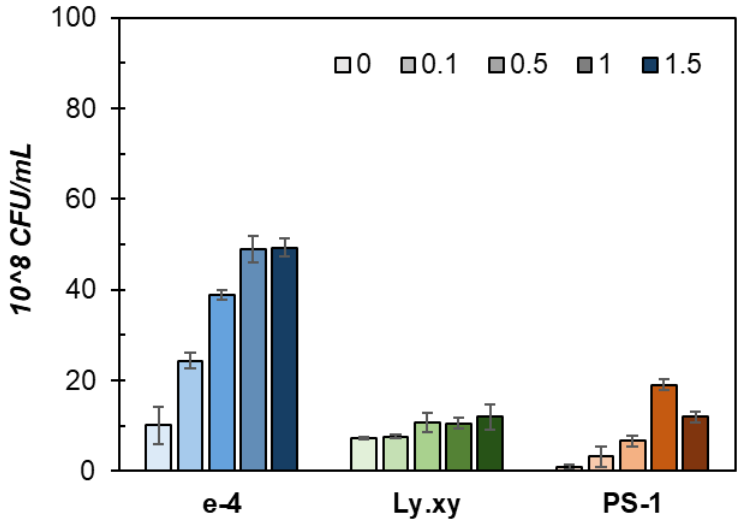

Figure 6. 1 Comparison of bacterial populations in culture media with 0.1, 0.5, 1.0, 1.5 % of HA as additive

6.3.2 Effects of HA on urease activity

Many studies have investigated the complex effect of HA on metal ions and enzymes for its application in a wide range, most of which focused on its inhibition effect when enzymes are involved. According to Yan Li et al. (2013), a small addition of HA enhanced the stability of plant-derived urease enzymes under certain conditions. However, effects of HA on bacterial urease activity are under research, thus it is needed to broaden the understanding of MICP application for organic soils rich in humic substances. This experiment started with a small HA addition, ranging from 0.1 to 1.5%, into culture media. From the results shown in Figure 6.2(a), we can see that e-4 had an optimal performance with a 0.1% addition of HA. And the other two strains' activity presented an increasing tendency with increasing addition of HA (see in Figure 6.2b&c).

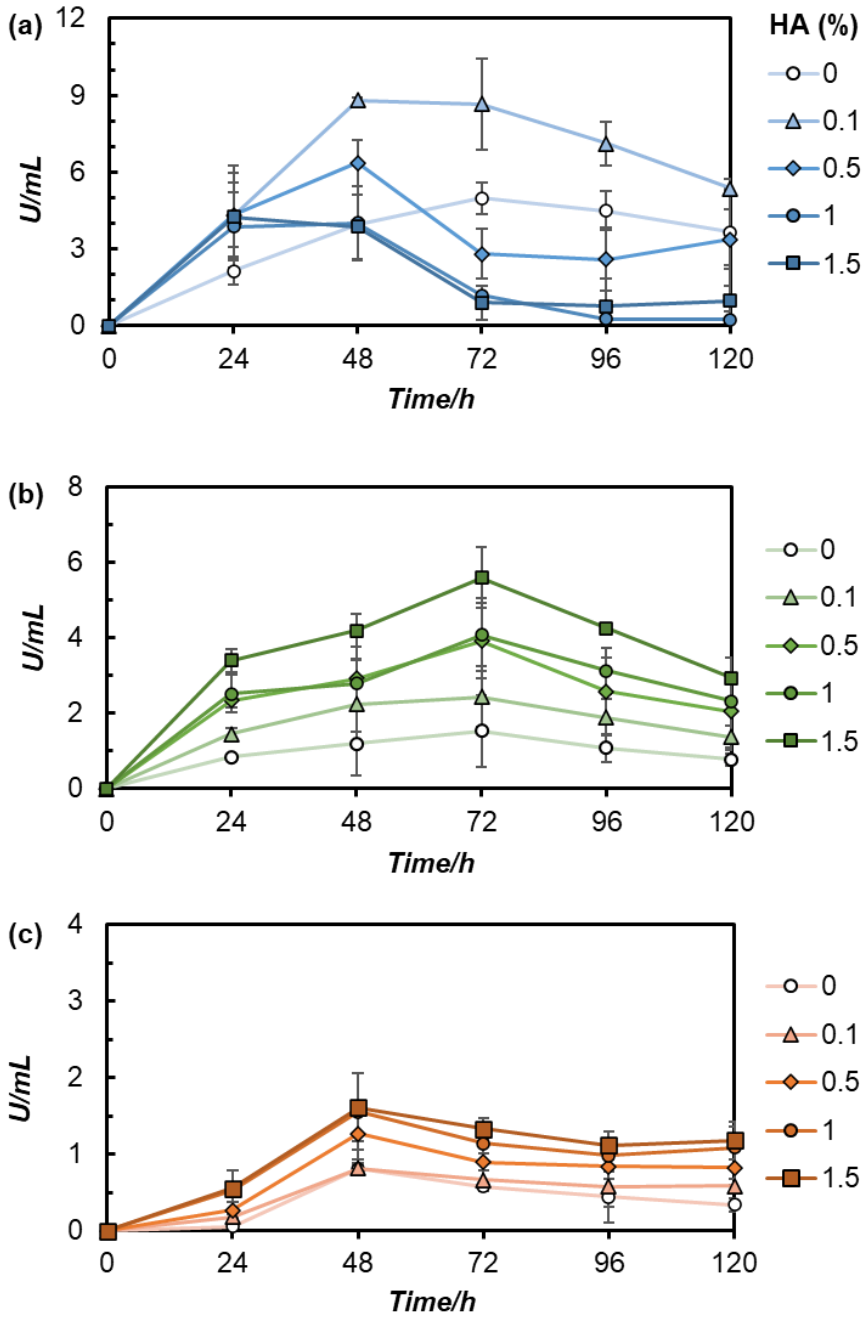

Figure 6. 2 Urease activity of three tested strains cultivated with 0, 0.1, 0.5, 1, 1.5% of HA addition: (a) e-4; (b) Ly.xy; (c) PS-1. Duplicate samples were prepared for each case

Most ureolytic bacteria tend to excrete small quantities of urease in an environment without significant substrate existing, leading to a small extracellular urease activity (Castro-Alonso et al. 2019). In this experiment, to figure out whether HA affects extracellular activity or not, the optimum cases of each tested strain were centrifuged, and the activity of the supernatant was determined at the same time as the total activity was tested. It was assumed that the total urease activity is the sum of intracellular and

extracellular activity. From the results shown in Figure 6.3, it seems that the HA addition has little effect on the production of extracellular urease in the bacterial culture process. The difference in extracellular urease ratio between HA-added cases and control cases is neglectable. On the other hand, three tested strains had quite distinguished extracellular urease ratio. PS-1 showed an extracellular activity of approximately 20%, which is the highest among the three strains. Ly. xy, in contrast, had the least extracellular activity ratio, about 3%. This ratio fell in between the former two strains in the case of e-4. This finding might give a reasonable explanation of the previous study on the influence of HA on MICP. Details are discussed in the next section.

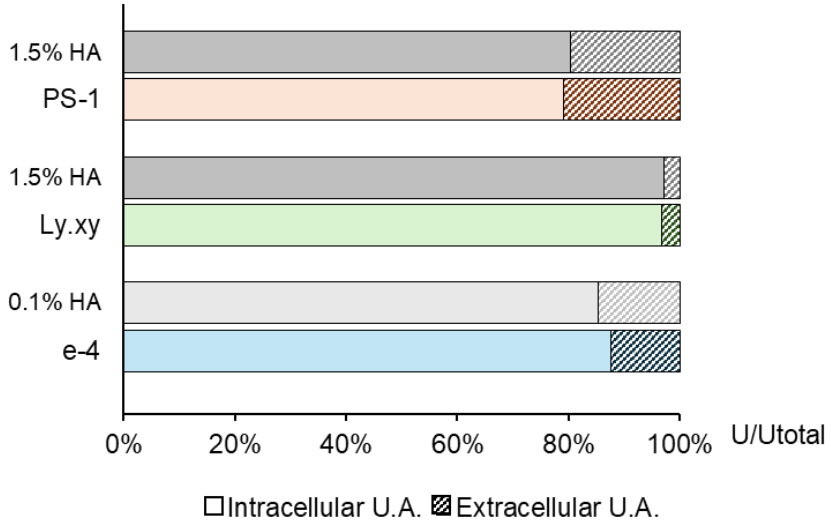

Figure 6. 3 Extracellular urease activity of three tested strains: a comparison between control cases and corresponding optimum cases

6.3.3 Discussion on effects of HA on bacteria

Figure 6.4 compares the stimulation effects of HA on three strains in the aspect of urease activity and growth. In the case of e-4, doubled activities were shown in 4% HA-added cases. However, cases with a relatively high addition tend to show an inhibited behavior after 48-h incubation, while a small addition of 0.1% could maintain the activity at a high level. More analysis is needed to figure out the mechanism. Ly. xy had a less complicated behavior, showing a similar tendency of stimulated activity with time. The growth of PS-1 was most greatly accelerated at first 24-hour cultivation, which might explain the dramatically increasing activity. But this effect decreased during the following cultivation hours. In terms of bacterial growth, Ly.xy was less affected and the other two strains were facilitated to

different degrees, indicating native species might much prefer HA in their habitat than species from the soil with less organic matter.

