Isolation and Characterization of Thermophilic Bacteria for Self - Healing Concrete

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ABSTRACT

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Microbial Induced Calcite Precipitation (MICP) derived from certain types of bacteria such as Bacillus sp. as crack healing agent in cementitious materials has been proven in previous works. The effect of bacteria-based self - healing agent application on mortar was studied to clarify possible applicability and benefit. This research paper describes the isolation and cultivation of bacteria, identification of bacteria species, creation of crack, injection of crack with bacteria solution and quantification of healing efficiency of Bacillus sp. (N7) and Bacillus sp. (N10). Besides that, different concentrations of Bacillus sp. N7 and Bacillus sp. N10 were injected into different size of crack openings in mortar samples. Bacillus sp. N7 and Bacillus sp. N10 were identified as Areurinibacillus Thermoaerophilus using 16sDNA identification and the size of the amplicons were 1500 bp. According to the data, the self-healing pattern does not always occur at the highest bacterial concentration. The best production of calcite is achieved at OD_{600} 1.0 for B. subtilis (N7) and Bacillus sp. (N10). XRD analysis showed that Bacillus sp. N7 and Bacillus sp. N10 could form calcite and vaterite phases, which were the major elements of calcium carbonate. From the observation, the variable calcite precipitation on cracks opening of mortar specimens at varying levels of bacterial concentrations is the explanation for the presence of the optimal bacterial concentration for a given bacterial type and mortar mix.

***Keywords**: Self-healing concrete, microbes, calcite precipitation*

INTRODUCTION

Concrete is the major component materials in construction industry due to its characteristic such as cheap, easily available and convenient to cast. Furthermore, it is fire resistant, robust, high compressive strength, and easier to handle than other construction materials. Concrete is weak in tension and cracks are inevitable in concrete (Vijay et. al., 2017). Cracks in concrete might shorten its lifespan. Various crack repair techniques exist, but they are both costly and time-consuming. Bacteria based in concrete is becoming one of the emerging methods for concrete's ability to self-healing in cracks. Recently, developing self-healing concrete technology has become an important objective for researchers in biotechnology and civil engineering sciences (Qian et. al., 2021; Nodehi et. al., 2022; Khaudiyal et. al., 2022). Mineral-producing bacteria mostly can be found in a variety of harsh environments, such soil, plants, and alkaline lakes, etc. They were able to survive in concrete paste because of their adaption to their surroundings. Microbially induced calcite precipitation (MICP) is a natural and environmentally friendly method to crack therapy. It also improves the tensile strength of the material (Jogi and Lakshmi, 2021). MICP can heal concrete fractures and reduce water and chloride ion permeability to damaging substances, allowing structures to last longer. Because of the broad range of applications, it's important to understand how microorganisms precipitate calcium carbonate (calcite) and the conditions that may limit their efficiency (Tang and Xu, 2021).

Bacillus sp. is known to be the most bacteria with the greatest ability to produce urease and spore (Ma et. al., 2020). *Bacillus* sp. is a non-pathogenic bacteria that thrives at a pH of 9.0 and has a high resistance for extreme conditions. Most research (Rezende et. al., 2021; Rauf et. al., 2020) have used a variety of approaches to introduce bacteria into concrete or mortar, which has limited our understanding of the mechanics of precipitation among microorganisms. Focusing on bacteria that could grow in the alkaline environments and live in extreme temperature (thermophilic), this study aims to determine the optimal concentration of *Bacillus sp.* (N7) and *Bacillus sp.* (N10) to produce urease enzyme for calcite precipitation. A thermophilic bacteria can survive at temperatures between 45°C and 122°C (Talaiekhozan et. al., 2014). The material used in this study is mortar. The bacterial solutions of *B. subtilis* sp. (N7) and *Bacillus* sp. (N10) were injected into the crack on surface of hardened mortar. Because of their high potential for living in strong alkaline conditions, susceptibility to activation upon exposure to water, and ability to live in high temperatures, thermophilic bacteria were chosen to be used in this research study. Through the urease activity, the bacteria work as a catalyst to convert ammonia to calcite, the crack's sealant material. The effect of bacterial development on mortars at various bacterium concentrations is also explored in this research.

