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Effects of Humic Acid on Process of Microbial Induced Carbonate Precipitation

M. Chen[1], S. Gowthaman[2], K. Nakashima[3], C. Takano[4], and S. Kawasaki[5]

[1] *Doctoral Student, Hokkaido University, Sapporo, Japan, email: meiqi.chen.g2@elms.hokudai.ac.jp*

[2]*Senior Lecturer, University of Jaffna, Kilinochchi, Sri Lanka, email: gowtham@tech.jfn.ac.lk*

[3]*Associate Professor, Hokkaido University, Sapporo, Japan, email: k.naka@eng.hokudai.ac.jp*

[4]*Assistant Professor, Hokkaido University, Sapporo, Japan, email: takano.chikara@eng.hokudai.ac.jp*

[5]*Professor, Hokkaido University, Sapporo, Japan, email: kawasaki@geo-er.eng.hokudai.ac.jp*

ABSTRACT

Biocementation research has been growing exponentially in recent years with more and more attention on sustainable techniques. Microbial Induced Carbonate Precipitation (MICP) cements soil particles by calcium carbonate formation microbially induced by urea hydrolysis. Most researches have been focusing on the stabilization of sandy soils, without considering the organic compounds that are 5% of natural soils on average. To investigate the effect of organic matter on MICP, humic substances (HS) should be taken into consideration. In general, this study attempts to figure out what is the effect of humic acid (HA), one fraction of HS, on bacterial growth and urease activity. In this study, three strains isolated from two different soils in Hokkaido were tested. With 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 varying degrees. In respect of urease activity, one strain showed an optimal performance with a 0.1% addition of HA, and the other two strains' activity showed an increasing tendency with increasing addition of HA. For three tested strains, no inhibition effects were found in the aspects of growth and activity. As for the mechanism, the stimulation effects could be attributed to the more efficient utilization of energy that is necessary for bacteria reproduction. As a baseline study, it could contribute to an insightful understanding of the MICP process when applying the technique in a field rich in organic matter.

Keywords: Humic acid, microbial induced carbonate precipitation (MICP), bacterial growth, urease activity

1 INTRODUCTION

In a typical soil profile, there is a layer of topsoil called humus that forms in soil when plant and animal debris break down further. The humus is particularly rich in organic soils, which consists of three main components: (i) humin, insoluble and resistant to biodegradation; (ii) fulvic acid (FA), soluble and susceptible to microbial action; (iii) humic acid (HA), only soluble alkalis and resistant to microbial action (Stevenson, 1995). Among the three components, HA is the most researched and widely applied in agriculture, population remediation, medicine, and pharmaceuticals. In agriculture, HA is well-known as one type of soil fertilizer due to its excellent cation exchange capacity from its various functional groups. Therefore, it plays an important role in the nitrogen cycle as it regulates some metabolic activities in soil microorganisms. For example, HA controls the speed of releasing nitrogen from urea, leading to the effective utilization of urea (Dong et al., 2009).

$$CO(NH_2)_2 + 2H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_3^{2-} \quad (1)$$

$$CO_3^{2-} + Ca^{2+} \xrightarrow{\text{Bacterial cell}} \text{Cell-}CaCO_3\downarrow \quad (2)$$

Biocementation generally refers to the application of microbial activities in the field of environmental and geological engineering, and microbial induced carbonate precipitation (MICP) is one of the most effective biocementation. Among various microorganisms and biochemical reactions involved in

cementation, MICP through ureolysis (mechanisms seen in equations above) is a major area of interest within the field of biocementation, particularly, for sand stabilization (Zhu & Dittrich, 2016). In recent years, not limited to sandy soils, more and more attention is being turned to other problematic soils, such as organic soils. One of the main obstacles in the conventional stabilization method is that humic substances in organic soils hinder the cementation process (Toda et al., 2020). Similarly, MICP by ureolysis for organic soil stabilization is also actually a challenging task, as HA inhibits urease under some conditions and its cation exchange capacity affects free calcium ions. Data from several studies including our previous studies have shown that biocementation could improve soil strength to a certain level, but not a satisfying one (Safdar et al., 2021; Gowthaman et al., 2021; Chen et al., 2021). However, few authors have focused on the mechanisms from the point of soil humic substances. The previous stage of this study found that the HA disrupts the crystallization of calcium carbonate, and it interacts with different ureolytic strains in a complicated manner (Chen et al., 2022). Currently, 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. 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.