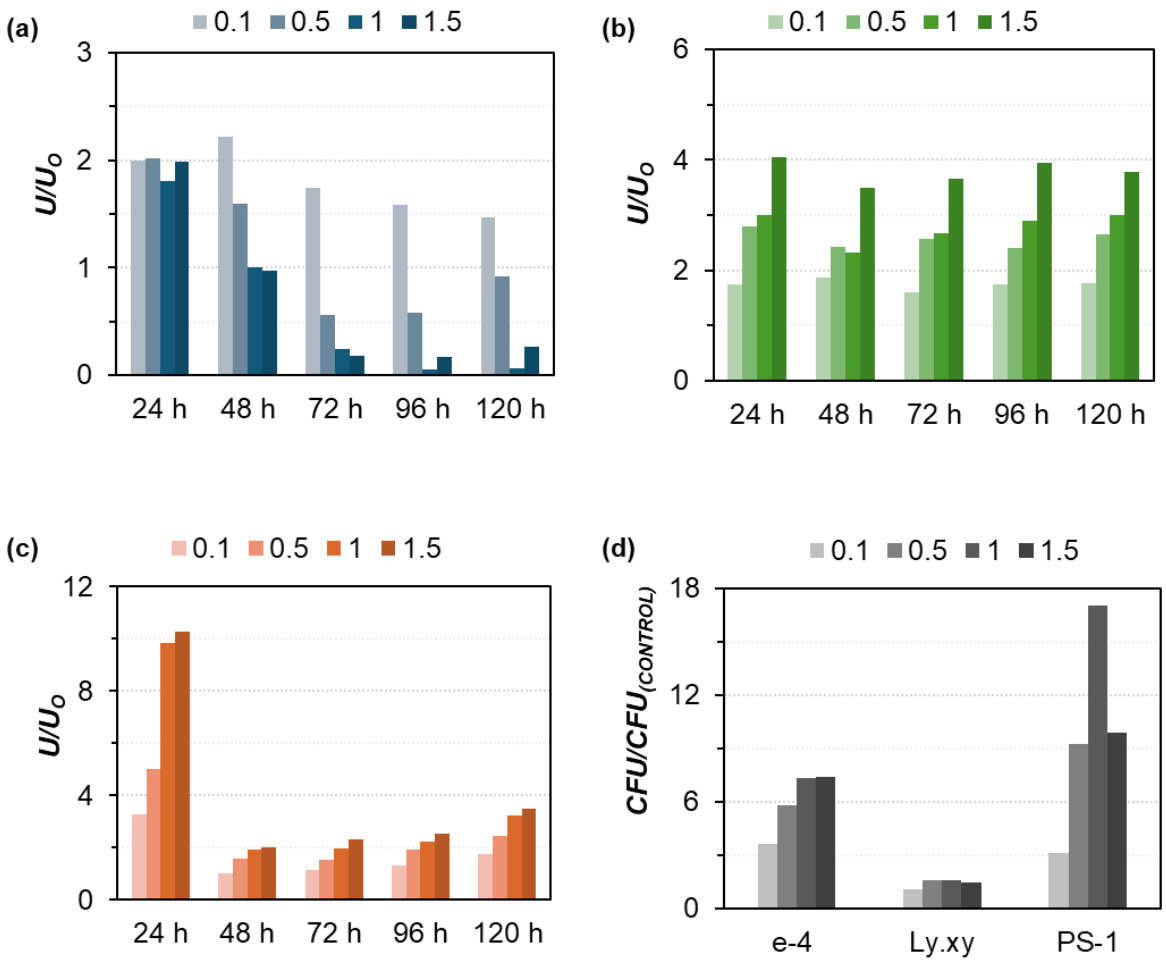

Figure 6. 4 Normalized effect of HA on bacteria (a) U.A. increase ratio of e-4; (b) U.A. increase ratio of Ly. xy; and (c) U.A. increase ratio of PS-1; (d) growth increase ratio of three strains

In the previous stage of this study, the influence of HA on calcium carbonate precipitation was investigated, mainly focusing on the calcium carbonate precipitation rate and morphology changes of carbonate precipitates. The results demonstrated that native strains from organic soils tend to have more tolerance to HA. It was difficult to give a clear explanation of those findings. With results from this study, it can be inferred that two strains, e-4 and PS-1, might have a longer survival period than Ly.xy in cementation media, leading to a higher carbonate precipitation rate. On the other hand, if those free

enzymes excreted from bacterial cells could be stabilized by appropriate additions of HA (Yan Li et al. 2013), the urease can still function properly even after most bacteria died off from starvation. By this inference, bacteria with relatively high extracellular urease activity and greatly HA-stimulated growth are more suitable for the MICP technique when stabilizing organic soils. To confirm it, a further experiment is designed to investigate the effects of varying HA addition on urease extracted from bacteria cells, which would be another challenging task since bacterial urease enzymes usually exhibit different structures and characteristics (Kappaun et al. 2018). Generally speaking, as a continuation of previous study, it further confirmed the importance of bacterial selection when considering their viability in real conditions.

6.3.4 Humic acid as amendment to improve MICP efficiency

A previous study on the influence of HA on plant-derived urease revealed that urease undergoes some conformational changes in an acidic environment and does not function normally. However, with pH increasing, the presence of HA particles tends to enhance the stability of urease by reducing the collision of enzyme proteins (Yan Li et al. 2022). Research in the agricultural field also revealed some negative effects of HA on urease activity, particularly, a blocked active site of urease enzyme was found when interacting with HA (X. Liu et al. 2019). During the cementation process, the pH showed a certain level of fluctuation, which means the effect of HA is also changeable. On the one hand, when the initial soil sample was buffered by a proper amount of HA, enzyme activity was partially inhibited. As the pH increased with ureolysis, urease enzymes showed an increase in stability and activity. In this case, the formation of calcium carbonate was partially hindered but not completely inhibited. On the other hand, when the HA addition is up to a certain amount, the urease enzyme usually does not function well, and the buffering capacity of HA makes the precipitation hard to occur. A possible mechanism was illustrated in Figure 6.5 based on existing studies on HA and urease (Li et al. 2013; De Melo, Motta, and Santana 2016; Adusei-Gyamfi et al. 2019; Li et al. 2022). Under acidic conditions, HA has fewer binding sites for metal ions, but in alkaline conditions, more binding sites create more complexation opportunities. However, this complexation is usually weak when common metal ions like calcium ions are bonded, which means the complexation does not significantly affect the occurrence of carbonate precipitation (Meiqi Chen et al. 2022). In this case, HA in the cementation process could serve as an aggregation site for calcium ions, probably leading to a more efficient formation of calcium carbonate (see Figure 6.5c). As mentioned in the previous sections, it has been found that small addition of HA affects the bacterial growth and bacteria urease activity positively in cultivation stage. The following sections discuss the influence of HA on biocementation process.

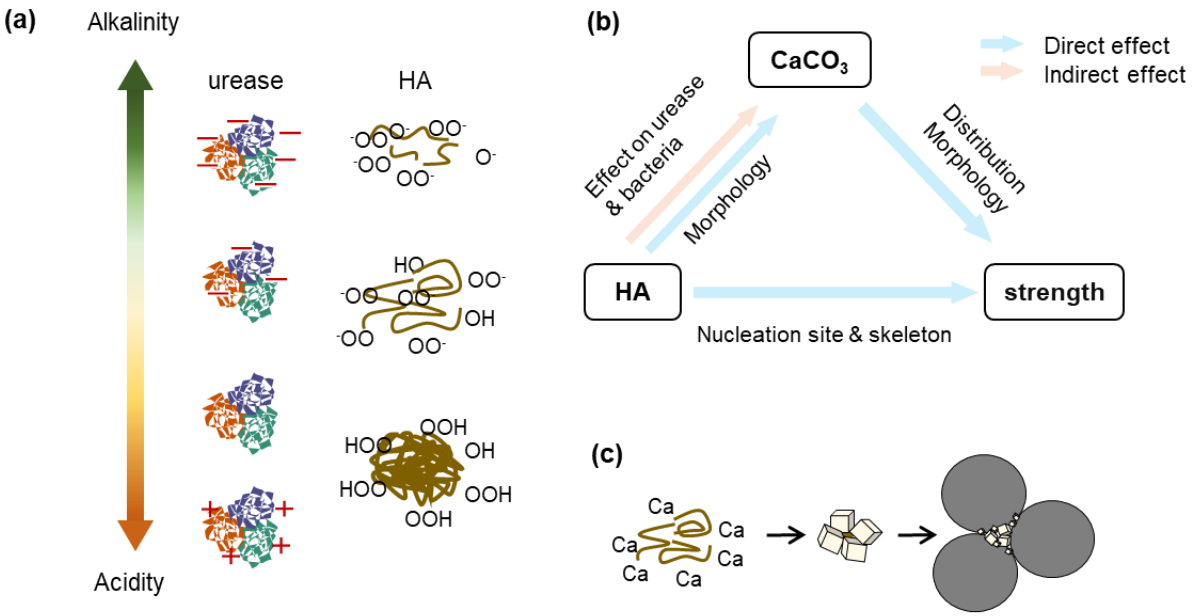

Figure 6. 5 (a) Comparison of general charge state of urease and HA under different pH level; (b) direct and indirect influence of HA on cementation effectiveness; (c) nucleation effect of HA. Modified from M. Chen et al. (2024)

(a) Effect of HA on calcium carbonate morphology

Calcium carbonate has three polymorphs, among which calcite is the most stable form, followed by aragonite and vaterite. Considering the long-term performance, the most stable form is preferred for biocementation applications. In our previous studies, HA has been found to direct influence the calcium carbonate morphology induced by bacteria, while the effect differs across species to species (Meiqi Chen et al. 2022). With small addition in the cementation media, precipitates tend to agglomerate to form a large aggregate. With the addition increasing, the crystallization is severely disrupted by HA, forming more amorphous calcium carbonate. Figure 6.6 shows the calcium carbonate formed by four selected strains in the presence of HA from previous chapters. Combined with previous findings, one similarity was found between cases of PS-1 (*Staphylococcus* sp.) and FU17 (*Glutamicibacter* sp.), which is that both bacteria were able to induce carbonate precipitation, not hindered by high addition of HA. Compared to other tested strains, these two strains are smaller in cell size, larger in bacteria populations, and are featured as strains that form aggregates frequently in bacterial cultures.