MEDIA PREPARATION

In this study, bacterial growth and cultivation were carried out on nutrient broth (NB) (Oxoid) and nutrient agar (NA) (Oxoid). An amount of 6.5 g of NB powder was mixed into 500 ml of distilled water and 7 ml of dissolved NB media was dispensed into universal bottle and sterilized in an autoclave machine at 121°C for 15 minutes at 15 psi. The NB broth was kept at room temperature until further used. For NA media, 28 g of NA powder was mixed with 1000 ml of distilled water. The mixture was microwaved for 10 minutes before sterilizing at 121°C for 15 minutes at 15 psi. The NA media was cooled to 45°C before being poured (approximately 20 ml) into each sterile Petri dish and stored at room temperature until further used.

Isolation and Identification of Bacteria

Thermophilic bacteria (N7 and N10) were isolated from a Malaysian hot spring. The isolation of thermophilic bacteria was accomplished through serial dilution of up to 10^8 water samples. A total of 100µl of diluted water samples were pipetted onto the surface of the NA plate and gently spread with a glass hockey stick before being incubated at 50°C for 24 hours (h) for pure colony observation. The pure colony was streaked onto the new NA plate using an inoculating loop, and the plate was incubated at 50°C for 24 h.

Gram Staining

A loopful of the bacterial culture was spread on a glass slide and was allowed to air dry. Then, the slide was flooded with crystal violet solution for one minute. The slide was washed off with tap water and drained. Gram's Iodine solution was added and flooded for one minute. The slide was rinsed and drained with tap water. Three drops of 95% of alcohol were flooded onto the slide for 10 seconds and washed off with tap water. Lastly, the slide was flooded with safranin solution for 30 seconds and discarded by wash off with tap water. The slide was visualized in using light microscope (LEICA) for bacteria characteristic.

Urease Test

Urease test was carried out to identify the ability of thermophilic *Bacillus sp.* N7 and *Bacillus sp.* N10 to hydrolyze urea to produce ammonia and carbon dioxide. Urease is a constitutively expressed enzyme that hydrolyzes urea to carbon dioxide and ammonia.

$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$ (Eqn. 1)

$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3$ (Eqn. 2)

The urea test media contains 2% urea and phenol red as a pH indicator. The colour changes from yellow (pH 6.8) to bright pink as the pH rises due to ammonia production (pH 8.2). This medium promotes bacterial growth and allows for the detection of urease activity for calcite formation (Eqn. 2).

Genomic and Bacteria Identification

For bacterial identification, *Bacillus sp.* N7 and *Bacillus sp.* N10 isolates were sent to a third-party sequencing company. The amplification samples were examined using electrophoresis on a 1% TAE agarose gel at 100V for 60 minutes. The primers used in this study were F27 and R1492 and the DNA marker was 1Kbp (Kilo base pair).

SPECIMEN PREPARATION

A total of 12 mortar specimens were made using the mortar mix design shown in Table 1. The specimens were divided into two series of mortar, 6 cubes and 6 prisms, each 40 x 40 x 40 mm in size. Then it was crushed in a steel mould. A thin metal plate (20 x 20 mm and 0.3 mm) thick was placed into each mortar specimen to produce an artificial crack for 24 hours until solidified. The aluminum plate was removed before the mortar specimens were placed in the water pond for 28 days to cure.

Table 1: Mixture Proportion of Mortar Specimen

Materials	Quantity (kg/m^3)
Cement	422
Sand	1266
Water	211

Bacteria Injection into Cracked Specimens

After curing period of 28 days, the cracked mortars were injected with different concentration of bacteria in each specimen as shown in Table 2. The bacteria solution was injected on the crack mouth using micropipette.

