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Screening of native ureolytic bacteria for self-healing in cementitious materials

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Abstract. In recent years, microbial calcium carbonate has been recognised for its potential to self-heal cementitious material by mimicking the natural biological systems of healing wounds. Thus, the inevitable microcracks and concrete pores can be filled with microbial calcium carbonate to prevent any aggressive chemical flow or water and prolong the life span of the structure. Recently, numerous studies have reported on bio-concrete incorporating bacterial species such as *B. sphaericus* and *B. pasteruii*, although there are limited studies on the *B. pseudomycoides* species. In this study, new native ureolytic bacteria were isolated from soil samples collected in Universiti Teknologi Malaysia. The morphology, characteristics, and ureolytic production of the bacteria were investigated through biochemical tests. The bacterial enhancement efficiency of the concrete compressive strength was also examined. The results demonstrated that the bacteria are gram-positive with encouraging characteristics such as endospore formation, which is required for application in harsh environments such as concrete. The native bacteria also demonstrated high urease enzyme productivity. Furthermore, the applicability of the bacterial spores and vegetative cells-urea solution as a healing agent in concrete was demonstrated by increasing concrete compressive strength by 10% and 15% compared to the control sample with the optimum cell concentration of 2×10^7 cell/ml. This finding was attributed to the precipitation of calcium carbonate in the pore volume and confirmed by X-ray diffraction (XRD).

1. Introduction

In general, concrete structures are damaged by the continued deterioration of its microstructure. This occurrence is linked to its low tensile strength, which provokes the progression of microcracks inside the concrete matrix. Typically, the microcracks create an easy path for water and harmful chemical materials that affect the durability of the structure [1, 2, 3] . This is a severely challenging issue for civil engineers, particularly with the increasing costs incurred from the rehabilitation and maintenance of concrete structures worldwide. Consequently, efforts to incorporate bacteria or polymers into the concrete were potentially established its usefulness without human intervention in recent years [4, 5, 6]. The concept of self-healing in affected cementitious material was derived from monitoring the remarkable natural biological system of healing damaged wounds in human skin through the blood clotting mechanism.

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Specifically, ureolytic bacteria has shown its ability to form spores in concrete, which enable it to survive the harsh concrete environment for many years until cracks occur. Subsequently, the nutrient spores activate, multiply, and produce urease enzyme with water, which in turn converts urea to carbonate ions [7]. The carbonate ions subsequently react with calcium ions to precipitate calcium carbonate on the cell wall of the bacteria, as shown in Figure 1. This microbial self-healing technique presents several benefits, including the long term, rapid and sustained crack repair properties. In addition, it is also environmentally friendly compared to chemical-based methods.

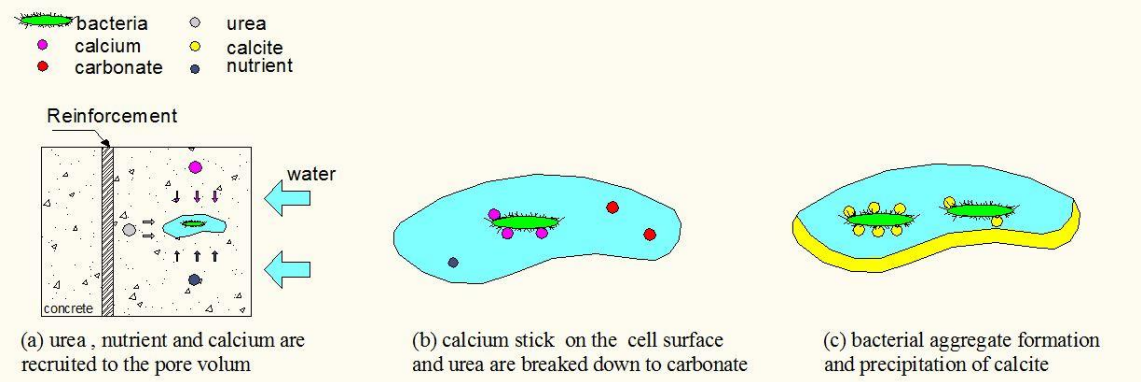


Figure 1. Microbial calcium carbonate evolution via ureolytic bacteria.