It should be mentioned that the HA also presents in the extracellular polymeric substances of bacteria cells, and it gives bacteria the ability to bind with cations (Shi et al. 2017). A possible explanation for the findings mentioned above is that the bacterial aggregates produce extracellular polymeric substances

that have a higher affinity to cation binding than the HA has. Therefore, instead of precipitating around nanoparticles of HA, more precipitation occurs surrounding bacterial aggregate.

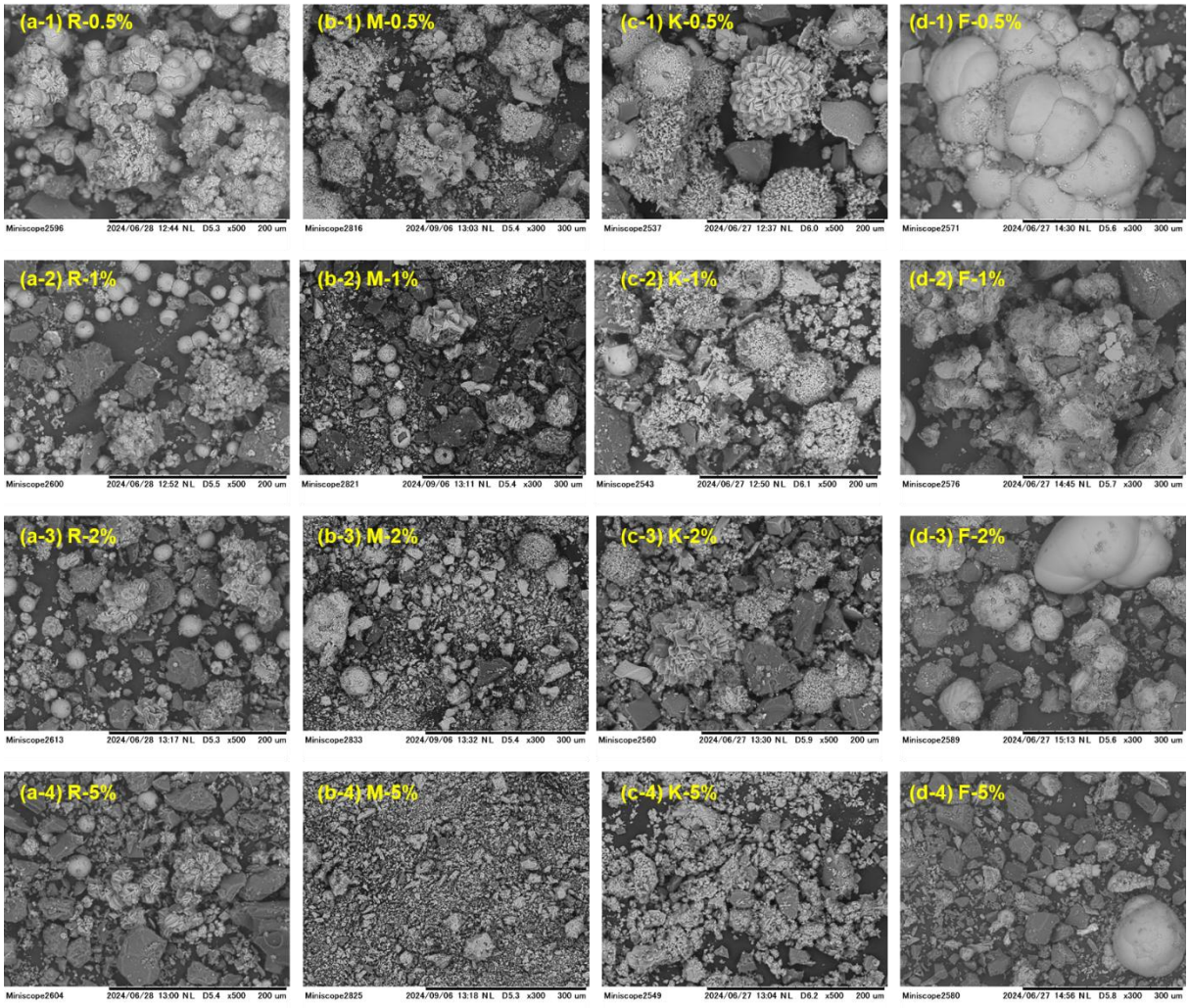

Figure 6. 6 SEM images of calcium carbonate induced by four strains in the presence of 0.5-5% of HA in precipitation test (a) RII-2; (b) MY2-9; (c) KK7; (d) FU17 (magnification: × 300)

(b) HA as nucleation sites and skeleton of soil matrix

At a macroscale level, with certain hardness, HA particles can be skeletons in soil matrix. Based on previous microscale observation, about 80% of HA particles have a diameter in the range of 20-150 μm, of which the average diameter (65 μm) has a similar grain size with silt composition, filling the voids in soil matrix. On the other hand, calcium ions can bridge HA molecules when complexing with HA, which further contributes to effective cementation since the aggregates of HA-Ca serve as nucleation sites. This means HA particles create high efficiency bonding between soil particles. Evidence can be observed in precipitates obtained from precipitation tests. Figure 6.7 depicts many calcium carbonate crystals surrounding the HA particles found in liquid precipitation tests of previous chapters. In one of our recent studies on the effects of HA on EICP, calcium carbonate precipitates were found filling the voids of clay structures in the soil. Calcium carbonate precipitates are indicated in yellow circles in Figure 6.8.

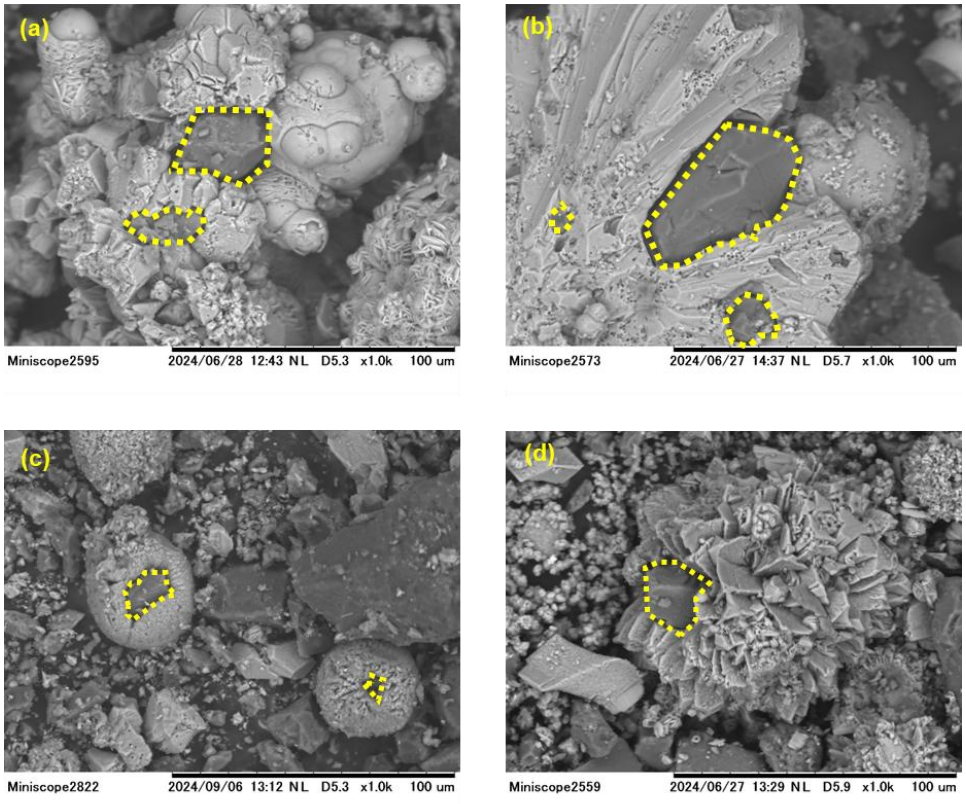

Figure 6. 7 SEM images of calcium carbonate forming surrounding HA particle in precipitation test. (a) RII-2 with 0.5 % HA; (b) FU17 with 0.5 % HA; (c) MY2-9 with 1 % HA; (d) KK7 with 2 % HA

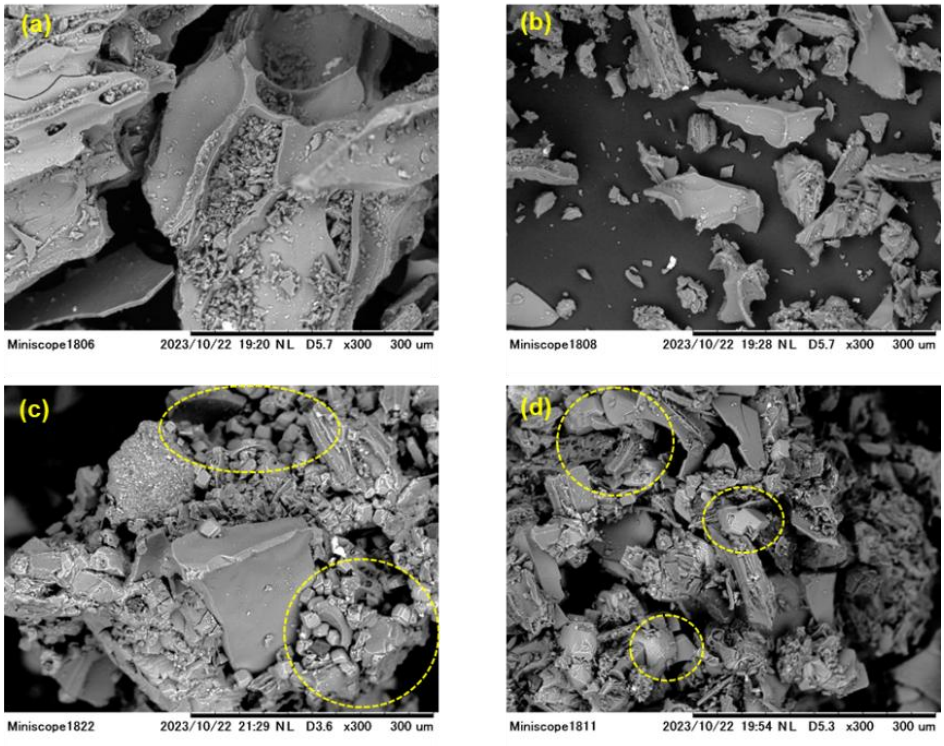

Figure 6. 8 SEM images of (a-b) untreated Wattsu soil; (c) soil with 4 % HA treated with e-4 urease; (d) soil with 4 % HA treated with Ly. xy urease. Modified from M. Chen et al. (2024)

