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Potential Concrete Healing Activity of *Bacillus marisflavi* Isolated in Vietnam

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The need to use self-healing concrete in inaccessible crack repairing positions has become an urgent issue. The concrete healing is a biological mechanism. Calcite crystals ($CaCO_3$) such as calcite, vaterite, and aragonite are responsible for concrete crack healing. In this research, *Bacillus marisflavi (*from Ben Tre province), an alkaliphilic spore-forming strain that was isolated from 106 soil and concrete samples in Vietnam, exhibits urease activity which accelerates the formation of calcite crystal in the presence of urea. An assay of the bacterial addition into the concrete demonstrates a significant increase of the aragonite mineral up to 70.4%, a higher compressive activity (14.66 MPa after 14 d, and 29.32 MPa after 28 d) compared to the control (11.3 MPa after 14 d, and 15.95 MPa after 28 d). These findings show that the isolated *Bacillus marisflavi* strain has potential in concrete healing applications.

1. Introduction

In the modern era, sustainable materials and renewable energy development are gaining great interest (Ramonet et al., 2022). Concrete and cement have wide applications in construction and storage reinforcement (Iorio et al., 2022). In the case of underground storage, concrete is easily in contact with water which increases water penetration into cracks on its surface. It is challenging to repair cracks in underground concrete. Concrete self-healing is an alternative method to tackle the cracking problem. Concrete healing is a biological mechanism based on the formation of calcite crystals ($CaCO_3$) to create bridges, connecting cracks appearing on the concrete surface. In this process, cations on the bacterial surface react with counter anions in the environment, recruiting precipitation under supersaturation conditions. Bacteria can metabolize anions and produce insoluble $CaCO_3$ which hardens on the surface of cracks. There are two groups of bacteria: ureolytic bacteria and non-ureolytic bacteria. Ureolytic bacteria possess urease that converts urea to ammonium (NH_4^+) and carbon dioxide (CO_2). Both byproducts create an alkaline environment that induces the deposition of calcite crystals acting as crack healers (Hoffmann et al., 2021).
The bacterial mineralization process involves three different mechanisms such as (i) direct mineralization (ii) passive mineral precipitation and (iii) biologically induced mineralization. In the latter mechanism, mineral precipitation often occurs in the presence of metabolism byproducts such as urea hydrolysis products. Bacteria are considered nucleation sites for CaCO3 crystal formation. Urease hydrolysis accelerates the precipitation via the modification of the environmental ion saturation state. It is possible to isolate ureolytic bacteria from nature. The isolated *Bacillus sphaericus* strains demonstrated effective $CaCO_3$ precipitation on limestone cubes (Dick et al., 2006).
Calcite, vaterite, and aragonite are the three primary polymorphs of $CaCO_3$. De Muynck et al. demonstrated that biofilms may act as templates to initiate the deposition of $CaCO_3$ (De Muynck et al., 2008). The use of urea and calcium improved the resistance of the limestone to water absorption due to $CaCO_3$ precipitation (De Muynck et al., 2010). The pH of concrete is as high as 11–13 even after it is completely cured (Bang et al., 2001). At the high pH condition, almost all bacteria will die, bacterial spores are destroyed, and only alkaliphilic spores remain intact and continue to germinate. In research by Jonkers, alkaliphilic spores in concrete can remain in a survival

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state for less than 4 months (Jonkers et al., 2010). The addition of *Bacillus sp*., isolated from commercially available cement, in cement and cured with urea/$CaCl_2$ showed improved compressive strength compared to the control cement (Achal et al., 2011).

These researches demonstrated the importance of $CaCO_3$ deposits in crack healing. Using commercial laboratory strain shows a limitation in application because of strain adaptation in the environment. In order to develop a "made in Vietnam" self-healing concrete, this paper focused on the isolation of non-pathogenic alkaliphilic spore-forming bacteria which remains survive in high pH conditions of the concrete. The bacteria were screened for ureolytic activity which is a prerequisite character for $CaCO_3$ crystal formation. The bacteria will have the potential for concrete healing activity.

