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# Evaluation of Calcium Carbonate Precipitation by *Bacillus* spp. Isolated from Stingless Bee Products

(Penilain Kerpasan Kalsium Karbonat oleh *Bacillus* spp. yang Dipencilkan daripada Produk Lebah Kelulut)

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ABSTRACT

Microbiologically Induced Calcium Carbonate Precipitation (MICCP) through urea hydrolysis is the most effective way to precipitate a high concentration of calcium carbonate ($CaCO_3$) within a short time. The MICCP process is used to remediate the micro-crack in the concrete. However, limited research has been conducted to determine $CaCO_3$ precipitation by bacteria, especially in Malaysia. Here, *Bacillus* spp. isolated from the Malaysian stingless bee products were evaluated for $CaCO_3$ precipitation. *Bacillus* spp. were selected for further study according to their ability to produce urease enzymes. The urease-positive *Bacillus* spp. were screened for $CaCO_3$ precipitation by culturing on both $CaCO_3$ precipitation agar and broth media. The survivability of the urease-positive *Bacillus* spp. in various temperatures, pH values, and NaCl concentrations were tested. Seven out of 11 *Bacillus* spp. were found as ureolytic bacteria. Among the ureolytic bacteria, bacteria belonging to the *Bacillus subtilis* species complex group showed the highest number of bacteria (36.4%) that are capable of precipitating $CaCO_3$. *Bacillus stratosphericus* PD6 and *B. aryabhattai* BD8 exhibited the largest $CaCO_3$ precipitation zones (15 mm). *Bacillus stratosphericus* PD6 also precipitated the highest amount of $CaCO_3$ (65 mg) and urease activity (0.197 U/mL). All the urease-positive *Bacillus* spp. were able to grow at 45 °C, pH (8 to 12), and 5% NaCl. Only *B. subtilis* BD3 can withstand high temperatures up to 55 °C and 15% NaCl concentration. In conclusion, *Bacillus* spp. isolated from stingless bee products showed the ability as the $CaCO_3$ precipitating bacteria; suggesting its potential application in self-healing concrete.

Keywords: *Bacillus* spp.; biomineralization; calcium carbonate precipitation; urease; ureolytic bacteria

ABSTRAK

Pemendakan Kalsium Karbonat Terinduksi Mikrobiologi (MICCP) melalui hidrolisis urea adalah cara paling berkesan untuk memendakkan kepekatan tinggi kalsium karbonat ($CaCO_3$) dalam masa yang singkat. Proses MICCP digunakan untuk memulihkan retakan mikro dalam konkrit. Walau bagaimanapun, kajian terhad telah dijalankan untuk menentukan pemendakan $CaCO_3$ oleh bakteria, terutamanya di Malaysia. Di sini, *Bacillus* spp. yang diasingkan daripada produk lebah Kelulut di Malaysia telah dinilai untuk pemendakan $CaCO_3$. *Bacillus* spp. telah dipilih untuk kajian lanjut mengikut kebolehan mereka menghasilkan enzim urease. *Bacillus* spp. yang positif urease telah disaring untuk pemendakan $CaCO_3$ dengan mengkultur pada media agar dan media kaldu pemendakan $CaCO_3$. Kebolehmandirian *Bacillus* spp. yang positif urease dalam pelbagai suhu, nilai pH dan kepekatan NaCl telah diuji. Tujuh daripada 11 *Bacillus* spp. ialah bakteria ureolitik. Antara bakteria ureolitik, bakteria yang tergolong dalam kumpulan kompleks spesies *Bacillus subtilis* menunjukkan bilangan bakteria tertinggi (36.4%) yang mampu memendakan $CaCO_3$. *Bacillus stratosphericus* PD6 dan *B. aryabhattai* BD8 didapati menunjukkan zon pemendakan $CaCO_3$ terbesar (15 mm).

*Bacillus stratosphericus* PD6 juga memendakan jumlah tertinggi $CaCO_3$ (65 mg) dan aktiviti urease (0.197 U/mL). Semua *Bacillus* spp. yang positif urease mampu tumbuh pada suhu 45 °C, pH (8 hingga 12), dan 5% NaCl. Hanya *B. subtilis* BD3 boleh menahan suhu tinggi sehingga 55 °C dan kepekatan NaCl 15%. Kesimpulannya, *Bacillus* spp. diasingkan daripada produk lebah Kelulut menunjukkan keupayaan sebagai bakteria pemendakan $CaCO_3$; mencadangkan penggunaan potensinya dalam pemulihan diri konkrit.

Kata kunci: *Bacillus* spp.; bakteria ureolitik; biomineralisasi; pemendakan kalsium karbonat; urease

## Introduction

Microbiologically Induced Calcium Carbonate Precipitation (MICCP) is a natural biological process in which microorganisms synthesize inorganic compounds as part of their metabolic activities (Dhami, Reddy & Mukherjee 2013). These microorganisms synthesize metabolic products such as carbonate ions ($CO_3^{2-}$) and that react with calcium ions ($Ca^{2+}$) in the environment, and resulting in the precipitation of calcium carbonate ($CaCO_3$) during the MICCP process. Among three major anhydrous polymorphs of $CaCO_3$, namely calcite, aragonite, and vaterite; calcite is considered the most thermodynamically stable (Ni & Ratner 2008). Various pathways of $CaCO_3$ precipitation have been discovered, including urea hydrolysis, ammonification, photosynthesis, sulfate reduction, methane oxidation, and denitrification (Braissant et al. 2007; Dupraz et al. 2004; Fujita et al. 2000; Reeburgh 2007; Rodriguez-Navarro et al. 2003; Van Paassen et al. 2010). However, the urea hydrolysis pathway is preferred for $CaCO_3$ precipitation due to its straightforward nature compared to other pathways (De Muynck, De Belie & Verstraete 2010).