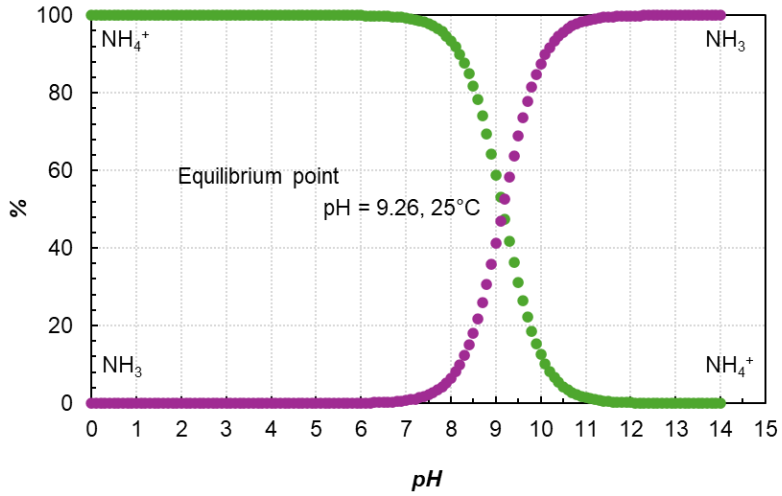

Figure 6. 9 Ammoniacal speciation in function of pH

(c) Buffering capacity of HA in the treatment process

As mentioned in the literature review, one challenge of particular concern in MICP research is that the undesired by-product, ammonia or ammonium, is harmful to ecosystems. Many studies have been working on this issue, and the posttreatment strategy now is attracting more and more interest. To minimize the release of ammonia during the treatment, measurements are necessary. Figure 6.9 presents the relationship between chemical speciation of NH_4^+/NH_3 with pH. In an acidic condition, most ammonium is dominated in the solution. When the pH goes up to an alkaline range, the portion of ammonia begins to increase with an increase in pH. The equilibrium point reaches at pH 9.26 at 25 °C. In most MICP studies using high activity bacteria (e.g., *S. pasteurii*), resulting from rapid hydrolysis of urea, the pH level increases fast and a large portion of ammonia escapes from cementation solutions during the treatment process.

Natural soils generally have a weak acidic property due to the composition of humic substances which serve as a buffer in the soil matrix. HA as a fraction of humic substances, has a great buffering capacity. Our previous findings have shown that with the presence of HA, the pH increase was much smaller than the blank cases. Figure 6.10 compares the relationship between pH and estimated calcium carbonate precipitation amount in cases with varying HA addition. Firstly, from the cases without HA addition (see Figure 6.10a), it can be seen that cases of four strains differ in the pH even when the calcium carbonate precipitation amount is similar. For example, when 75 % of the calcium ions precipitated, the pH of RII-2 cases reached more than 7.5, followed by FU17, KK7, and MY2-9. It suggests that these bacteria have modified the environment in some ways. The ability of bacteria to modify the environment surrounding them has been investigated by many studies. Ratzke and Gore (2018) had revealed that bacteria manipulate the pH environment which leads to their survival or extinction, and pH buffer can greatly reduce the negative effect of this kind of manipulation. It also explains the enhanced bacterial growth observed in the culture media with HA as an additive.

For the cases with HA, there is a clear trend that the peak pH of each case is gradually decreasing with the increase in HA addition. With precipitation almost fully completed, the peak pH was about 7.2 in the case with 5 % HA, and it decreased to 6.8 when the HA addition is up to 15 % (see Figure 6.10 b-d). In solution of pH 6.8, more than 99.5 % are ammonium ions, which greatly reduces the emission of ammonia gas. In the field of agriculture, the addition of HA was used to reduce the ammonia loss from soils (Ahmed, Aminuddin, and Husni 2006). To achieve a more ecofriendly MICP treatment, the ammonium should be kept in the soil before posttreatment is conducted. It should be mentioned that too much HA can lead to failure of treatment, therefore, the amount of HA should be adjusted according to the corresponding performance of employed bacteria.

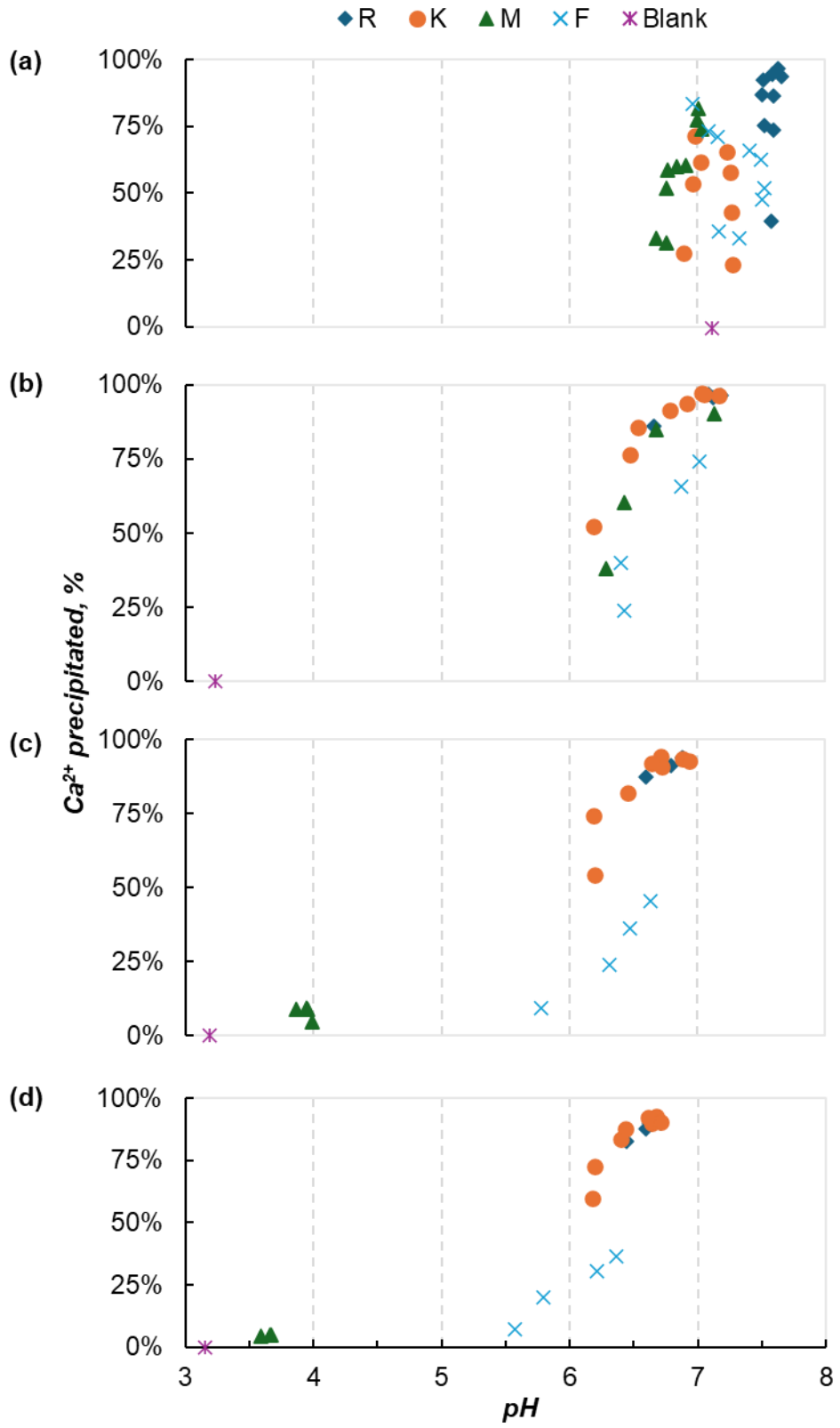

Figure 6. 10 Relationship between pH and estimated calcium carbonate precipitation amount with (a) 0 % HA; (b) 5 % HA; (c) 10 % HA; (d) 15 % HA in liquid precipitation tests

(d) Bacterial survivability in cementation media with HA

The results in previous sections have found that the addition of HA in culture media can significantly enhance the growth and urease activity of three tested bacteria. How it influences the bacteria in cementation media is still unclear. In the previous chapter, several strains were examined in the precipitation test with varying addition of HA. How many bacterial cells remained viable after 168-hour incubation was evaluated by plate counting. Figure 6.11 presents the survival of two strains in tests with HA addition up to 15 %. KK7 was characterized as an acidic tolerant species in previous characterization tests. In this result, the survival of KK7 in cementation media after 168 hours was about 10%, which was greatly enhanced by HA. In the case of RII-2, instead of dying with time, the bacterial population seems to be increasing after incubation. Existing studies have confirmed that many soilborne bacteria can utilize HA as energy sources. It is very likely that this strain might be able to make use of HA to reproduce in the cementation media. Ideally, this strain can be employed to stabilize organic soils or other sediments rich in organic matters, as its applicability has been proved in sand column solidification tests of previous chapter.