2 MATERIALS AND METHODOLOGY

2.1 Chemical reagents and culture method

The HA is a commercial product purchased from FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan. Figure 1 presents an SEM image of HA particles. The culture medium for bacteria is NH_4-YE (ATCC 1376), with 15.75 g of Tris-aminomethane, 10 g of ammonium sulfate, 20 g of yeast extract, and 20 g of agar (for plate only) in 1 L of medium. Bacterial colonies are inoculated to a 5 m L-preculture test tube from an agar plate. After 24-hour incubation, 1 mL of preculture is transferred to 100 mL of a main-culture flask in a shaker at 160 rpm under the optimal temperature for bacteria.

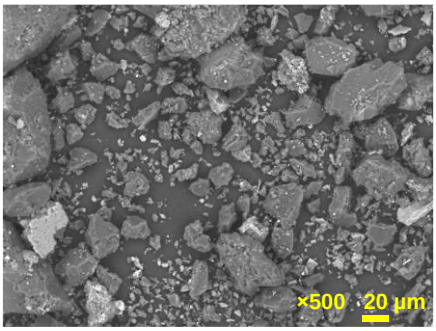


Figure 1. *Scanning electron microscope image of HA particles (Miniscope TM 3000, HITACHI)*

2.2 Characteristics of tested strains

This study 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 1. *Basic information about three tested strains*

Strain ID	Source	Closest taxon	Gram-stain	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.

2.3 Quantificational measurements

The bacterial population could be monitored indirectly by using a spectrophotometer when quantification is not necessary. However, with HA content up to 1%, the fast-settling HA particles in the suspension make it impossible to measure the optical density accurately. Considering the difficulty of evaluating the bacteria characteristics when insoluble HA is added into the main culture, methods to quantify the bacteria population and bacterial urease activity should be selected carefully. In this experiment, plate count is adopted for bacterial quantification. Some determination methods of urease activity are based on some colorimetric tests that detect ammonia concentration to calculate the urease activity of samples, by which it is difficult to obtain reliable data. Therefore, an activity evaluation test using electrical conductivity (EC) is adopted.

2.3.1 Plate count

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 (Madigan et al., 2021).

2.3.2 Electrical conductivity measurement

In the hydrolysis process of urea, ion concentrations increase with time, which results in increasing the EC of solutions. Therefore, a quick determination of urease activity is using a conductivity meter to monitor the conductivity changes with time (Whiffin et al., 2007). To establish the standard curve (Hydrolyzed urea versus EC), standard urea solutions with varying concentrations were prepared and hydrolyzed by Jack bean-derived urease, then EC was measured until the hydrolysis was completed. This experiment uses a compact EC meter (LAQUAtwin EC-33) produced by HORIBA, Ltd. With 3-point calibration, the accuracy is ±2% full scale. For testing, 0.5 mL of bacterial culture is added into a beaker with 0.1 mol/L urea solution placed in a water bath (Thermal Robo TR Series, AS ONE Corporation) at 25 °C. One sampling of 0.12 mL is taken from the bacteria-urea solution and measured at 5-minute intervals (0, 5, 10, 15-min). The figure below presents the calibration curve.