$CaCO_3$ precipitation is a chemical process that is ruled by four key factors: (i) calcium concentration; (ii) the concentration of dissolved inorganic carbon; (iii) pH; and (iv) availability of nucleation sites (Hammes & Verstraete 2002; Phillips et al. 2013). In this process, microorganisms produce urease enzymes to catalyze the formation of $CaCO_3$. The demand for $CaCO_3$ is significant worldwide, primarily due to its wide range of application, particularly in bulding construction (Dhami, Reddy & Mukherjee 2014). It is extensively used to enhance the strength of concrete in construction projects (Krishnapriya, Venkatesh Babu & Prince Arulraj 2015). Repairing demaged concrete exposed to micro-cracks and pores can be costly, and traditional repair systems often rely on chemical-based materials that can have adverse environment impacts. Therefore, implementing a more sustainable approach such as self-healing concrete with bio-concrete technology becomes imprerative to enhance concrete strength and quality.

Microorganisms, mainly bacteria, are widely used to precipitate $CaCO_3$ (Hammes et al. 2003). As urea hydrolysis is a common and the most effective pathway to precipitate a high concentration of $CaCO_3$, ureolytic bacteria have been used widely to promote the strength of concrete. Moreover, spore-forming and alkali-resistant bacteria with ureolytic activity are the most favourable bacteria used in the bio-concrete application (Wiktor & Jonkers 2011). Numerous spore-forming and ureolytic bacteria including *Bacillus aerius* (Siddique et al. 2016), *B. alkalinitrilicus* (Wiktor & Jonkers 2011), *B. cereus* (Alshalif et al. 2019), *B. megaterium* (Andalib et al. 2016), *B. pseudofirmus* (Alazhari et al. 2018), *B. subtilis* (Khaliq & Ehsan 2019), *Lysinibacillus sphaericus* (Algaifi et al. 2018), and *Sporosarcina pasteurii* (Algaifi et al. 2020) have been indentified for their ability to precipitate $CaCO_3$.

This study aimed to evaluate the precipitation of $CaCO_3$ by *Bacillus* spp. isolated from stingless bee products. Currently, there is limited research on $CaCO_3$-precipitating bacteria in Malaysia, highlighting the need for concerted efforts to identify and study these bacteria, which deserve scientific attention. The present investigation focused on testing for urease activity, $CaCO_3$ precipitation capability, and their survivability under harsh conditions. Evaluating the $CaCO_3$ precipitation ability of *Bacillus* spp. serve as an initial step towards understanding their potential application in self-healing concrete, particularly in the context of Malaysia, paving the way for future research in this area.

## Materials and Methods

### Source of Bacteria, Media and Growth Condition

All *Bacillus* species (*B. altitudinis* BD4, *B. stratosphericus* PD6, *B. safensis* BD9, *B. subtilis* BD3,

*B. velezensis* PD9, *B. oleronius* PD3, *B. pseudomycoides* HM2*, B. cereus* HD1, *B. toyonensis* PD13, *B. aryabhattai* BD8, and *B. nealsonii* PD4) isolated from stingless bee products (Ngalimat et al. 2019) were obtained from Enzyme and Microbial Technology Research Centre (EMTech, Universiti Putra Malaysia). *Escherichia coli* ATCC 25922 and *Proteus vulgaris* ATCC 13315 were obtained from the Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, UPM. *Lysinibacillus sphaericus* LMG 22257 was obtained from Universiti Sains Malaysia. Aseptically, a single colony of bacteria was grown onto 10 mL nutrient broth (Merck, Darmstadt, Germany) and incubated at 37 °C with shaking at 180 rpm overnight. For long-term preservation, bacterial culture was preserved in nutrient broth using 20% (v/v) glycerol and stored at -80 °C.

### PHYLOGENETIC TREE ANALYSES

The 16S ribosomal RNA genes were obtained from National Center for Biotechnology Information database under accession number: KY773597 (*B. altitudinis* BD4), KY773588 (*B. stratosphericus* PD6), KY773601 (*B. safensis* BD9), KY773596 (*B. subtilis* BD3), KY773579 (*B. velezensis* PD9), KY773585 (*B. oleronius* PD3), KY773605 (*B. pseudomycoides* HM2), KY773580 (*B. cereus* HD1), KY773592 (*B. toyonensis* PD13), KY773600 (*B. aryabhattai* BD8), KY773586 (*B. nealsonii* PD4), CP017560 (*L. sphaericus* LMG 22257), and CP009072 (*E. coli* ATCC 25922). Phylogenetic tree analysis was computed using the MEGA X software (Kumar et al. 2018) and according to the neighbor-joining method (Saitou & Nei 1987) and the *p*-distance method (Nei & Kumar 2000) with 1,000 bootstrapped (Felsenstein 1985). *E. coli* ATCC 25922 was chosen as an out-group.

### DETERMINATION OF UREOLYTIC BACTERIA

The production of urease by bacteria was investigated following the methodology described by Mohammadizadeh et al. (2019). The screening for urease-producing bacteria was conducted using the urea broth medium (Merck, Darmstadt, Germany) at pH 6.8 ± 0.2 in a 28-mL universal bottle. Briefly, a single colony of each bacterium was inoculated into 10 mL of urea broth and incubated for 24 to 48 h at 37 °C. The colour changes of the urea broth from pale yellow to pink indicated positive urease activity.

### MICCP ANALYSES

The $CaCO_3$ precipitation test in solid and broth states was performed based on the method described by Hammad, Talkhan and Zoheir (2013) with slight modifications. In the solid state, calcite precipitation agar (CPA) containing ($gL^{-1}$): nutrient broth, 2% (w/v) urea, 2.85% (w/v) calcium chloride, 0.212% (w/v) sodium bicarbonate, 1% (w/v) ammonium chloride, and 2% (w/v) bacteriological agar was prepared. For screening of $CaCO_3$ precipitation, 20 µL of bacterial culture (absorbance reading of 0.5 at $OD_{600nm}$) was inoculated on the centre of the CPA plate. The cultures were incubated at 30 °C for 7 days. The plates were examined for the precipitation zone, and the diameter was measured.