2. Materials and methods

2.1 Sampling site and sampling procedure

The chosen sampling sites have inclement climate conditions: Ho Chi Minh City is an urban area with daily fluctuating temperatures. The temperatures of provinces including An Giang, Binh Phuoc, Binh Thuan, and Quang Ngai usually are high. Long An and Ben Tre provinces which are located near the Pacific Ocean, are often flooded by high tides. The soil samples were collected 5 cm below ground level at the dry site to enhance the probability of spore-forming bacteria isolation. Concrete samples consisted of concrete blocks. Bacteria in samples were immediately isolated. Soil samples and concrete samples were collected from various locations, as described in Table 1.

Table 1: The number of soil and concrete samples in various sites

Place	Ho Chi Minh (HCM)	Long An (LA)	Ben Tre (Btr)	An Giang (AG)	Binh Phuoc (BP)	Binh Thuan (BT)	Quang Ngai (QN)
Number of samples	17	2	36	4	19	9	19

2.2 Sampling Bacterial isolation method

Soil and broken concrete samples were immersed in a saline buffer of 0.85 % NaCl (Xilong, China). The solutions were diluted in a 10-fold dilution series to collect isolated colonies using the spread plate method on Luria-Bertani (LB) Broth (Himedia, India). Isolated bacteria were subcultured multiple times and Gram-stained.

Alkaline pH tolerance test

The pH condition of concrete is alkaline. Only bacterial spores survive in such conditions. For this purpose, spore-forming bacteria strains were selected by subjecting bacterial culture to heat shock 70 ºC for 1 h to kill all living bacteria, and the spores remained intact. Selected strains were then cultured in LB within 36 h, at room temperature to induce spore formation. The culture was heat shocked at 70 ºC 1 h to kill living bacteria. Spores were harvested by centrifugation, immersed in a pH=13 solution for 24 h, and plated on dishes to estimate the spore germination rate after alkaline pH treatment.

2.3 Urease test

The urease test was conducted using Stuart's Urea Broth (composition for 1 L): yeast extract (0.1 g) (Himedia, India), urea (20 g) (Xialong, China), potassium phosphate monobasic (9.1 g) (Xialong, China), potassium phosphate dibasic (9.5 g) (Xialong, China), phenol red (0.01 g/L) (Shanghai Zhanyun Chemical, China), pH 6.8 (Brink, 2010). Isolated strains were cultured at room temperature for 24 h. The bacteria were inoculated into Stuart's Urea Broth eppendorf tubes at 35 ºC, and the broth color changes in the broth were observed after 48 h. Ureolytic bacteria will use urea to produce ammonia. Ammonia alkalizes the medium and causes the color change of phenol red to bright pink. If the bacteria were positive for urease, the broth would turn bright pink.

2.4 $CaCO_3$ formation test

Urease-positive bacteria decompose urea into NH_4^+ and CO_3^{2-} in the presence of $Ca(OH)_2$ (0.014 M) (Xilong, China), white precipitation forms in the test tube after 24 h of incubation at room temperature. White precipitation indicates the formation of $CaCO_3$.

2.5 Concrete block sample

Concrete blocks with a measurement of 40x40x160 mm were prepared according to Vietnam standards (TCVN 6016:1995). The concrete blocks were prepared using the machine with a mold to ensure similarity among the samples. The samples were left in open air for 24 h, immersed in water for 14 or 28 d, and then measured for compressive strength. The composition of concrete is introduced in Table 2.