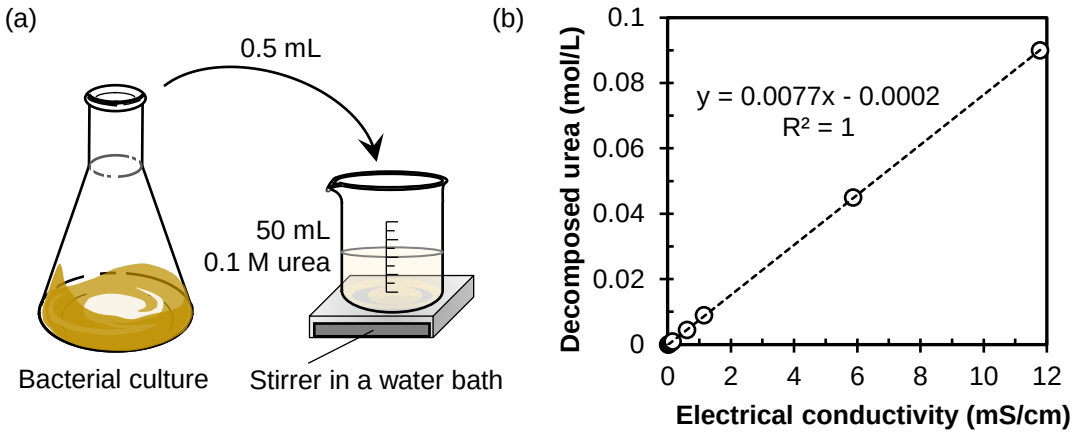


Figure 2. *Determination of urease activity by measuring EC. (a) experimental setup; (b) calibration curve: the relationship between decomposed urea and EC.*

2.4 Terms

U.A. refers to urease activity in general; U_o is the urease activity of control case; U_{total} includes both intracellular activity and extracellular activity; CFU refers to colony forming unit that estimates the number of viable cells in a sample under specific conditions.

3 RESULTS AND DISCUSSION

3.1 HA as the only carbon source

Various studies have confirmed that many soil microbes could make use of HS as sources of carbon, nitrogen and energy (Lipczynska-Kochany, 2018). Tikhonov et al. (2010) found 115 out of 170 isolates can grow on a medium with small concentration of HA as the only carbon. In this experiment, replacing yeast extracts with HA in NH_4-YE agar media, 0.01-1% of HA was added to make NH_4-HA agar media for investigating the growth of three strains. Unfortunately, the results showed that none of the tested strains can grow on media with HA as the only carbon source.

3.2 Effects of HA on bacterial growth

In general, it is believed that the presence of HS has a double side effect on bacterial growth and activity, both stimulation and inhibition (Yang et al., 2021). In this experiment, with 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 3 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 tested strains in this study, the HA might be an indirect external energy and carbon source for them (Lipczynska-Kochany, 2018).

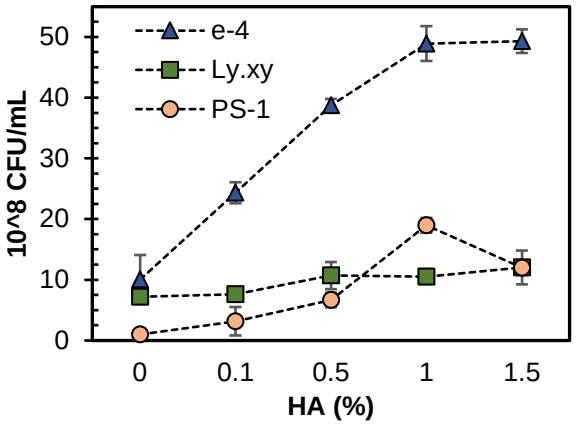


Figure 3. *Colony forming unit of three tested strains counted by plate counting method (e-4, after 48-h cultivation; Ly.xy and PS-1, after 24-h cultivation). Error bars: standard deviation for triplicate samples.*

3.3 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 Li et al. (2013), 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 4a, 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 (seen in Figure 4b&c).