In the broth state, nutrient broth-urea/calcium chloride (NB-U/Ca) medium containing nutrient broth ($gL^{-1}$) supplemented with 2% (w/v) urea and 2% (w/v) calcium chloride was prepared. An aliquot of 30 mL of NB-U/Ca medium was inoculated with 2% (v/v) of bacterial culture standardized at the absorbance reading 0.5 at $OD_{600nm}$. The culture was incubated at 30 °C for 7 days with shaking at 130 rpm. The resulting $CaCO_3$ minerals were collected on a filter membrane with a pore size of 11 µm, and washed two times with distilled water to remove cells and culture medium. The filter paper containing the precipitant was oven dried at 60 °C for 48 h and weighed (Golovkina et al. 2020; Hammad, Talkhan & Zoheir 2013). The weight of the precipitant ($W_c$) was determined from the equation: $W_c = W_{fc} - W_f$, where ($W_{fc}$) is the weight of filter paper containing precipitant while ($W_f$) is the weight of empty filter paper.

### EXAMINATION OF TEMPERATURE TOLERANCE, ALKALITOLERANCE, AND HALOTOLERANCE

The bacterial cultures were inoculated onto a nutrient medium (Merck, Darmstadt, Germany). Different temperatures (40, 45, 50, 55, and 60 °C) were used to test temperature tolerance. Various pH (pH 8, 9, 10, 11, and 12) and NaCl concentrations (5, 10, and 15% [w/v]) were used to test alkalitolerance and halotolerance, respectively.

### PHENOL-HYPOCHLORITE ASSAY

The phenol-hypochlorite assay method was performed according to the method described by Chahal, Rajor and Siddique (2011) with slight modifications. The culture filtrates (10 µL) were added to the mixture containing 40 µL of 0.1 M potassium phosphate buffer (pH 8.0)

and 100 µL of 0.1 M urea solution. The mixture was incubated at 37 °C for 5 min. Then, 40 µL of phenol nitroprusside solution (Sigma Aldrich, St. Louis, MO, USA) and 40 µL of alkaline hypochlorite solution (Sigma Aldrich, St. Louis, MO, USA) were added and incubated at 37 °C for 25 min. The optical density was measured at 626 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). The calibration curve was prepared using ammonium chloride as a standard. One unit (U) of urease is defined as the amount of enzyme hydrolysing 1 µmol urea/min (Natarajan 1995). The enzyme assays were performed in triplicates. *E. coli* ATCC 25922 was used as a negative control in this test.

### STATISTICS

The NCSS software version 2020 (NCSS, LLC., Kaysville, Utah, USA) was used for statistical and dendrogram analyses. The experiments (determination of ureolytic bacteria, $CaCO_3$ precipitation test in the solid and broth states, physiological tolerance tests, and urease activity) were carried out in 3 replicates. The numerical data from related experiments were compared using a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test. Significant results have been considered at $P \leq 0.05$. Hierarchical clustering was constructed using qualitative and quantitative data from urease, $CaCO_3$ precipitation and physiological tolerance tests.

## RESULTS

### SCREENING OF UREOLYTIC BACTERIA

Ureolytic bacteria were screened using urea broth medium and incubated for 24 and 48 h at 37 °C as shown in Figure 1. Among all the tested bacteria, *B. altitudinis* BD4, *B. stratosphericus* PD6, *B. safensis* BD9, *B. subtilis* BD3, *B. pseudomycoides* HM2, *B. toyonensis* PD13, and *B. aryabhattai* BD8 showed positive results by changing the colour of the medium from yellow to pink within the incubation of 24 to 48 h. On the other hand, *B. velezensis* PD9, *B. oleronius* PD3, *B. cereus* HD1, and *B. nealsonii* PD4 showed negative results after 48 h since no color change in the media.

Among the positive bacteria, *B. stratosphericus* PD9, *B. safensis* BD9, and *B. aryabhattai* BD8 demostrated rapid ureolytic activity, showing a colour change within 24 h of incubation. Conversely, *B. altitudinis* BD4, *B. subtilis* BD3, *B. pseudomycoides* HM2, and *B. toyonensis* PD13 exhibited slow ureolytic activity, requiring 48 h of incubation for the colour change to occur. These finding indicated that *B. stratosphericus* PD9, *B. safensis* BD9, and *B. aryabhattai* BD8 possess higher urea hydrolysis abilities, likely attributed to their efficient production of the urease enzyme. This was evident from their rapid and intense pink colour development, which is comparable to the positive control, *P. vulgaris* ATCC 13315 within 24 h incubation period.

### DETERMINATION OF MICCP BY BACTERIAL CULTURES

The urease-positive bacteria were further tested for their capabilities in precipitating $CaCO_3$ on the calcite precipitation agar (CPA). CPA is a solid medium used to screen bacteria that can precipitate $CaCO_3$ through ureolysis. The formation of $CaCO_3$ on CPA appeared as a white precipitant within and around the growth area. Here, all of the urease-positive bacteria which were *B. altitudinis* BD4, *B. stratosphericus* PD6, *B. safensis* BD9, *B. subtilis* BD3, *B. pseudomycoides* HM2, *B. toyonensis* PD13, and *B. aryabhattai* BD8 formed white $CaCO_3$ precipitant; appeared as distinct circular zone around the growth area on CPA after 7 days of incubation at 37 °C (Figure 2(A)). *Bacillus stratosphericus* PD6 and *B. aryabhattai* BD8 have exhibited the largest $CaCO_3$ precipitation zone, which was 15.00 ± 1.41 mm, followed by *B. safensis* BD9 (14.00 ± 1.41 mm), *B. subtilis* BD3 (14.00 ± 0.71 mm), and *B. toyonensis* PD13 (14.00 ± 0.71 mm). While, *B. pseudomycoides* HM2 and *B. altitudinis* BD4 exhibited the smallest $CaCO_3$ precipitation zone, which was 13.00 ± 1.41 and 11.00 ± 0.71 mm, respectively. As for the positive control, *L. sphaericus* LMG 22257 exhibited 14.00 ± 0.71 mm for the $CaCO_3$ precipitation zone.