The improvement of compressive strength is one of the most crucial indicators and evidence of bacterial concrete (bio-concrete) based on the many different bacteria species in the literature. For example, the compressive strength of concrete specimens incorporating 10^5 cells/ml of *B. megaterium* BSKAU, *B. megaterium* MTCC 1684, *B. licheniformis* BSKNAU and *B. flexus* BSKNAU were enhanced by 12.1, 16.1, 10.6 and 6.1%, respectively, which were higher than the control specimens at 28 days under tap water curing [8]. Similarly, *Lysinibacillus* sp. I13 demonstrated the potential for application in concrete by increasing the compressive strength (up to 34.6%), compared to the control specimens [9]. The study that incorporated 10^5 cells/ml of *B. ACRN5* associated with urea and calcium ions reduced chloride penetration and increased the compressive strength of cement-water mixture by 14.94% after 36 days of curing [10]. Therefore, many investigations have been evaluting concrete incorporating different species of bacteria such as *B. pasteruii*, *B. megaterium*, *B. sphaericus*, *B. subtilis* and *B.thuringiensis*. However, other bacteria species, such as *B. pseudomycoides,* was not yet utilized in the literature. Thus, the focus of this study is to introduce new native tropical bacteria, namely *B. pseudomycoides*, and examine its suitability in a concrete environment based on the compressive strength of both the control and bacterial concrete.

2. Experimental work

2.1 Bacterial isolation and colony morphology

The native *B. pseudomycoides* species was isolated from a soil sample in Universiti Teknologi Malaysia (UTM) using the streak plate technique on nutrient agar as well as further identified using 16S rRNA sequencing. The pure colony was subcultured on a new agar plate to examine its characteristics such as colony morphology. Colony morphology is a description of the appearance of the colonies since each bacterium has a distinct shape, size, and colour. In the present study, the target colony was observed with the naked eye, as colony morphology is not challenging to recognise. In addition, gram staining test was carried out to examine the thickness of the bacteria cell wall [11]. The cell wall is one of the main features used to differentiate its capacity to withstand harsh conditions [12]. Previously, two structural formats of cell walls have been categorised, namely; gram-negative and gram-positive. Gram-positive has a thicker cell wall, which is constructed with peptidoglycan that makes it extremely robust and highly interactive to cations and resistant to severe environments such as concrete.

In particular, the types of the bacterial cell wall were identified based on their ability to stain and examined under a microscope in several steps. Firstly, a single colony was isolated from the agar plate using the inoculating loop and then swirled in a water drop on a clean glass slide. This was subsequently heated by cautiously waving the slide over the flame repeatedly. Secondly, the smear slide was flooded with crystal violet (purple colour) for one minute before rinsing with tap water. Thirdly, the smear slide was flooded again with Gram's iodine solution, which acted as a mordant to bind with the crystal violet for one minute before finally rinsing with tap water. Fourthly, ethanol (95%) was applied to the smear slide for a few seconds until decolourisation before it was then rinsed with water. Subsequently, the slide was exposed to safranin (pink colour) for one minute before washing. Finally, the bilbous paper was utilised to wipe the slide before examining it under a microscope.

2.2 Endospore test

The endospore-forming ability of the isolated bacteria was revealed under a light microscopic using spore staining technique [8]. Bacteria smear on the glass slide was prepared by heat fixing. This was subsequently covered with a filter paper and saturated with Malachite green colour (0.5%), followed by heating until steam was detected from the slide surface for 3 minutes. After that, the filter paper was removed, and the slide was thoroughly decolourised by rinsing with tap water, followed by staining with diluted safranin. Finally, it was immediately examined under a microscope after washing and blotting the slide.

2.3 Ureases test and microbial calcium carbonate examination

To examine the ureolytic activity, the isolated bacterial species were inoculated in 15 mL of fresh broth (LB) in a flask in triplicate. It is important to note that the nutrient broth (LB media) and equipment must be autoclaved at 100 °C for 15 minutes before use. Next, the new media was supplemented with 1 µg/mL of phenol red pH indicator and 10 g/L of urea, adjusted to 6.8 pH and incubated at 37 °C for 24 hrs. The presence of urease enzyme was detected through the change in the colour of the media from yellow to pink [13].