Table 2: Concrete composition in detail

	Control	Concrete + bacteria
Cement PC40 (g)	450	450
Standard sand (g)	1350	1350
Water (g)	225	_
Bacterial culture (10^8-10^9 spores/g)	_	225
Urea (g)	_	2
Yeast extract (g)	_	4

2.6 X-ray diffraction (XRD) and concrete compressive strength measurement

XRD is used to identify the minerals composing. Analysis of airborne imaging spectrometer data can directly map mineral occurrences by detecting diagnostic spectral absorption bands, the shape and position of which are determined by the individual mineral structure. 1 g of the sample was ground into a fine powder which was then placed in a tray and analyzed using XRD (D8-ADVANCED).
The compressive strength was measured by Tecnotest, Modena, Italy. Compressive strength is calculated from the failure load divided by the load-bearing cross-sectional area and is reported in MPa unit.

3. Results and discussion

3.1 Screening spore-forming bacteria

The spore-forming bacteria are able to survive in harsh conditions such as high pH of concrete. A quantity of 106 isolated bacteria strains was cultured for 36 h. Then they were heat-shocked at 70 ^oC for 1 h in order to eliminate living bacteria and placed onto Petri dishes, which were later incubated at room temperature for 24 h. 34 of 106 isolated bacteria strains showed spore-forming capacity: Ho Chi Minh City (9/17), Long An (0/2), Ben Tre (1/36), An Giang (1/4), Binh Phuoc (4/19), Binh Thuan (7/9) and Quang Ngai (12/19) (Table 3). The results showed that sample sites close to the ocean such as Long An and especially Ben Tre provided less spore-forming bacteria strain than other sites. Among 36 isolated bacteria strains from Ben Tre, only one strain can produce spores.

Table 3: The number of spore-forming strains in various sites

Place	Ho Chi Minh (HCM)	Long An (LA)	Ben Tre (Btr)	An Giang (AG)	Binh Phuoc (BP)	Binh Thuan (BT)	Quang Ngai (QN)
Number of spore-forming bacteria strain	9	0	1	1	4	7	12

3.2 Screening of alkaline tolerance strain

Spores from the isolated spore-forming bacteria strains were incubated in a solution with a pH of 13 for 24 h at room temperature. The cultures were then spread onto Petri dishes to evaluate the spore germination rate. The number of observed bacterial colonies correlated with the number of spores that remained intact under high pH conditions (Table 4).

Table 4: Spore germination rate of 34 selected bacteria strains after 24 h incubation in pH of 13

Strain	HCM.1.1.1	HCM.2.1.1	HCM.2.1.2	HCM.2.1.4	HCM.2.3.3	HCM.2.3.5	HCM.3.1.4
Spore germination rate (%)	2.38 ± 0.06	2.15 ± 0.22	2.25 ± 0.22	2.03 ± 0.14	2.17 ± 0.32	2.26 ± 0.13	1.84 ± 0.12
Strain	BTr.1.1.2	AG.1.1.1	BP.1.1.4	BP.1.2.4	BT.1.1.1	BT.2.2.1	BT.2.2.3
Spore germination rate (%)	2.89 ± 0.1	1.95 ± 0.15	1.22 ± 0.19	1.03 ± 0.15	2.39 ± 0.1	2.65 ± 0.18	2.5 ± 0.13
Strain	BT.2.2.4	BT.2.2.5	BT.2.2.6	QN.2.1.1	QN.2.2.2	QN.2.3.1	QN.2.3.2
Spore germination rate (%)	1.5 ± 0.1	1.9 ± 0.18	2.7 ± 0.1	1.18 ± 0.14	1.34 ± 0.08	1.22 ± 0.24	1.09 ± 0.21
Strain	QN.2.4.1	QN.2.4.3					
Spore germination rate (%)	1.24 ± 0.17	1.35 ± 0.05					

The majority of bacteria strains showed spore germination rates ranging from 1 % to 2.5 %. In Ben Tre, only 1/36 isolated bacteria strains can produce spores. And that strain (BTr.1.1.2) showed the highest spore germination rate at 2.89 %. Spores from 7/9 isolated strains from Ho Chi Minh, 1/1 strain from An Giang, 2/4 strains from Binh Phuoc, 6/7 strains from Binh Thuan, and 6/12 strains from Quang Ngai were alkaliphilic.