Overall, the *Bacillus* spp. showed different sizes of $CaCO_3$ precipitation zones, which may result from their capabilities in producing urease to induce $CaCO_3$ precipitation through MICCP. To further determine the weight of $CaCO_3$ precipitant, bacteria were subjected to the $CaCO_3$ precipitation test in broth state using NB-U/Ca medium. After the inoculation of bacterium into NB-U/Ca, a white precipitant was observed in the medium (Figure 2(B)). The $CaCO_3$ precipitant was collected, filtered and weighed after 7 days of incubation at 37 °C. In this test, *B. stratosphericus* PD6 (65.0 ± 2.0 mg) precipitated $CaCO_3$ the most compared to the other *Bacillus* spp. tested. The precipitation was also higher than the positive control, *L. sphaericus* LMG 22257 (55.0 ± 1.0 mg). This indicated that this bacterium was more efficient in precipitating $CaCO_3$ through urea hydrolysis.

Noteworthy, based on the phylogenetic tree analysis, three bacterial groups were detected under the *Bacillus* clade: (i) *B. subtilis* species complex group; (ii) *B. cereus* group; and (iii) *B. megaterium* group (Figure 3A). Approximately 36.4% of the bacterial species (*B. altitudinis* BD4, *B. stratosphericus* PD6, *B. safensis* BD9, and *B. subtilis* BD3) belonging to the *B. subtilis* species complex group showed the highest number of bacteria that were capable of producing urease enzyme and precipitating $CaCO_3$. This is followed by 18.2% (*B. pseudomycoides* HM2 and *B. toyonensis* PD13) from the *B. cereus* group and 9.0% (*B. aryabhattai* BD8) from the *B. megaterium* group.

### ANALYSIS OF TEMPERATURE TOLERANCE, ALKALITOLERANCE, AND HALOTOLERANCE OF BACTERIAL CULTURES

The urease-positive bacteria were further tested to analyse their characteristics in harsh conditions; based on three parameters including temperatures, pH values, and NaCl concentrations (Table 1). *Bacillus* spp. which were *B. altitudinis* BD4, *B. stratosphericus* PD6, *B. safensis* BD9, *B. subtilis* BD3, *B. pseudomycoides* HM2, *B. toyonensis* PD13, and *B. aryabhattai* BD8 can tolerate the temperature until 45 °C. Only *B. altitudinis*

**A**

| Bacteria | Urease test |
|---|---|
| *B. aryabhattai* BD8 | + |
| *B. safensis* BD9 | + |
| *B. stratosphericus* PD6 | + |
| *B. subtilis* BD3 | + |
| *B. toyonensis* PD13 | + |
| *B. pseudomycoides* HM2 | + |
| *B. altitudinis* BD4 | + |
| *B. nealsonii* PD4 | - |
| *B. velezensis* PD9 | - |
| *B. cereus* HD1 | - |
| *B. oleronius* PD3 | - |

**B**

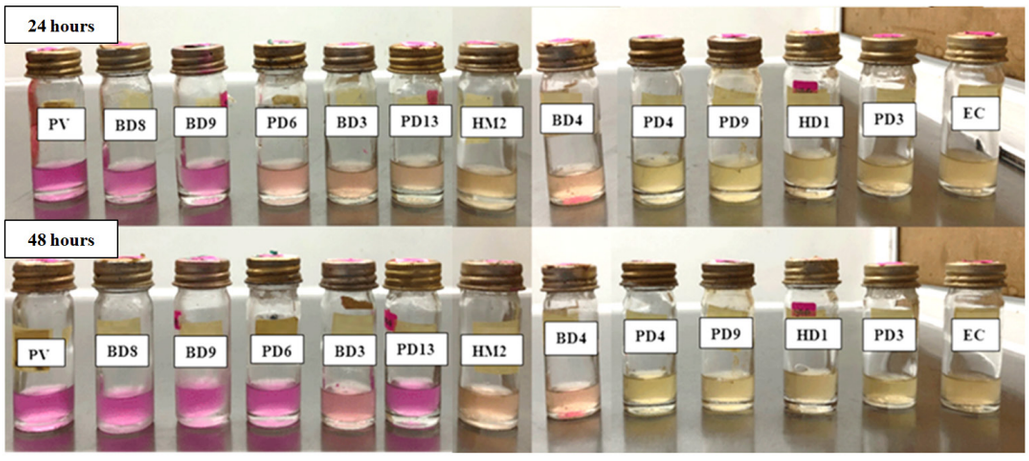


FIGURE 1. Urease assay of *Bacillus* spp. (A) Qualitative urease assay of *Bacillus* spp. Note, '+' = positive urease test and '-' = negative urease test. (B) Qualitative urease assay of *Bacillus* spp. Ureolytic bacteria was screened using UB within 24 and 48 h incubation at 37 °C. Note, *B. aryabhattai* (BD8), *B. safensis* (BD9), *B. stratosphericus* (PD6), *B. subtilis* (BD3), *B. toyonensis* (PD13), *B. pseudomycoides* (HM2) and *B. altitudinis* (BD4) showed positive result, whereas, *B. nealsonii* (PD4), *B. velezensis* (PD9), *B. cereus* (HD1) and *B. oleronius* (PD3) showed negative result. In this test, *P. vulgaris* ATCC 13315 (PV) was used as a positive control, whereas, *E. coli* ATCC 25922 (EC) was used as a negative control