On the other hand, the microbial calcium carbonate precipitation and *B. pseudomycoides* species were grown on a 100 ml flask filled with 25 ml broth supplemented with 2% calcium nitrate and 2% urea, which is known as urea-calcium-broth (U–Ca broth). The growth condition was preserved at 37 °C by shaking at 150 rpm for seven days. Later, it was transferred into a centrifuge tube to harvest the bacteria by centrifuging the media at 10,000 rpm for 10 minutes, followed by overnight drying in an oven at 70 °C. Finally, the bacterial aggregate formation was analysed with x-ray diffraction (XRD) to identify the bio-product.

2.4 Bacterial growth test

This test is critical to monitor the behaviour of bacteria in terms of the number of bacterial cells and the required time to achieve optimum density. In general, bacterial growth is categorised into four main phases [14]. In this study, 25 ml of autoclaved LB broth was inoculated with the target overnight culture and grown for 48 hrs at 37 °C by shaking at 150 rpm. Later, 1 ml was taken each hour to measure the optical density using a spectrophotometer at OD600 during the first 12 hours. The other measurement was repeated every 6 hrs.

2.5 Compressive strength of concrete

The influence of ureolytic activity on self-healing concrete was evaluated in terms of concrete compressive strength. Firstly, the control concrete mixture was designed to achieve a compressive strength of 30 MPa at 28 days, based on the specifications of the UK Department of Environment (DOE). Secondly, the bio-concrete mix was incorporated with a bio-healing agent, which included the nutrient, bacteria, urea, calcium and bacteria solution. Two types of bacteria solution were prepared; spores and urea-vegetative solution. Next, 2% urea and 2% calcium nitrate were mixed with water for the urease enzyme activity and its function in bacterial aggregate formation, respectively. It was accompanied by

nutrient (0.3% yeast extract) for bacterial growth. For the bacterial solutions, both spores and urea-vegetative cells, were diluted with mixing water to obtain the target cell concentrations. In other words, a part of the bacterial solution was only replaced by a part of mixing water on the condition that the water-cement ratio must be fixed.

2.6 Preparation of bio-healing agent

To prepare the bacteria spores, the required flasks were first filled with autoclaved LB broth with pH 8, followed by inoculation of the bacteria in laminar flow. Next, 0.01 g/L of $MnSO_4.H_2O$ was added into the media to enhance the sporulation before incubation [15]. The culture was then incubated in a 30 °C incubator at 100 rpm for 14 to 30 days to ensure that at least 90% of the vegetative cells were converted into spores. Moreover, the bacterial cells were harvested to isolate them from the media by centrifuging the media for 4mins at 5000 rpm at 20 °C. The spores were subsequently re-suspended in a saline solution before washing at least twice using distilled water. The spore solutions were stored in a 4 °C room for further use with a concentration of 10^9 cells/ml.

3. Results and discussion

3.1 Characterisation of bacterial strains

The colony morphology of the isolated pure strain was distinguished by its ability to form chains of cells and was creamy white, as shown in Figure 2 (a). Furthermore, the native bacteria had a positive result; hence, it was classified as gram-positive. Gram-positivity greatly influences communication with its surrounding environment by creating a quixotic crystal nucleation site for calcite precipitation. Specifically, the gram-positive bacteria appeared in purple as shown in Figure 2 (b). The presence of a thick layer of peptidoglycan in the bacterial cell wall leads to the appearance of the purple colour. Therefore, it retains the crystal violet, which was stained.

On the other hand, the endospore staining test indicated that the bacteria belonged to the family *Bacillus*, which are characterised by their capability to form spores. This is because the vegetative cells appeared in pink, whereas the endospore appeared in green as shown in Figure 2 (c). The spore is essential in the concrete environment since the temperature may reach up to 70 °C during cement hydration along with the exposure to both chemical and mechanical stress during mixing [8].