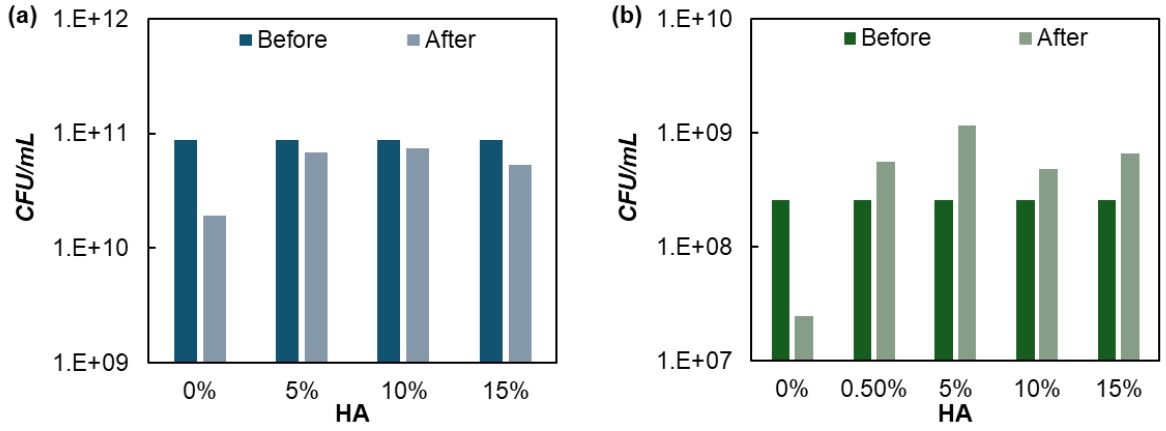

Figure 6. 11 Bacterial survival after 168-hour precipitation test-a comparison of before and after the test. (a) KK7; (b) RII-2

6.3.5 Recommendations for further investigation

Taken together, our studies have revealed that HA can have positive effects on MICP effectiveness. In terms of bacterial growth and survival in cementation media, HA is a potential energy source, and as a buffer, it can enhance the survival of bacteria to some extent. For direct improvements, HA particles can be skeletons in soil matrix and serve as nucleation sites to facilitate the precipitation of calcium

carbonate. It buffering capacity also greatly contributes to a minimized generation of ammonia gas. Therefore, we propose that HA can be a useful amendment for MICP improvements. In further investigation, the effect of HA should be evaluated in the soil solidification test. More broadly, some findings provided us with new solutions to other biocementation applications. For instance, for surface treatment to control fugitives, amendment of HA to soil surfaces can greatly increase cementation efficiency since the purpose is to cement the top layer of soil. HA has many advantages as an additive for geotechnique. Moreover, the emission of undesirable byproduct, ammonia, can be suppressed by HA since it has buffering capacity. Considering the effect of treatment in the long term, the amendment of HA can increase the water-holding capacity of the soil and probably improve the vegetation condition of barren land in the future.

In summary, HA has several applications in MICP technique. Firstly, it can be an additive into culture media, which enhances bacteria growth and activity. For treatment process, HA can be mixed into inorganic soils to improve the cementation effectiveness, meanwhile, minimize the emission of ammonia into atmosphere. Widely used in agriculture, it is a mature commercial product that is available everywhere. The addition of HA can also increase the cost efficiency of MICP technique.

6.4 Conclusions

In this chapter, the influence of HA was investigated in terms of the effects on bacterial growth, urease activity, and direct effects on biocementation process. The findings in this study provide insights into the improvement of organic soils by MICP technique and have significant implications for the understanding of how to make use of HA as amendment for improvement of inorganic soil through biocementation. The following conclusions can be drawn.

I. Small addition of HA can greatly increase the bacterial growth rate at the beginning stage of cultivation, meanwhile, increase the urease activity. With the presence of HA in cementation media, the survival of bacteria was significantly increased, which partially explains the long-lasting precipitation observed in previous tests.

II. During the biocementation process, HA can serve as skeletons for soil matrix and nucleation sites for calcium carbonate precipitation and buffer the cementation media to maintain a weak acidic level, minimizing the risk of ammonia emission. Therefore, HA has the potential as an amendment for MICP technique aiming for various application purposes.

Chapter 7 Conclusions and recommendations

7.1 Summary of conclusions

This dissertation presents the investigation of airborne bacteria for MICP applications. The main objective of this research is to explore the occurrence of ureolytic airborne bacteria in Japan, to evaluate the feasibility of applying airborne bacteria for biocementation, and to develop methods for the improvement of biocementation towards a more compatible and versatile technique.

This work mainly consists of five parts.

(i) Chapter 2 presents a literature review on challenges of MICP technology from three aspects: i) raw materials used in the treatment; ii) Compatibility of target soil and durability of treated soil; iii) Regulatory issue, survivability and adaptability of bacteria. Seeking for a solution to the challenges related to the utilization of bacteria, the potential of airborne bacteria for biocementation applications was investigated.

(ii) Chapter 3 describes the sampling of airborne bacteria from different climate zones in Japan, discussed the occurrence of ureolytic bacteria in different sampling sites. After several rounds of screening, selected candidates from numerous isolates were identified by gene sequencing, revealing a high similarity in taxonomy between samples from different sites. In this study, ureolytic bacteria were identified according to the results of a simple urease test. Furthermore, urease-positive percentage changes when different culture media were used for sampling. Therefore, it should be noted that the ureolytic bacteria discussed in this study are subject to certain limitations. Overall, the urease-positive percentage identified in air samples from different areas is approximately 10-20% of tested colonies. Samples collected from sites with dense vegetation tend to have more colonies than samples from open land, indicating that plants might be a major source of airborne bacteria. The gene identification of selected strains found that about half of them are spore-formers, which suggests that these isolates might drift in the air as a dormant form instead of a living cell. Others are mostly from the same family, concluding many species that are ubiquitous in environments.

(iii) Chapter 4 demonstrates the basic characteristics of some selected strains, including their temperature dependence, pH dependence, adaptability to cementation media with varying concentration and varying calcium sources, and performance with the presence of humic acid in the precipitation test. Some isolates from different climate zones showed regional features. For example, one isolate from the Okinawa sample was found not able to grow under 20 °C, and one Sporosarcina strain from Sapporo has optimal growth under 20 °C but no growth when the culture temperature is up to 35 °C. In the characterization tests, typical ureolytic bacteria from *Sporosarcina* or *Bacillus* genus exhibited an

excellent precipitation rate, but their survivability is rather disappointing. What's interesting is that most of the non-spore forming strains exhibited an extradentary tolerance to harsh conditions in cementation media. At the end, two candidates were examined in sand solidification tests, suggesting a high applicability compared to existing studies. Generally speaking, some selected strains have great potential in MICP applications, and to make full use of their potential, more efforts are needed to further improve the efficiency of precipitation.

(iv) Chapter 5 investigates improvement of precipitation efficiency under changeable conditions by mixing two strains from different genera. After trying different mixing methods and mixing ratios for the mixed strains, selected pairs were then examined in a series of precipitation tests. The tests were conducted under low temperature, high concentrations of cementation media, and high concentrations of humic acid. It was found that mixed strains exhibited a higher efficiency of carbonate precipitation than the mono strains did, in particular, when the environment was not favorable for their survival. Pairing of different species differs in precipitation efficiency. Among the tested pairs, the best pair in our observation is the combination of *Lederbergia* sp. with *Glutamicibacter* sp., while the mechanism of their interaction in the precipitation process is not elucidated. Further investigation is needed to gain a more comprehensive understanding of bacterial interactions.

(v) Chapter 6 demonstrates the influence of HA on bacterial growth, urease activity, and cementation process. With a small addition of HA in the culture media, bacterial reproduction was greatly stimulated, as well as the bacterial urease activity. In the precipitation test, HA particles can serve as nucleation sites for calcium carbonate formation, at the same time, enhancing the survival of bacteria in the long term. Combing the findings in previous chapters, the potential of HA as amendment for improving the cementation effectiveness and reducing ammonia emissions was discussed. Considering its wide application in the field of agriculture, HA can be a helpful and cost-effective amendment for MICP technology.

This chapter summarizes the conclusions of each part of the dissertation and provides recommendations for further investigation into this topic.

7.2 Guideline for applications

Bacteria with great diversity were isolated from air samples collected from different climate zones in Japan. They were found to be versatile in many aspects for engineering applications. Based on the findings, the application guidelines are briefly outlined for a more effective and versatile MICP technique.

i) Selection of bacteria according to regional features

To achieve a high efficiency treatment, the environmental factors should be taken into consideration. For instance, Hokkaido, as a typical cold region in Japan, its temperature requires specific bacteria that could work in a relatively low temperature. Generally, urease activity of mesophilic bacteria decreases with a decrease in temperature. To improve the time efficiency of treatment, one general strategy is to employ ureolytic bacteria with cold tolerance. According to our findings in Chapter 5, mixed strains can greatly enhance the precipitation rate at low temperatures. Therefore, mixed strains can be an option for cold region applications. In particular, mixed strains are a better option for soils with unique chemical and physical properties.

ii) Stabilization of organic soils through biocementation

Several strains identified in this study have a high tolerance to HA, which suggests that these strains might be appropriate for organic soils stabilization. For instance, RII-2 has a high urease activity in pH range of 4-9 (preference to weak alkalinity), temperature range of 20-35 °C (preference to relatively high temp.), while it can tolerate HA addition up to 15 %. Moreover, the addition of HA in cementation media enhanced the survival and reproduction of the bacteria. Another strain exhibited a higher activity in acidic conditions than alkaline conditions, indicating that its urease synthesis might be regulated by the pH level of surroundings. Similarly, this strain has excellent tolerance to high concentrations of HA and survives better in the cementation media with the assistance of HA. In this study, these two strains are believed to be good candidates for organic soil stabilization through MICP technique.