Table 2: Different Bacteria Concentration Applied on Cracked Mortars

Specimen	N7 OD_{600}	N10 OD_{600}
Specimen 1	Control	Control
Specimen 2	0.2	0.2
Specimen 3	0.4	0.4
Specimen 4	0.6	0.6
Specimen 5	0.8	0.8
Specimen 6	1.0	1.0

XRD Analysis

XRD analysis was carried out using PANalytical X'Pert PRO XRD instrument (USA) at 40kV and 30 mA. XRD analysis was carried out to characterize the crystalline phases of the calcium carbonate crystals formed in the concrete. The XRD patterns for calcium carbonate crystals were detected on the inner surfaces of the concrete cracks shown in (Figure 5).

RESULTS AND DISCUSSIONS

Identification of Bacteria

Two isolates (*Bacillus sp.* N7 and *Bacillus sp.* N10) of thermophilic isolates were grown NA media by dilution streaking method. After 24h incubation, *Bacillus sp.* N7 and *Bacillus sp.* N10 showed the yellowish, irregular, raise and undulate shaped colonies were formed after 24h incubated at 50°C. Figure 1 (a) show the *Bacillus sp.* N7 and (b) *Bacillus sp.* N10 on the NA plate media after a 24h incubation at 50°C.

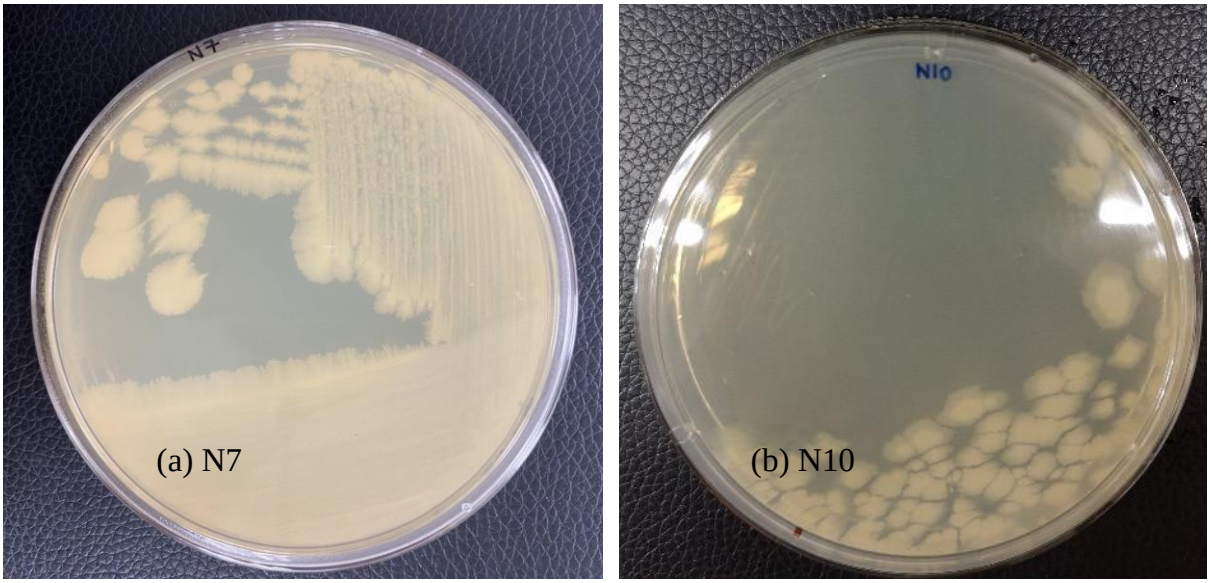

Figure 1: (a) N7, (b) N10

Gram stain observation was observed by using light microscope (LEICA) with 1000x magnification. From the observation, both isolates were a Gram positive bacteria. The morphology of *Bacillus sp.* N7 and *Bacillus sp.* N10 were rod in shaped with purplish blue in colour. Figure 2a and Figure 2b show a morphology of Gram-positive bacteria *Bacillus sp.* N7 and *Bacillus sp.* N10. Gram positive bacteria have thick peptidoglycan coatings in their cell walls, allowing them to survive in their environments (90% of cell wall) and the morphology of the bacteria was purple in colour. Gram negative bacteria have thin peptidoglycan coatings (10% of wall thickness) and a high lipid content, and the morphology observed was pink in colour.