TABLE 1. Analysis of temperature tolerance, alkalitolerance and halotolerance of *Bacillus* spp. Note, '+' = positive and '-' = negative

| Bacteria | Temperature (°C) | | | | | pH | | | | | NaCl concentration (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | 5 | | 10 | | 15 | |
| | 40 | 45 | 50 | 55 | 60 | 8 | 9 | 10 | 11 | 12 | 24h | 48h | 24h | 48h | 24h | 48h |
| *B. altitudinis* BD4 | + | + | + | + | - | + | + | + | + | + | + | - | - | - | - | - |
| *B. stratosphericus* PD6 | + | + | - | - | - | + | + | + | + | + | - | + | - | - | - | - |
| *B. safensis* BD9 | + | + | - | - | - | + | + | + | + | + | - | + | - | - | - | - |
| *B. subtilis* BD3 | + | + | + | + | - | + | + | + | + | + | + | - | + | - | + | - |
| *B. pseudomycoides* HM2 | + | + | + | + | - | + | + | + | + | + | + | - | - | - | - | - |
| *B. toyonensis* PD13 | + | + | - | - | - | + | + | + | + | + | - | + | - | - | - | - |
| *B. aryabhattai* BD8 | + | + | - | - | - | + | + | + | + | + | - | + | - | - | - | - |

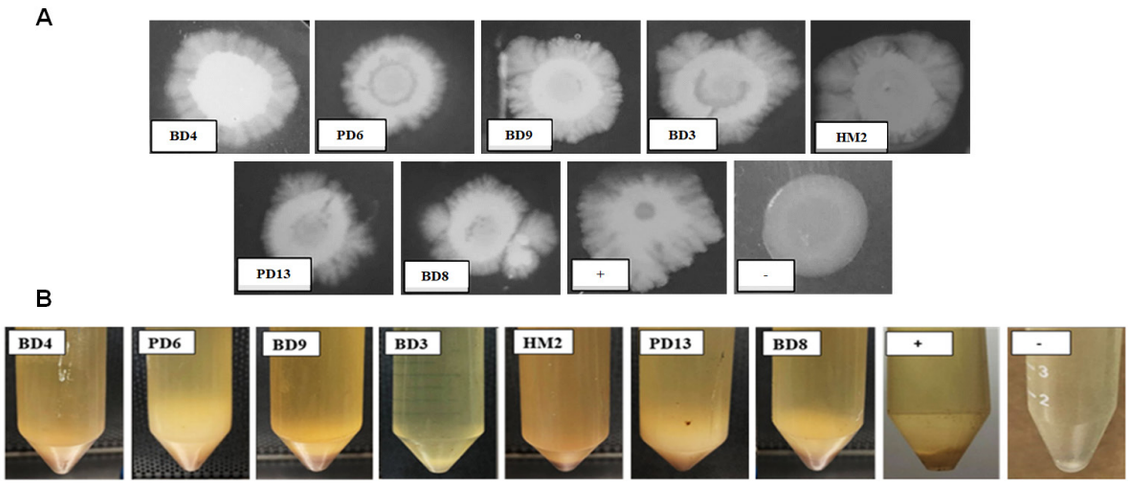


FIGURE 2. Determination of $CaCO_3$ precipitation by *Bacillus* spp. (A) The $CaCO_3$ precipitation after 7 days of incubation on the calcite precipitation agar (CPA). (B) The $CaCO_3$ precipitation in NB-U/Ca medium at 30 °C for 7 days with shaking at 130 rpm. Note, *B. altitudinis* (BD4), *B. stratosphericus* (PD6), *B. safensis* (BD9), *B. subtilis* (BD3), *B. pseudomycoides* (HM2), *B. toyonensis* (PD13), *B. aryabhattai* (BD8) formed white $CaCO_3$ precipitant at the bottom of the tube. In this test, *L. sphaericus* LMG 22257 (+) was used as a positive control, whereas, *E. coli* ATCC 25922 (-) was used as a negative control

BD4, *B. subtilis* BD3, and *B. pseudomycoides* HM2 can tolerate high temperatures up to 55 °C. Nevertheless, all the *Bacillus* spp. tested cannot survive in a temperature of 60 °C. As for the alkalitolerance, *Bacillus* spp. were tested for their ability to tolerate different pH values ranging from 8 to 12. All the *Bacillus* spp. tested can tolerate from pH 8 to 12. Moreover, the capabilities of *Bacillus* spp. in tolerating different NaCl concentrations ranging from 5 to 15% also were tested. The growth of *B. altitudinis* BD4, *B. stratosphericus* PD6, *B. safensis* BD9, *B. subtilis* BD3, *B. pseudomycoides* HM2, *B. toyonensis* PD13, and *B. aryabhattai* BD8 were observed on the nutrient agar supplemented with 5% of NaCl. The growth of *B. altitudinis* BD4, *B. subtilis* BD3, and *B. pseudomycoides* HM2 were detected within 24 h incubation. In contrast, the growth of the remaining four *Bacillus* spp. was observed within a 48 h incubation period. Interestingly, *B. subtilis* BD3 was tolerant to NaCl up to 15% concentration.