For the treatment of organic soil, the treatment strategy is one of the most challenging issues due to the low permeability of the soil. Instead of grouting, premixing might be necessary for a uniform cementation. In this case, one-time mixing should be effective enough to meet the requirements of treatment. A relatively high concentration of cementation media may be a better option. Therefore, the selected bacteria should have certain tolerances to high concentrations of cementation media. Based on our findings, mixing two strains with acidic tolerance has great advantages in this kind of situation.

iii) HA as amendment for MICP technology

Adding a small amount of HA (~1 %) into culture media can enhance the bacterial growth and urease activity, increasing the cost efficiency of culture media. At the same time, the addition of HA into culture media may assist in enhancing the adaptation of employed bacteria in an organic-rich soil environment, which leads to higher survivability and better performance of bacteria in the treatment process. For the stabilization of inorganic soils, e.g., prevention of wind erosion of barren land, the amendment of HA (~ 5 %) can greatly enhance the cementation effectiveness. Considering the long-term effect, with the amendment of HA, MICP treatment can be combined with vegetation stabilization. As an efficient fertilizer and bio-stimulant, HA can increase the water and cation retention capacity of soil, which greatly promote plant growth, contributing to the stabilization of surface soils through vegetation. This can be an ecofriendly erosion control strategy in the desert.

7.3 Recommendations for future work

7.3.1 Exploration of airborne bacteria

a) Adoption of new cultivation methods

It has been several centuries since we discovered the existence of bacteria using microscopes, while there are still many mysteries surrounding them. With the development of technology, gene analysis has become a good tool for us to understand what these bacteria could do from a gene level. However, the link between what these bacteria potentially can do and what these bacteria actually respond to different cultivation environments is still unclear. In this sense, the traditional method based on bacterial cultivation is still necessary for characterization of bacteria. On the other hand, the current cultivation method has a lot of limitations that should be investigated further. For instance, by changing the culture media, bacteria of great difference can be isolated from one sample. To explore the unknown bacteria in the environment, some researchers are focusing on the development of new cultivation methods. So far, agar plate is widely used for cultivation of microbes. Agar, as a gelling agent, is generally believed to be non-toxic and not easily metabolized by microorganisms. Recent studies have shown that some bacteria are not able to growth on agar, and agar can be toxic to microorganisms in specific conditions (Davidson et al. 2024). Therefore, development of new solid culture media without agar is necessary for the exploration of unknown species in the environment.

b) Extra-resistant spores

Spores are believed to have great resistance to harsh conditions like desiccation, UV radiation, nutrient deficiency, and they can remain in a dormant state for years and regain active growth in minutes (Setlow

2014). However, the resistance level varies across species, and the current knowledge of spore tolerance is still insufficient (Setlow and Christie 2023). It means that it is possible to find spore-formers with excellent resistance in the biocementation process. It is an essential feature in the process of commercializing ureolytic bacteria. Storage as spore forms and direct utilization of spore forms could significantly simplify the treatment process, thus increasing efficiency.

c) Non-ureolytic pathway through airborne bacteria

In this study, a few trials of sampling were conducted using calcium carbonate precipitation agar (CPA and B4 agar media). Given sufficient incubation time, many colonies with visible calcium carbonate precipitation formation were observed. Considering the time efficiency of precipitation, these colonies in the media were not examined further. Based on existing studies, more than 50% of environmental bacterial isolates tested to date can induce precipitation of calcium carbonate on B4 medium, and given favorable condition, this percentage can be greatly increased (Marvasi et al. 2012). Through metabolizing organic salts of calcium, calcium carbonate is formed slowly. Since the majority of bacteria are able to induce carbonate precipitation using calcium acetate, it could be an ecofriendly cementation pathway for other application purposes.

d) Airborne bacteria for various applications

Specific airborne bacteria utilizing pigment as a protection from UV radiation can be applied to surface treatment without shading, which would be good candidates for surface restoration of stone historic heritage. Several *Glutamicibacter* isolates in this study can produce yellow pigments, which is also found in many other strains in this genus (Sumi et al. 2019). One of them pigments in a light-dependent manner as a protection strategy (see Appendix D).

7.3.2 Sophisticated design of mixed strains for biocementation

Using multiple strains for cementation treatment was found to have many merits for applications. In this study, strains from four different genera were mixed in varying ratios and examined in calcium carbonate precipitation tests. The results found that with a small addition of slow precipitators in fast precipitators, the precipitation efficiency can be greatly increased, while adding fast precipitators into slow precipitators leads to a reduction in precipitation. Limited by the number and taxonomic diversity of tested strains, it is difficult to generalize the conclusion. Therefore, to design a pair with more predictable MICP performance, it is necessary to understand the mechanisms of interactions between different bacteria.

As mentioned before, existing studies have found that some microbes can produce extracellular polymeric substances as nano-bacteria structure that acts as nucleation sites for biomineralization to avoid cell entombment by biomineralization process (Bontognali et al. 2008). Bacteria with this kind of survival strategy have absolute advantages in biocementation applications. In this study, isolates from *Glutamicibacter* genus are very likely to have similar behavior in cementation media. As it is closely related to *Arthrobacter* species, the strain has a typical feature that form aggregates in culture media due to large production of extracellular polymeric substances (see Appendix D). And this kind of aggregation is relatively robust to environmental stress, specifically, it has a more resistance to metal toxicity (J. Tang et al. 2018). In this case, *Glutamicibacter* species can be assistance of high activity bacteria, e.g., *S. pasteurii*, in the cementation process, increasing the survival rate of these bacteria and crystal sizes of calcium carbonate. Besides, the genetic factors involved in MICP process are still unclear, which could be a promising research topic. If the factors can be clarified, it would be helpful for selecting bacteria with defined tasks (e.g., complementary to each other) and contributing to the development of high-performance mixed strains. It is unfortunate that this study did not include soil solidification test to evaluate the performance of mixed strains. To confirm the feasibility of this hypothesis, future investigations should be focused on the utilization of mixed strain with *Glutamicibacter* species in soil solidification tests.

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Appendix A Precipitation capacity and survivability of TF7-26

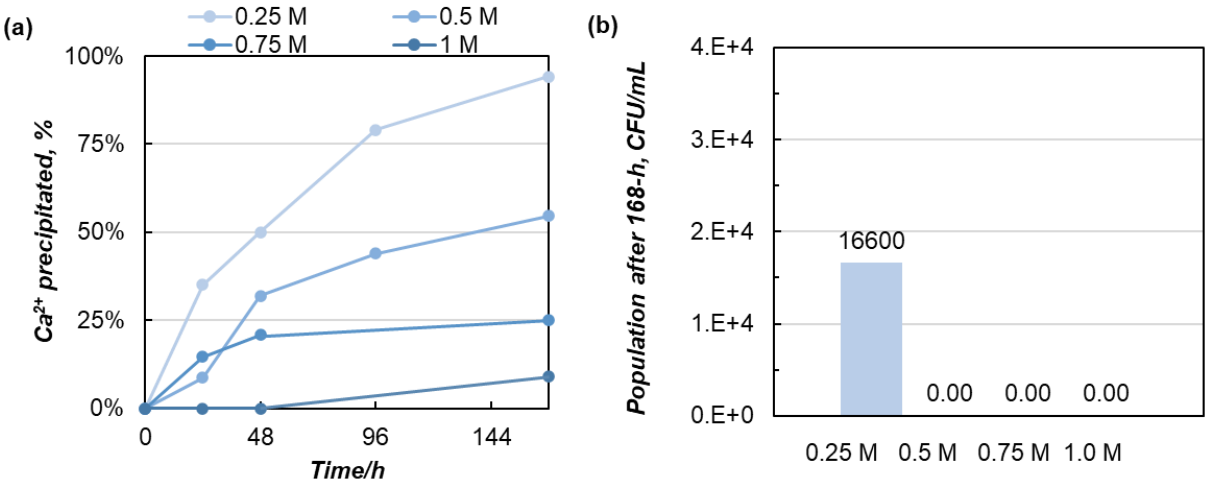

Figure A. 1 (a) Monitoring of carbonate precipitation with varying concentrations of cementation media- TF7-26. (b) Survival after 168-h incubation