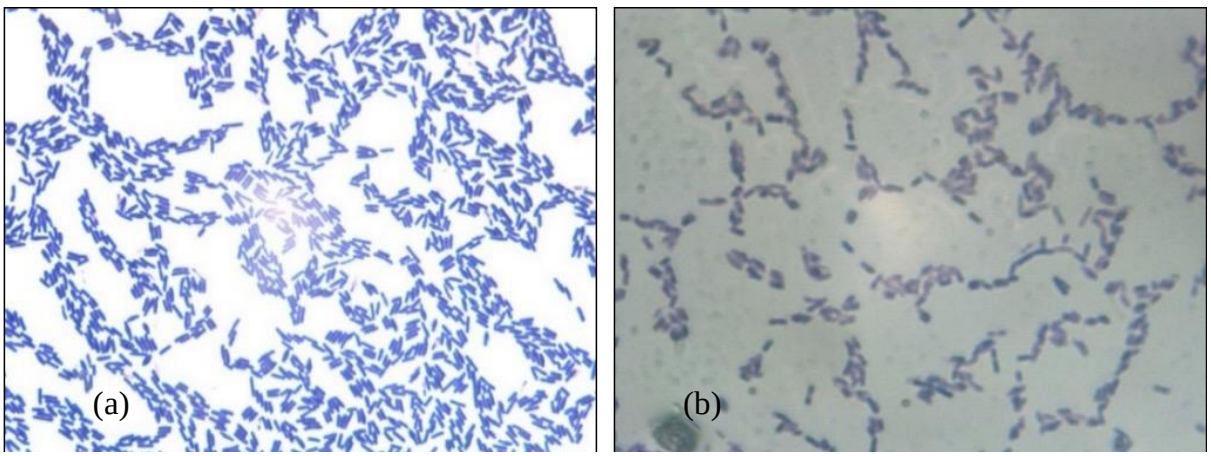

Figure 2: Purple-stained gram-positive of (a) *Bacillus sp.* N7 and (b) *Bacillus sp.* N10

Figure 3 shows the urease test findings. This approach was used to identify bacteria capable of producing calcite via urea activity. After 24 hours at 50°C, both *Bacillus sp.* N7 and *Bacillus sp.* N10 bacteria cultures turned pink (fuchsia). Thus, it is a positive reaction of urease activity for *Bacillus sp.* N7 and *Bacillus sp.* N10. The pH of the control sample culture media was less than 7, indicating that the organism was urease negative. Based on the results, it can be expected the higher calcite production as a result of urease activity.