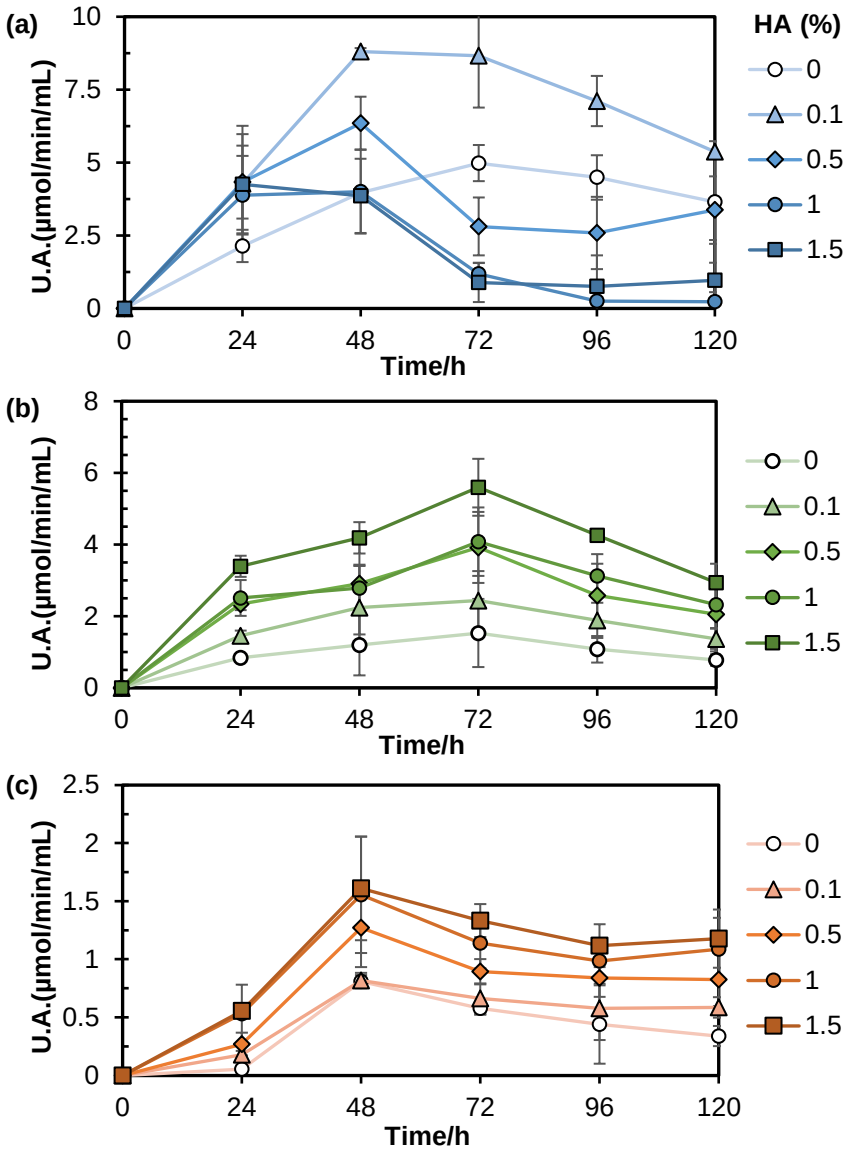


Figure 4*. Urease activity of three tested strains cultivated with 0, 0.1, 0.5, 1, 1.5% of HA addition: (a) e-4; (b) Ly.xy; (c)PS-1 (duplicate samples were prepared for each case).*

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 5, 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, on the contrary, had the least extracellular activity ratio, about 3%. This ratio fell in between of 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 subsequent section.

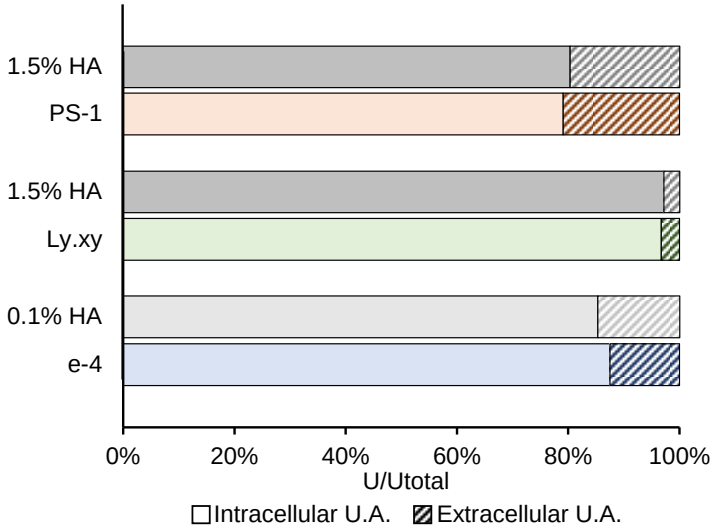


Figure 5. *Extracellular urease activity of three tested strains: a comparison between control cases and corresponding optimum cases.*

3.4 Discussion

Figure 6 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 at 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.