## UREASE ACTIVITY

Cluster analysis based on urease, $CaCO_3$ precipitation, and physiological tolerance tests has been conducted (Figure 3(B)). Dendrogram generated has found that the *Bacillus* spp. tested were grouped into two different clusters. Cluster 1 was dominated by *B. aryabhattai* BD8, *B. stratosphericus* PD6, and *B. safensis* BD9, while cluster 2 dominated by *B. toyonensis* PD13, *B. altitudinis* BD4, *B. pseudomycoides* HM2, and *B. subtilis* BD3. To note, *Bacillus* spp. belonged to cluster 1 have both rapid ureolytic activity and exhibited among the highest weight of $CaCO_3$ precipitant. Therefore, *B. stratosphericus* PD6, *B. safensis* BD9, and *B. aryabhattai* BD8 were selected for further study to determine their urease activity using phenol-hypochlorite assay. The urease activity measured in *B. stratosphericus* PD6, *B. safensis* BD9, *B. aryabhattai* BD8 after 7 days of incubation were $0.197 \pm 0.001$, $0.166 \pm 0.002$, and $0.139 \pm 0.001$ U/mL, respectively. The highest productivity was obtained at 144 h of incubation (Figure 4).

Based on the growth curve (Figure 4), a gradual increase in urease activity for *B. stratosphericus* PD6, *B. safensis* BD9, and *B. aryabhattai* BD8 in the first 20 h was observed, indicating the first log phase of bacterial growth and reproduction. Here, the urease activity of the *Bacillus* spp. tested was slowly increased along with the incubation period. During the second log phase (from 20 to 96 h), the urease activity kept increasing due to the exponential reproduction rate that led to ammonia's mass production (Bachmeier et al. 2002). In the third stationary phase (96 to 144 h), the urease activity remained stable

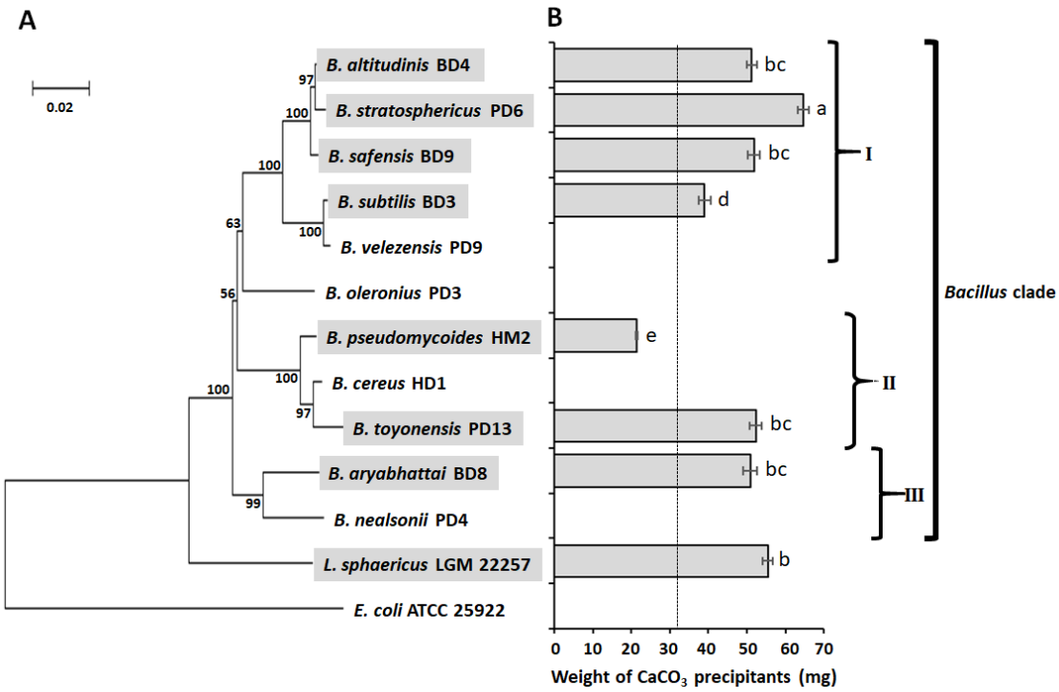


FIGURE 3. Clustering analysis of *Bacillus* spp. (A) Neighbor-joining tree of bacterial tested. The optimal tree with the sum of branch length = 0.40056292 is shown. The analysis involved 13 nucleotide sequences where a total of 1,349 positions in the final dataset was computed. Here, *E. coli* ATCC 25922 was used as an outgroup. The bacteria able to produce urease enzyme are highlighted in the grey box. (B) The weight of $CaCO_3$ precipitants by bacteria after 7 days of incubation in NB-U/Ca medium. The weight was qualified from three independent experiment (error bars). The weight of $CaCO_3$ was significantly different from those of negative control using ANOVA test. The boundary average of the weight of $CaCO_3$ precipitant is indicated by the dashed line. An analysis of mean test ($\alpha = 0.05$) also indicates that the weight of $CaCO_3$ precipitant of the negative control are significantly below the mean threshold of the measured values. Means with different superscript letters (a-e) differ significantly at $P \leq 0.05$ (One-way ANOVA) significance level. Note: (I) *B. subtilis* species complex group; (II) *B. cereus* group; and (III) *B. megaterium* group