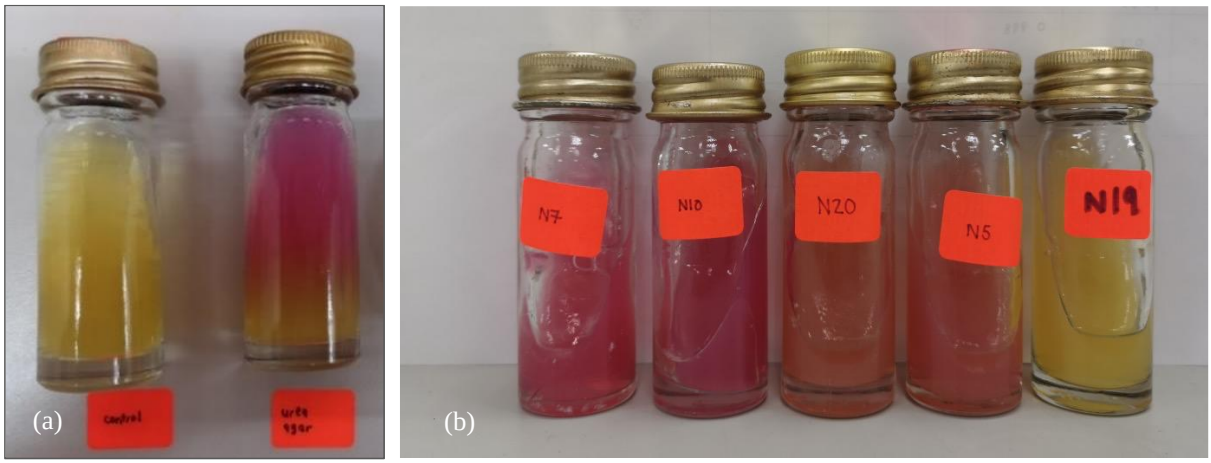

Figure 3: Positive and negative reaction of urease activity of (a) Control sample and (b) *Bacillus sp.* N7 and *Bacillus sp.* N10 isolates

Genomic and Bacteria Identification

The samples were examined by electrophoresis with 1% of TAE agarose gel at 100V for 60 min. The DNA fragments length were compared with 1Kbp (Kilo base pair) DNA marker. The DNA size of *Bacillus sp.* N7 and *Bacillus sp.* N10 were approximately 1500 bp in size. N7 and N10 were recognized as *Areurinibacillus Thermoaerophilus* based on the blast results on the NCBI Genbank Database website. Figure 4 shows an image of PCR gel electrophoresis of N7 and N10.

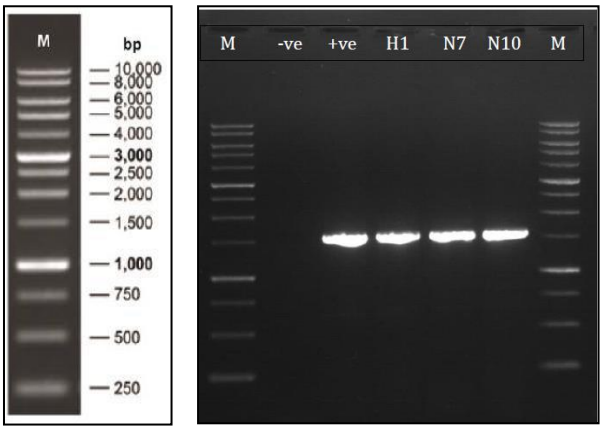

Figure 4: Image of PCR Gel Electrophoresis of N7 and N10.

Formation of Calcite in Cracked Specimens

The crack closing was visualized after 100 days of bacteria injection into mortar specimens by using Microscope Digital at 1000x magnification. The formation of white precipitation was observed at the crack mouth of the specimens. The amount of calcite precipitation was different in each specimen with different OD_{600} concentration as shown in Figure 5 *Bacillus sp.* N7 and Figure 6 *Bacillus sp.* N10. The highest production of calcite was achieved at OD_{600} 1.0 for both *B. subtilis* (N7) and *Bacillus* sp. (N10).