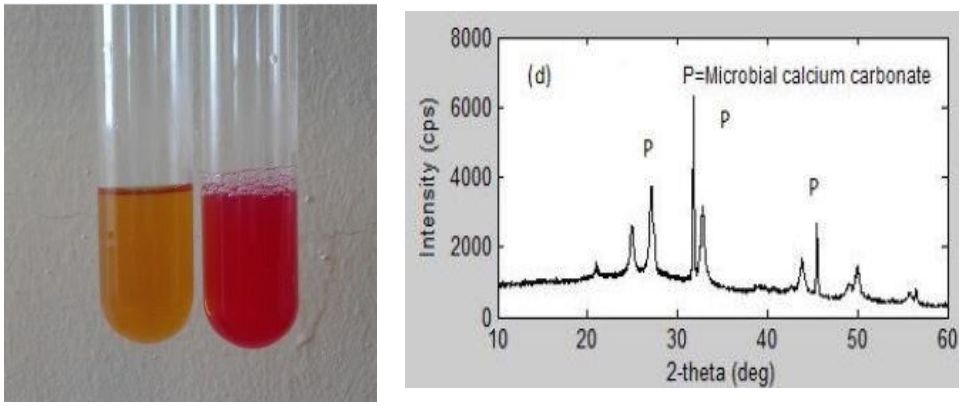
(a) (b) (c)

Figure 2. Bacterial strain identification.

3.2 Ureolytic activity and calcium carbonate identification

After confirming that the bacteria belong to the *Bacillus* genus due to the positive outcome of both gram stain and end spore tests, a preliminary screening was also conducted. This was to ensure that the bacteria are under the ureolytic bacteria strain by showing its capability to induce urease enzyme through both urease test and calcium carbonate identification using XRD, respectively. First, the urease test revealed

the capability of the bacterial strain in releasing the urease enzyme. The purpose of the urease enzyme was to utilise urea as sources of nitrogen and energy by converting urea into carbonate and increasing the ambient pH. The presence of the urease enzyme was detected by the change in the colour of the medium from yellow to red, as shown in Figure 3 (a). Secondly, the results of XRD quantitative analysis of the biochemical compound precipitation ($CaCO_3$), which is shown in Figure 3 (b), revealed that the calcium carbonate bio-product was identified as vaterite (V) with a smaller amount of calcite (P). Vaterite and calcite are the most common crystalline polymorphs of calcium carbonate produced through microbial urea hydrolysis [16]. In this research, both native bacteria primarily formed vaterite crystals instead of non-ureolytic bacteria, where the calcium carbonate crystal was chemically formed by the reaction of urea and calcium ions.



(a) (b)

Figure 3. Ureolytic activity test.

3.3 Compressive strength of concrete

The indigenous isolated *pseudomycoides* species have shown its suitability to be used in concrete. The compressive strength of the concrete was enhanced by incorporating the optimum concentration of the bacterial cells. It was clear that the compressive strength of the bio-concrete increased slightly with lower cell concentrations of 5×10^6 and 10^7, compared to the control samples, as shown in Figure 4. The enhancement of the bacterial concrete strength was approximately 6% and 12% respectively, while the compressive strength of the control concrete was 36.1 MPa at the age of 28 days. In the same context, the highest increase of bio-concrete compressive strength incorporated with a urea-vegetative solution was obtained at a cell concentration of 2×10^7 cell/cm^3, which were approximately 15% in comparison with the control mix. The positive results were associated with the formation of microbial calcium carbonate due to the metabolic process of the bacteria. In particular, the bacterial aggregates would fill both the concrete pores and microcracks (which appeared during cement hydration), thus resulting in enhanced concrete strength. This metabolic process coincided with previous research, which reported rapid crystal formation, owing to a variety of ureolytic bacteria strains [17].

Moving on, a bacterial cell concentration of greater than 2×10^7 decreased strengths, in which a compressive strength of 34.2 MPa was detected at a cell concentration of 10^8, compared to 36.1 MPa in the control mix at 28 days. The noticeable negative change of strength was associated with the replacement of excessive LB broth with water which was in line with the previous studies [18]. Specifically, this phenomenon was attributed to the negative impact of immoderate chemical ions induced by the metabolic activity of the ureolytic bacteria on the compressive strength of the concrete, which hindered the process of cement hydration