Appendix B Calcium carbonate morphology

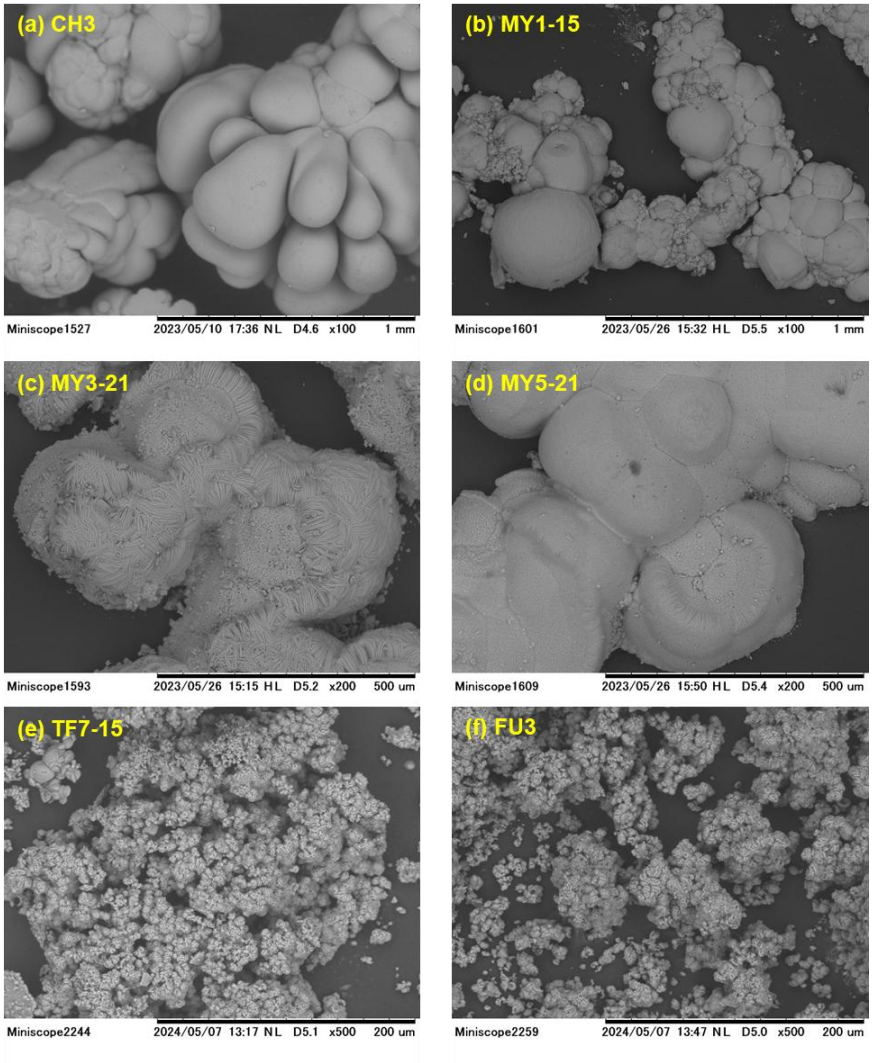

Figure B. 1 Calcium carbonate induced by (a-d) four *Glutamicibacter* strains and (e-f) two *Lederbergia* strains

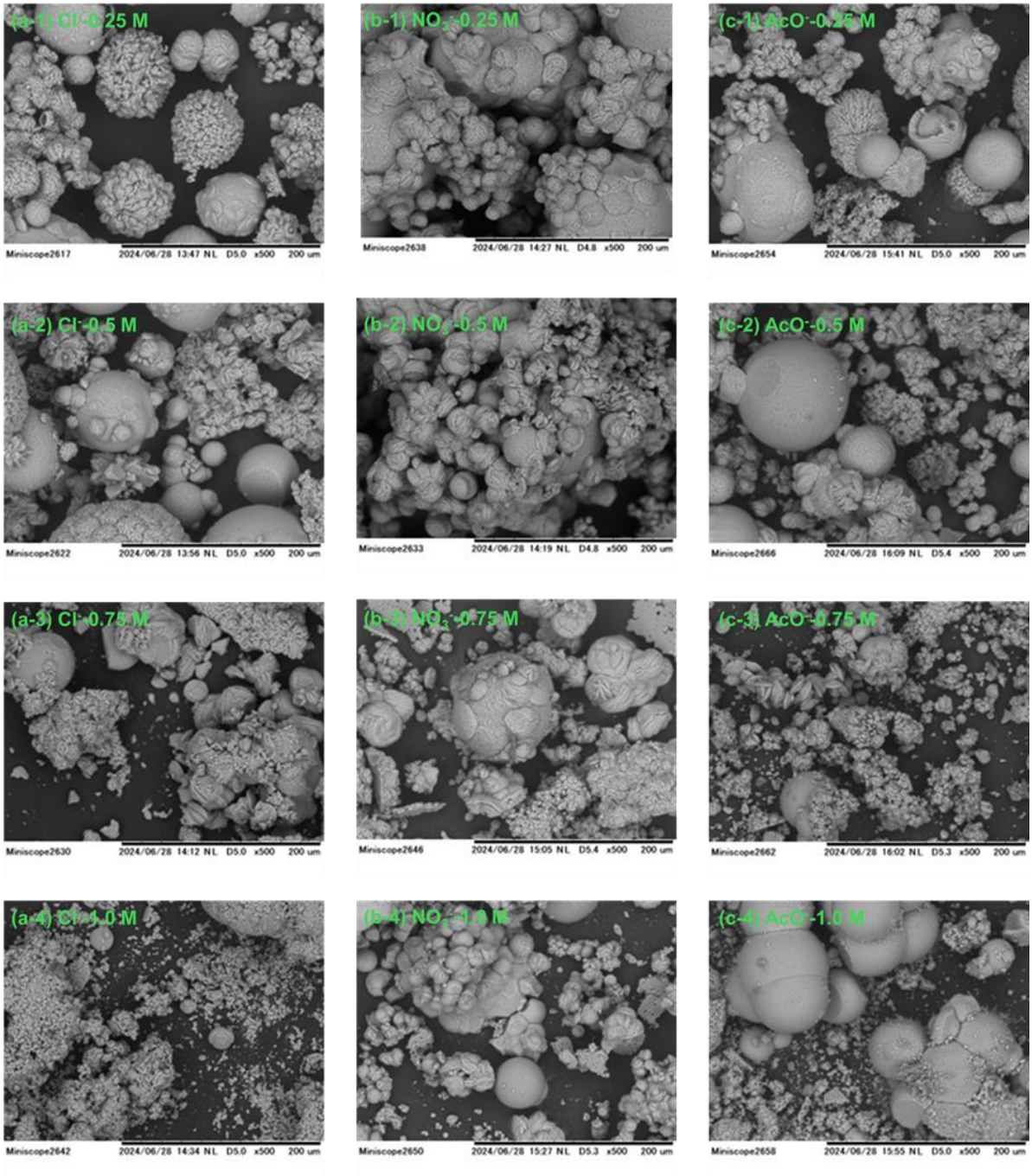

Figure B. 2 Calcium carbonate induced by MY2-9 strain with varying calcium salts and concentrations of cementation media. (a) calcium chloride; (b) calcium nitrate; (c) calcium acetate

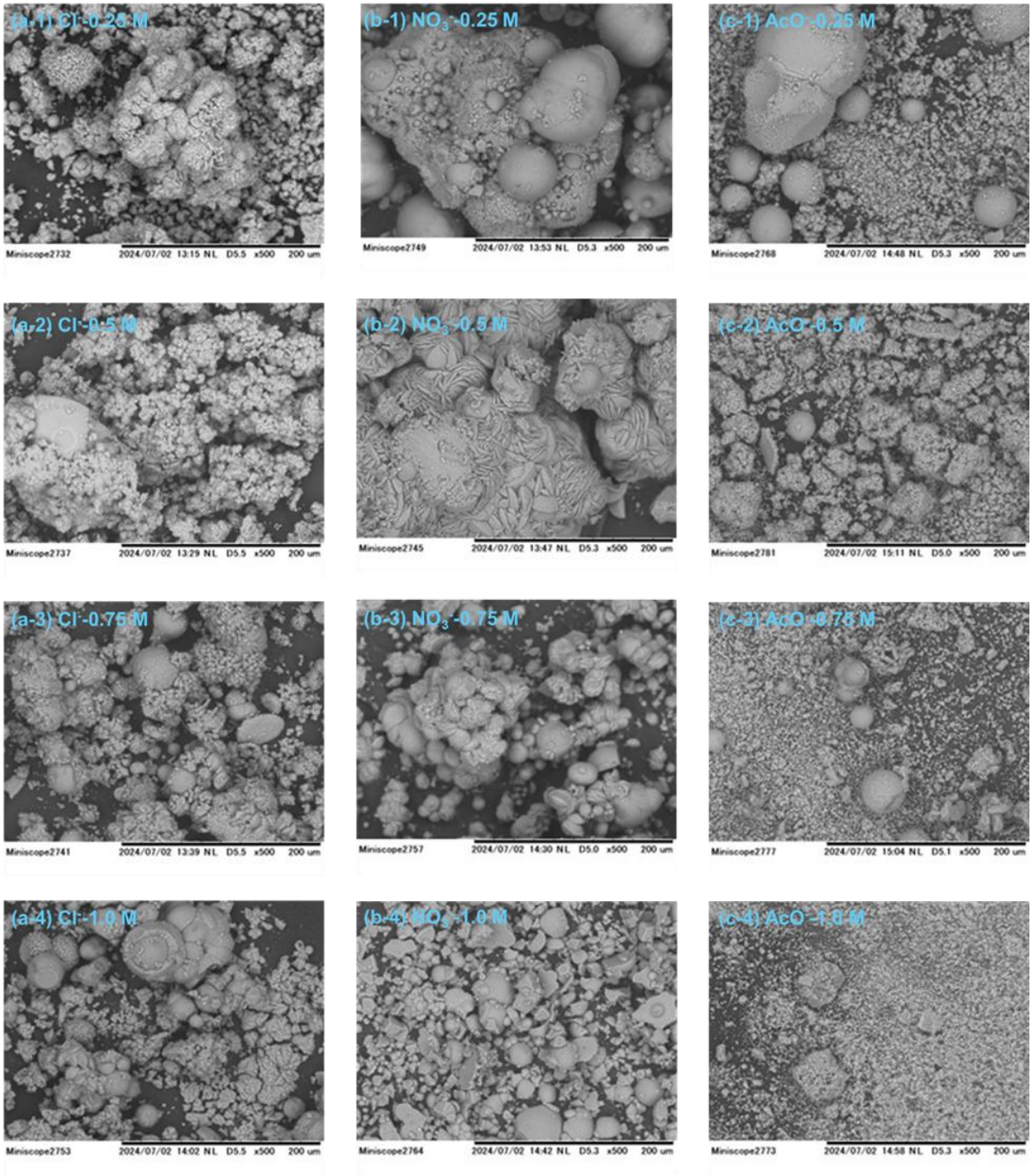

Figure B. 3 Calcium carbonate induced by KK7 strain with varying calcium salts and concentrations of cementation media. (a) calcium chloride; (b) calcium nitrate; (c) calcium acetate