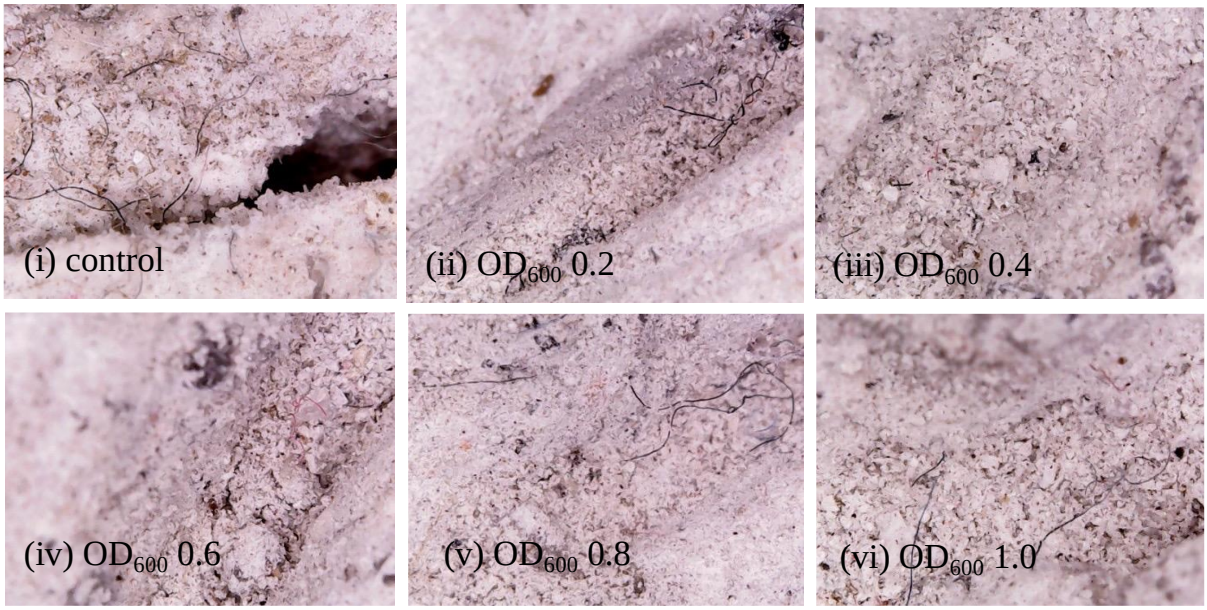

Figure 5: Image of Calcite Precipitation of *Bacillus sp.* N7

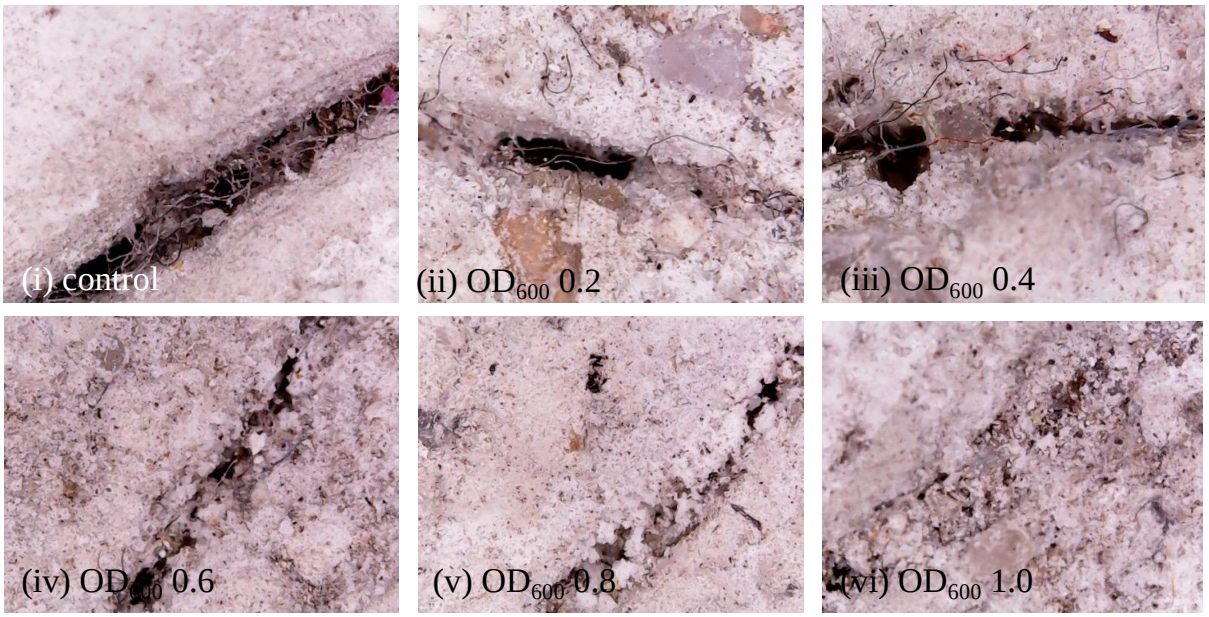

Figure 6: Image of Calcite Precipitation of *Bacillus sp.* N10

XRD Analysis

The XRD patterns for calcium carbonate crystals (dominated peak) were detected on the inner surfaces of the concrete cracks shown in (Figure 7). Both calcite and vaterite phases were found in all bacteria isolates. The ratio between calcite and vaterite phases was shown to be different for each bacteria sample. Based on the result obtained, two thermophilic bacteria *Bacillus sp.* N7 and *Bacillus sp.* N10 (*Areurinibacillus thermoaerophilus*) showed similar peak ratio between calcite (29.3°) and veterite (25°) as compared with the standard (Calcium carbonate) powder ((Figure 7 (a)(b)). The XRD pattern of *Bacillus sp.* N7 and *Bacillus sp.* N10 showed that each of bacteria isolates were able to produce calcite and vaterite phases. These two phases were the main elements of calcium carbonate. Besides that, the peak ratio showed the similar peak to calcium carbonate standard.

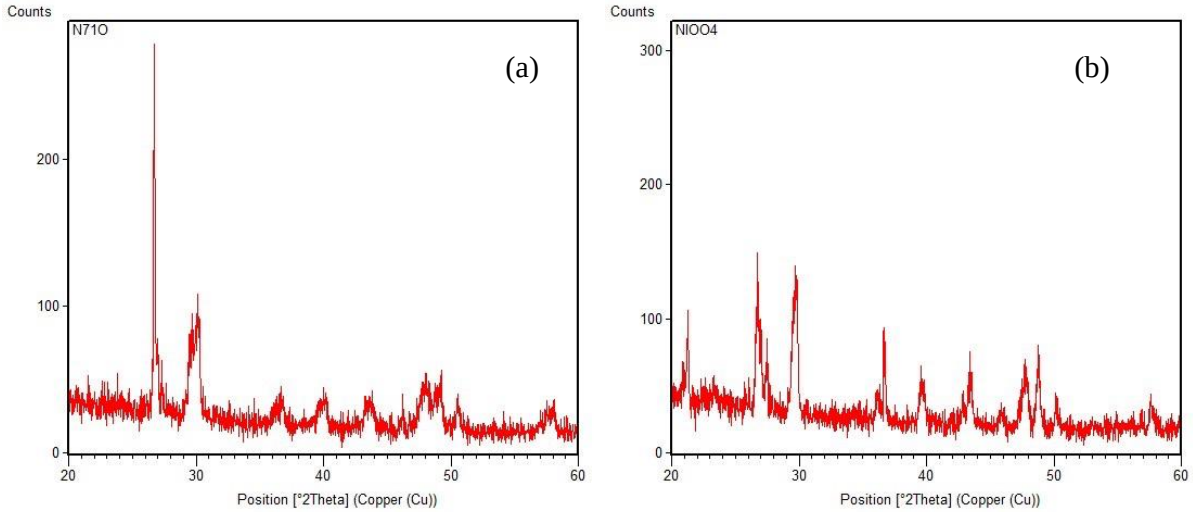

Figure 7: XRD pattern of calcium carbonate crystals formed by (a) *Bacillus sp.* N7 (b) *Bacillus sp.* N10

CONCLUSIONS

The ability of two thermophilic bacteria isolates (*Bacillus* sp. N7 and *Bacillus* sp. N10) to seal cracks in mortar specimens was investigated in this study. *Bacillus* sp. N7 and *Bacillus* sp. N10 were able to produce calcite at all concentrations tested, however the OD_{600} 1.0 resulted in the highest calcite precipitation on the cracks opening of the mortar specimens. The variable calcite precipitation observed on cracks opening of mortar specimens at varying levels of bacterial concentrations may be the reason for the development of the ideal bacterial concentration for a certain bacterial type and mortar mix.

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AUTHOR CONTRIBUTION & CONFLICT OF INTEREST

All authors contributed in the conception and design of study, acquisition and analysis of data and drafting the manuscript. The authors confirm that they have no conflict of interest from this research, except that the publication of this article will advance their CV.

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