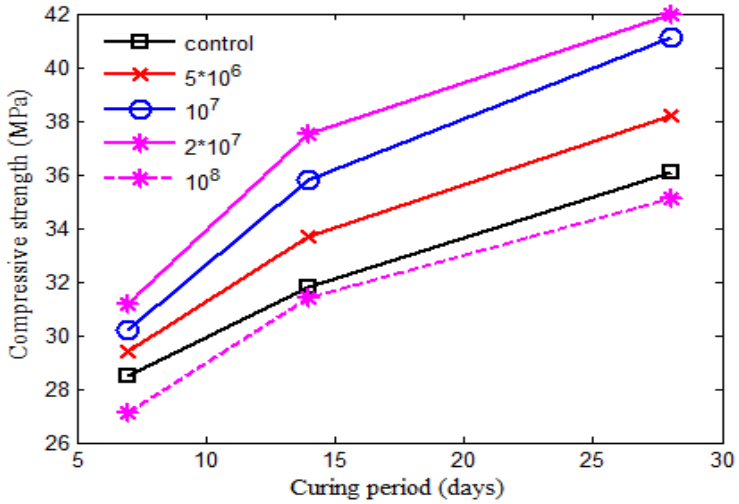


Figure 4. Enhancement of concrete strength with different bacterial cells concentration.

Apart from the bio-concrete incorporated with urea-vegetative cells solution, Figure 5 shows that there was also a convergent increase in the compressive strength of the concrete series that was separately incorporated with bacterial spore solution.

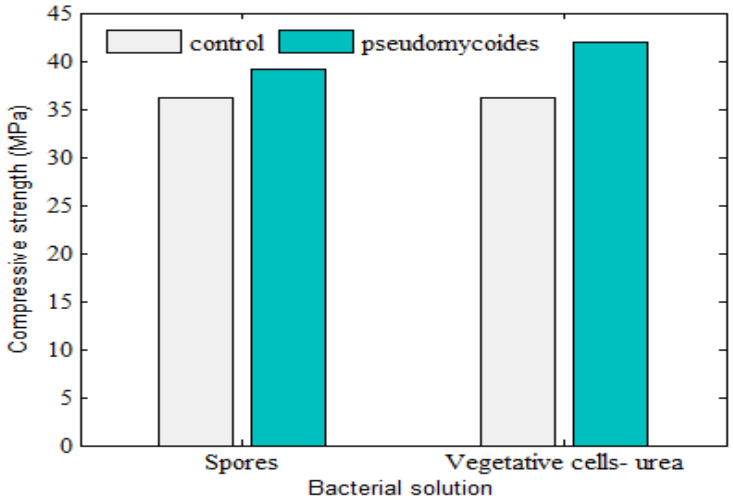


Figure 5. Effect of the different bacterial solution on concrete strength at 28 days.

The enhancement of concrete strength was around 10% in comparison with control specimens. The results confirmed that the urea-vegetative cells solution has a better positive result compared to other solutions, which were attributed to the availability of carbonate ions. In other words, the spore solution has a limited function due to the harsh environment inside concrete pores. this finding was in agood agreement with prior studies which indicated that the ureolytic activity encountered challenges inside the concrete pores such as high pH of 13, limitation of oxygen and nutrient as well as the decreasing of pores size due to continuous cement hydration[19].

Meaning that the ability of the bacteria to proliferate and hydrolyse urea in a concrete crack mouth is better than the concrete pore since the crack zone has lower pH. In addition, the native microbe would be better exploited for self-healing cracks to prolong the concrete life span, which is the primary purpose of incorporating the bacteria inside the concrete matrix.

4. Conclusion

Harnessing bacterial aggregate to improve the particular characteristics stimulated the interest of durable and green buildings in recent years. In this research, new indigenous tropical bacteria demonstrated its ability to form spores and induce microbial calcium carbonate by urea hydrolysis activity. The influence of concrete incorporating the bacteria spores and vegetative cells–urea solution has been examined through compressive strength with different cell concentrations. Bacterial cell concentration of 2×10^7 cell/ml was detected as optimum to increase the compressive strength up to 10% and 15% respectively, in comparison with the control mix. The observed results were attributed to the formation of bacteria aggregate that fills the pores and obvious microcracks inside the concrete. Furthermore, XRD analysis confirmed that the precipitated material was recognised as calcium carbonate due to ureolytic activity.

5. Acknowledgements

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