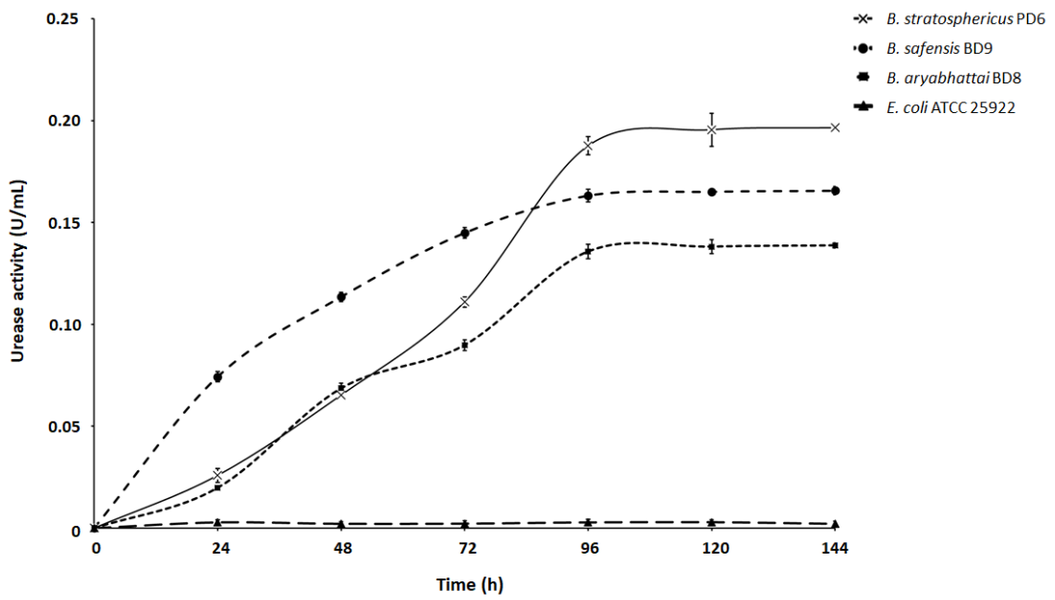


FIGURE 4. Urease activity of *Bacillus* spp. The urease activity was determined from three independent experiment (error bars). The urease activity was significantly different from those of negative control using ANOVA test ($P \leq 0.05$)

at a constant rate for all the bacteria as they entered the stationary phase, where the reproduction still occurred but not at a similar speed as in the log phase. After 144 h, it was estimated that the urease activity might be declined as the bacteria entered the death phase due to the depletion of nutrients.

Overall, among *Bacillus* spp. tested, *B. stratosphericus* PD6 could induce the highest amount of $CaCO_3$ precipitation and has the highest urease activity, followed by *B. safensis* BD9 and *B. aryabhattai* BD8. Results suggested that *B. stratosphericus* PD6 was more efficient than other *Bacillus* spp. tested and potentially act as the best candidate as $CaCO_3$-precipitating bacteria.

## DISCUSSION

The capability of *Bacillus* spp. isolated from the stingless bee products to hydrolyze urea using the urease enzyme was tested in the urea broth medium. Urease produced by some microorganisms is a hydrolytic enzyme that attacks nitrogen and carbon bonds in amide compounds such as urea and forms the alkaline end-product, ammonium (Mitchell & Grant Ferris 2005). Urease production is important in inducing $CaCO_3$ precipitation by the MICCP bacteria. The formation of $CaCO_3$ precipitation by the MICCP bacteria is crucial in prolonging the concrete service life. $CaCO_3$ produced by the microorganisms could heal the cracked concrete surface and, thus, increase the strength and durability of the concrete (Vijay, Murmu & Deo 2017).

The MICCP bacteria can be added to the cement mixture and nutrients during the erection of a new structure to generate self-healing concrete or bio-concrete (Amran et al. 2022). This creates a proactive approach that enhances the self-healing capabilities of the bio-concrete for potential micro-crack reparation. The MICCP bacteria will remain dormant within the concrete and be activated when a micro-crack appears due to external factors such as structural loading, thermal stress, and shrinkage (Huang & Zhou 2022). Subsequently, the MICCP bacteria initiate the repair process by utilizing calcium lactate (or another calcium source) and producing calcite to fill and seal the micro-crack.

Additionally, the maintenance workers can also inoculate the MICCP bacteria whenever new micro-cracks are detected, as proven by previous research that successfully remedies concrete cracks using bio-concrete (Luhar, Luhar & Shaikh 2022). This method allows for the targeted application of the MICCP bacteria to specific areas requiring repair. The bacteria can be injected into the cracks via a solution, enabling them to utilize calcium sources, including urea, and facilitate the formation of $CaCO_3$. As a result, the newly formed microcracks in the concrete can be sealed effectively. This process allows ureolytic bacteria to utilize available calcium sources and actively contribute to forming $CaCO_3$, thereby aiding in sealing newly formed micro-cracks in concrete (Luhar, Luhar & Shaikh 2022).

Until now, *L. sphaericus* LMG 22257 has been recognized as a superior MICCP bacterium that can hydrolyze urea from various sources, including soil, water, animals, and even humans (Azmi et al. 2018). This bacterium has high urease activity that eventually leads to the high production of $CaCO_3$ precipitation. Here, ureolytic bacteria from the *B. subtilis* species complex group (*B. altitudinis* BD4, *B. stratosphericus* PD6, *B. safensis* BD9, and *B. subtilis* BD3), *B. cereus* group (*B. pseudomycoides* HM2 and *B. toyonensis* PD13) and *B. megaterium* group (*B. aryabhattai* BD8) were found able to precipitate $CaCO_3$. *Bacillus* spp. from the *B. subtilis* species complex group (Huynh, Imamoto & Kiyohara 2019; Joshi, Kumthekar & Ghodake 2016; Khaliq & Ehsan 2019; Nosouhian, Mostofinejad & Hasheminejad 2015; Pachaivannan et al. 2020; Sarkar et al. 2015; Shahid et al. 2020; Siddique et al. 2016), *B. cereus* group (Alshalif et al. 2019; Dhami, Reddy & Mukherjee 2013; Shahid et al. 2020), and *B. megaterium* group (Andalib et al. 2016; Dhami, Reddy & Mukherjee 2014, 2013) were found to be able to precipitate $CaCO_3$.