3.3 Screening for ureolytic bacteria and $CaCO_3$ formation test

Out of the 34 strains tested for urease production activity - 5 strains (HCM.2.3.3, HCM.2.3.5, BTr.1.1.2, BT.1.1.1, BT.2.2.3) showed persuasive results. These strains were later tested for $CaCO_3$ formation in the presence of $Ca(OH)_2$. Under these conditions, urease helps to form white $CaCO_3$ precipitation, which appears at the bottom of the tube. The CO_3^{2-} ions generated during metabolism combine with Ca^{2+} ions to form stable precipitation, which is responsible for the concrete healing process. Among the 5 strains, BTr.1.1.2 and BT.2.2.3 showed massive white precipitation in comparison with the others (Table 5).

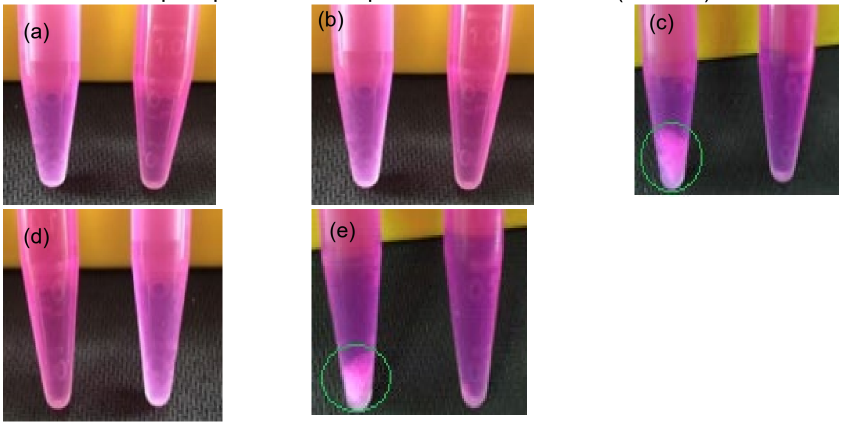

Figure 5: $CaCO_3$ formation test in 5 ureolytic bacteria strains (a) HCM.2.3.3, (b) HCM.2.3.5, (c) BTr.1.1.2,(d) BT.1.1.1 and (e)BT.2.2 .3

3.4 Compressive activity

Strains BTr.1.1.2 and BT.2.2.3 were selected to prepare concrete blocks for further study of compressive activity on the 14th and 28th days after water immersion. The result showed that the concrete samples with the addition of BTr.1.1.2 exhibited significantly higher compressive strength with an increase of 38.8 % after 14 d and 78.8 % after 28 d in comparison with the control. The compressive samples with the addition of BT.2.2.3 were lower than the control after 14 d and slightly increased after 28 d in comparison with the control (Table 6). The result demonstrated that by the time BTr.1.1.2 increased compressive strength more effectively than BT.2.2.3 didn't have a great impact on the nature of concrete. Each strain added to the concrete may have a different effect on compressive strength. The reasons could be (i) the surface charge of each strain, (ii) ureolytic activity, or (iii) adaptation of each strain in a concrete environment. The contrasting effect has been also observed in *B. sphaericus* in mortar where it reduced the compressive strength by 15 %-34 % (Wang et al., 2014) but it increased the compressive strength at 7 and 28 d (Achal et al., 2013).