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 ($CaCl_2$ & urea) solution, 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 (Li et al., 2013), the urease can still function properly even after most bacteria died off from starvation. By this inference, bacteria with a relatively high extracellular urease activity and a 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.

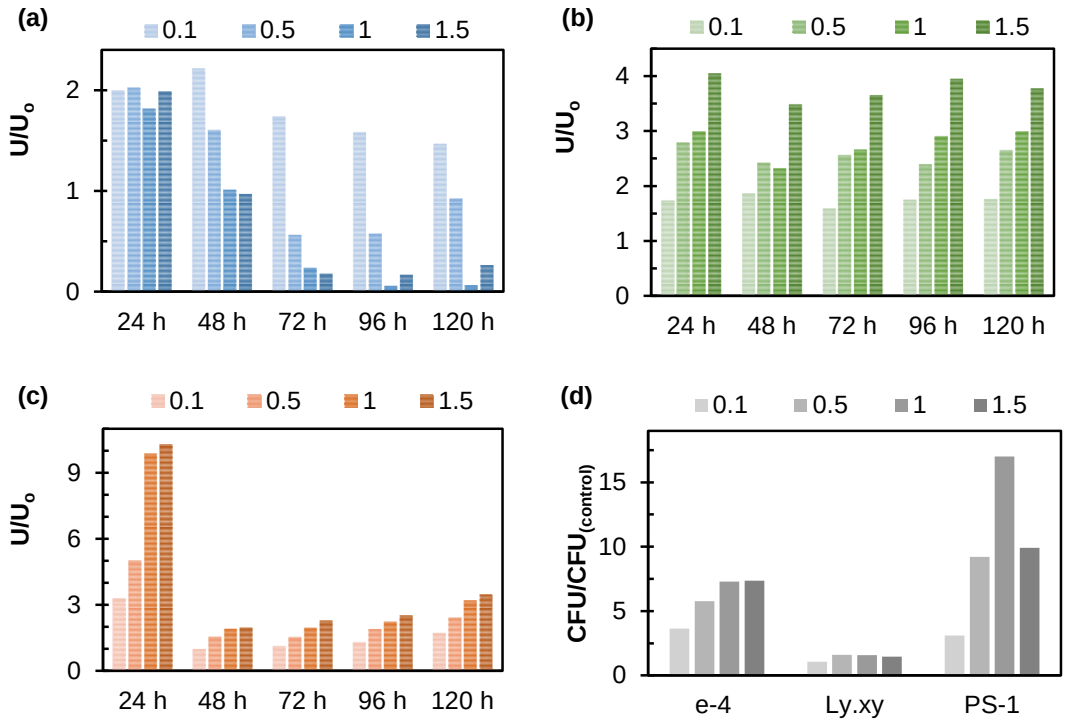


Figure 6. *(a) U.A. increase ratio of e-4; (b) U.A. increase ratio of Ly. xy; and (c) U.A. increase ratio of PS-1; (d) growth increase ratio of three strains*

4 CONCLUSIONS

This study was designed to investigate the effects of HA on the growth and urease activity of three ureolytic bacteria to obtain a deeper understanding of MICP application for organic soils. The results of this investigation show that all three strains were stimulated in the aspect of growth, while the activity might peak at a certain point depending on the strains. Extracellular urease activity of each strain differs, which may lead to a distinct performance in an aqueous environment with HA due to its direct effects on urease. Although the present study was limited in terms of tested strain number, it provides valuable insights into the impact of soil humus on biocementation, suggesting microbial selection should be a crucial factor when applying the technique to some extreme conditions.

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