Generally, concrete is known for its harsh environment for the bacteria to survive primarily due to its high pH values ranging from 11 to 13 resulting from calcium hydroxide formation (Jin, Yu & Shui 2018). Even for the alkaliphiles, concrete is still considered a hostile environment due to lack of moisture and nutrients, diverse temperatures and possible exposure to ultraviolet light that may affect the bacterial metabolic activity, making them and their spores susceptible to death. Previously, bacteria that can be used in the bio-concrete through MICCP have been proposed to survive in high temperatures by producing endospores (Chen et al. 2019). In fact, the temperature has affected the bacterial growth more than the pH value. Hence, it is crucial to control the temperature during the MICCP through precooling and post-cooling process or choosing the right cementitious materials that can minimize the heat-generating potential of the concrete (Deng & Wang 2018). Moreover, the bacteria must also withstand the pressure and heat loss due to mixing and hardening concrete. Spore-forming bacterium, namely *B. subtilis*,

was reported to survive in high temperatures (70 °C) and precipitate $CaCO_3$ (Manikandan & Padmavathi 2015). In this study, all *Bacillus* spp. tested were toleratant to temperature 45 °C, while, only *B. altitudinis* BD4, *B. subtilis* BD3, and *B. pseudomycoides* HM2 could tolerate high temperature up to 55 °C.

As the nature of concrete is highly alkaline, the discovery of an alkalophilic bacterium with the ability to precipitate $CaCO_3$ is needed. Concrete was made up of dry materials, and the pH value of the mixture was up to pH 13 (Behnood, Tittelboom & Belie 2016). Thus, not all bacteria can survive in the concrete environment due to high internal pH. The bacteria cell may die, or their enzyme can be denatured under highly alkaline conditions (Irwan & Othman 2013). Noteworthy, *Bacillus* spp. such as *B. alkalinitrilicus* (Wiktor & Jonkers 2011), *B. cohni* (Sierra-beltran, Jonkers & Schlangen 2014), and *B. pseudofirmus* (Alazhari et al. 2018) were reported able to thrive in high alkaline conditions and precipitate $CaCO_3$. Since *Bacillus* spp. was able to form an endospore in the hostile environment, this bacterial genus can remain dormant while waiting for a better condition to germinate. The germination occurs once the concrete starts to crack due to contact with water and oxygen (Dhami, Reddy & Mukherjee 2014). As bacteria germinate, ammonium ions ($NH_4^+$) and $CO_3^{2-}$ production from bacterial urease activity made the condition more alkaline. This will favour the precipitation of $CaCO_3$ with the presence of $Ca^{2+}$. Thus, the $CaCO_3$-precipitating bacteria must withstand alkaline conditions to induce $CaCO_3$ precipitation through MICCP. In this test, *Bacillus* spp. tested was found can tolerate from pH 8 to 12.

In addition, the structure of the buildings in high water environments, such as underground basements and marine structures, were more prone to corrosion of steel reinforcement. This was due to the salts that entered through the cracks of the concrete and caused steel corrosion (Aburawi & Swamy 2008). Salt and concrete did not act well together since chemicals containing chlorides tended to be slightly acidic, which may deteriorate the concrete's bond. Salt in concrete caused the concrete structure to become less durable and not resistant to infiltration by water, sulfates, chlorides, and carbon dioxide (Wegian 2010). Salt can ultimately increase the concrete's permeability, making it more prone to deterioration (Binici et al. 2008). Therefore, the $CaCO_3$-precipitating bacteria needed to withstand high salt concentrations to quickly produce more $CaCO_3$ precipitant, thus preventing more salt solution from entering the concrete, which caused deterioration. According to Williams et al. (2010), seawater salinity is approximately 3.5%. From this experiment, all *Bacillus* spp. tested able to tolerate up to 5% of salt concentration.

In addition of their ability to survive in extreme conditions, bacteria that precipitate $CaCO_3$ must also exhibit high urease activity to precipitate a high amount of $CaCO_3$ (Krishnapriya, Venkatesh Babu & Prince Arulraj 2015). Noteworthy, the standard concrete formulation does not include urea. However, introducing urea to the concrete formulation has been reported to improve the ureolytic activity of MICCP *Bacillus* (Hammad, Talkhan & Zoheir 2013). Urea is a nitrogen source for the MICCP bacteria and is essential for their ureolytic activity. The urea solution can be added to the concrete by surface application or injection into existing cracks along with the MICCP bacteria. The presence of urea generates the production of urease by MICCP bacteria that catalyzes the hydrolysis of urea into $NH_4^+$ and $CO_3^{2-}$ (Wang et al. 2014). This, in turn, increases the pH in the bacteria's vicinity, resulting in alkaline conditions favorable for $CaCO_3$ precipitation. Furthermore, the negatively charged cell wall of bacteria draws $Ca^{2+}$ from the environment, which causes the precipitation of $CaCO_3$ in micro-cracks due to the reaction between $Ca^{2+}$ and $CO_3^{2-}$ (Zhang et al. 2019).

In this study, *B. stratosphericus* PD6 exhibited the highest urease activity (0.197 U/mL) and $CaCO_3$ precipitation compared to other *Bacillus* spp. tested. It should be noted that previous studies have reported ureolytic bacteria like *S. pasteurii* isolated from soil, exhibiting urease activity exceeding 10 U/mL (Chahal, Rajor & Siddique 2011). As ureolytic bacteria are typically found in soil, which is enriched in urea compared to the stingless bee products (Damayanti et al. 2019), the bacteria isolated from stingless bee products may have limitation in producing significant amount of urease enzyme.

## Conclusions

In conclusion, this study represents the first investigation of $CaCO_3$ precipitation by *Bacillus* spp. isolated from Malaysian stingless bee products. The tested *Bacillus* spp. tested demostrated the ability to produce urease, facilitate $CaCO_3$ precipitation, and withstand harsh environmental conditions typically found in concrete environment (e.g., temperature up to 45 °C and pH ranging from 8 to 12). These finding indicate that *Bacillus* spp. isolated from stingless bee products hold potential agent for self-healing concrete. Further reseach

focusing on evaluating the effectiveness of *Bacillus* spp., particularly *B. stratosphericus* PD6, in remediation of concrete cracks would be valuable. Such studies could provide insights into their application for self-healing concrete, offering promising solutions for enhancing the durability and longevity of concrete structure.

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