Table 6: Compressive strength test

	Control (MPa)	Concrete + BTr.1.1.2 (MPa)	Concrete + BT.2.2.3 (MPa)
Day 14th	11.3 ± 0.31^a	14.66 ± 0.57^b	9.09 ± 0.19^c
Day 28th	15.95 ± 0.89^a	29.32 ± 1.23^b	17.34 ± 0.78^a

3.5 $CaCO_3$ mineral composition analysis

The XRD spectral results showed the presence of $CaCO_3$ in both the control and the sample with bacteria. Overlaying the 2 XRD spectra, the aragonite mineral in the bacteria-supplemented sample was much higher than in the control reaching up to 70.4 %. This indicates that the bacterial strain BTr.1.1.2 has the ability to heal cracks through high-level production of $CaCO_3$. The presence of calcite mineral crystals in the concrete plays an important role in improving its mechanical properties. When compared to the standard spectrum of $CaCO_3$, aragonite was present (peaks A) (Figure 1).

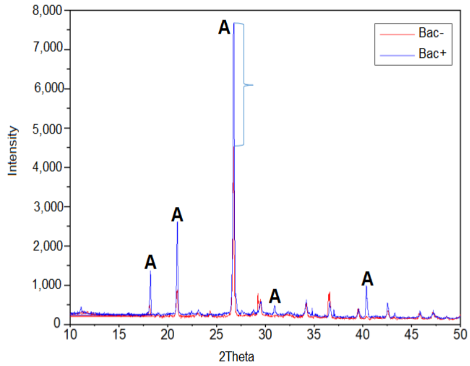

Figure 1: XRD spectrum of control concrete block (red) and concrete block with BTr.1.1.2 (blue)

3.6 Identification of BTr.1.1.2

BTr.1.1.2 was sent to the identification of the bacterial strain. The sequencing of its 16S RNA showed 100 % match to *Bacillus marisflavis*. The sequence is shown in Figure 2.

>HM02386-F
TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGATCGA
TGGGAGCTTGCTCCCTGAGATCAGCGGCGGACGGGTGAGTAACACGTGGGTAACC
TGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACACCTA
CCCCCGCATGGGGGAAGGTTGAAAGGTGGCTTCGGCTATCACTTACAGATGGACC
CGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAA
GTGCCGTTCGAATAGGGCGGCGCCTTGACGGTACCTAACCAGAAAGCCACGGCTA
ACTACGTGC

Figure 2: Sequence of 16S RNA of BTr.1.1.2

B. marisflavi has been isolated from agricultural soil in India (Gupta et al., 2020) and from seawater in the Yellow Sea (Wang et al., 2015). It has various applications such as nanoparticles synthesis with ovicidal, larvicidal, and pupicidal activity (Thermal and Balasubramanian, 2021); carotenoid production (Wang et al., 2015); cyanide remediation (Mekuto et al., 2016); and cadmium remediation (Yu et al., 2020). Ureolytic *Bacillus* species have been reported as concrete healing factors such as *B.subtilis* (Khushnood et al., 2020), *B.megaterium* (Krishnapriya and Babu, 2015), and *B.sphaericus* (Chaerun et al., 2020). In this paper, *B.marisflavi* was isolated from soil in Ben Tre province, Vietnam, and exhibits potential for concrete healing activity that can be applied to the green material industry.

4. Conclusions

The study focused on the isolation of non-pathogenic bacteria capable of repairing cracks in concrete. A total of 106 strains were isolated from 7 different cities/provinces, among them, BTr.1.1.2 was identified as *Bacillus marisflavi*, isolated in Ben Tre province. The strain exhibited characteristics, including Gram-positive, ureolytic, alkaliphilic spore-forming, and capable of forming aragonite crystal as $CaCO_3$. The aragonite crystal amount in the concrete sample supplemented with *Bacillus marisflavi* reached 70.4% higher than in the control. The concrete samples supplemented with bacteria exhibited a significantly higher compressive strength compared to the control with an increase of 38 % at day 14 and 78.8 % at day 28 after water immersion. Spores from *Bacillus marisflavi* are tolerant to alkaline conditions of concrete and easy to produce on a large scale. Realizing the need for the development of "made in Vietnam" self-healing concrete, these findings showed the potential of *Bacillus marisflavi* in the application of self-healing concrete which improves durability and reduces maintenance cost, especially, underground crack healing.

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