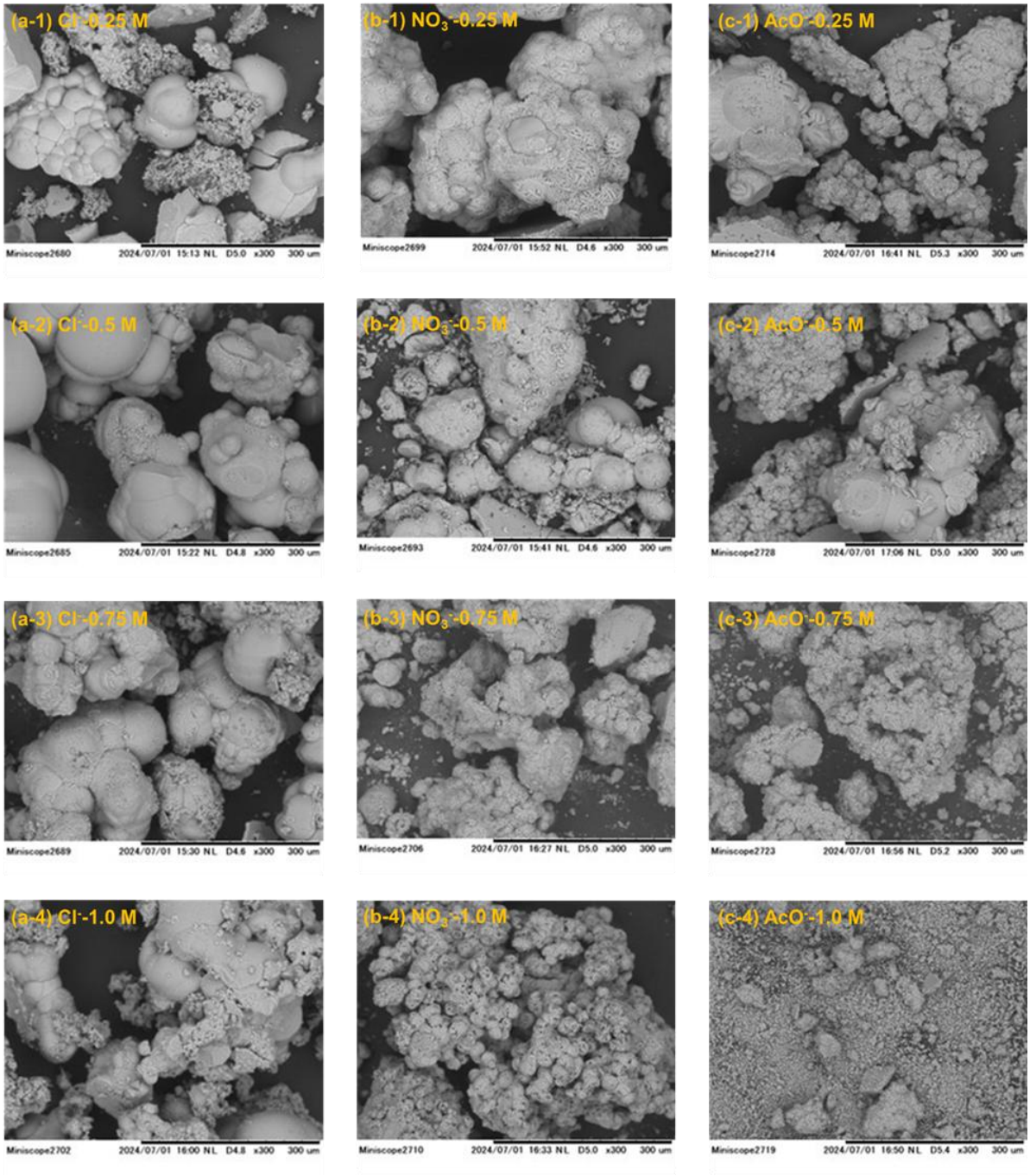

Figure B. 4 Calcium carbonate induced by FU17 strain with varying calcium salts and concentrations of cementation media. (a) calcium chloride; (b) calcium nitrate; (c) calcium acetate

Appendix C Effect of temperature shifts on precipitation

$$*\%\ increase\ or\ decrease = \%precipitated\ (15°C - 35°C) - \%precipitated(25°C)$$

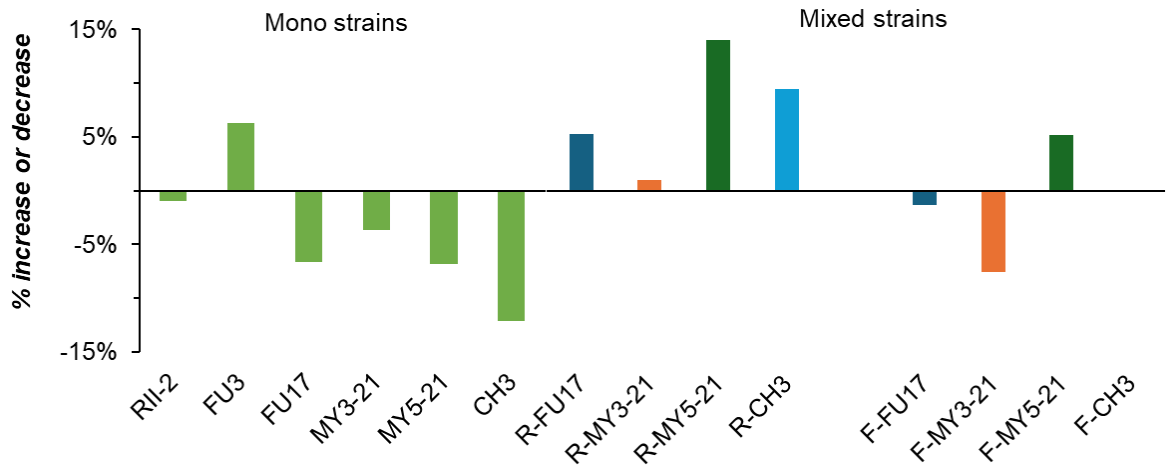

Figure C. 1 Effect of temperature shifts on calcium carbonate precipitation-a comparison of mono strains with mixed strains

Appendix D Pigmentation and agglomeration behavior of three *Glutamicibacter* strains

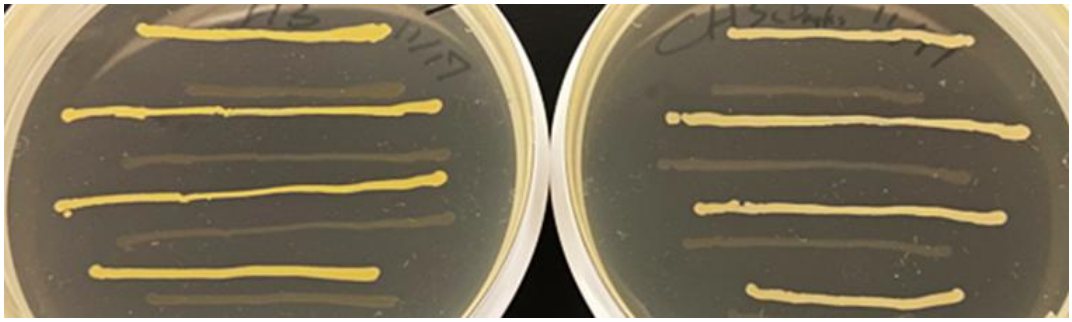

Figure D. 1 Light-inducible pigmentation of CH3, yellow pigment produced under light condition (left) and white colonies under dark condition (right)

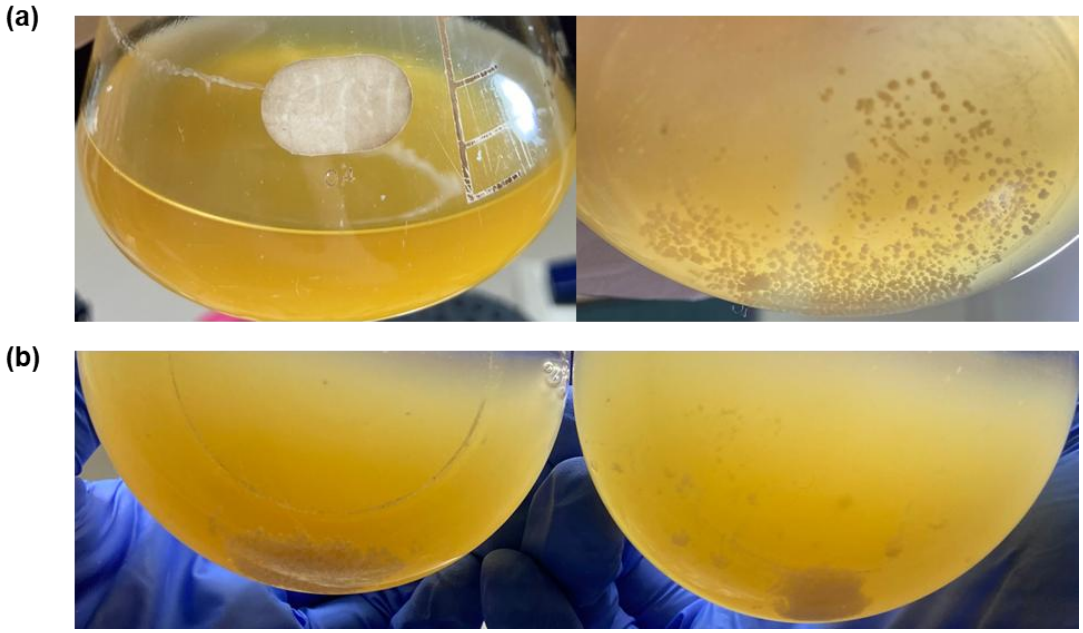

Figure D. 2 Images of bacterial agglomeration (a) MY3-21 cultivated at 35 °C; (b) FU17 cultivated at 20 °C (left) and 30 °C (right)