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Research Article

Preparation of calcite-precipitating bacteria-embedded magnesium phosphate cement for self-healing application

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ABSTRACT

The present study was undertaken to check the feasibility of magnesium phosphate cement (MPC) for the immobilization of calcite-precipitating bacteria. An aqueous route of MPC synthesis was followed using magnesium phosphate $Mg_3(PO_4)_2$ powder and ammonium phosphate solution. The Fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) analysis confirmed the synthesis of MPC. The thermal decomposition analysis (TGA) showed decomposition of struvite between 50–60 °C - Paenibacillus sp. NCIM 5410 was used due to its urea hydrolysis ability. pH 9 was found to be optimum for urea hydrolysis. The urea hydrolysis steadily decreased with an increase in temperature from 30 °C to 60 °C. The hydrolysis was seen to increase with an incubation time of up to 72 h and subsequently reduced. The bacteria showed 90% urea hydrolysis at pH 9, 30 °C temperature, and after 72 h. The bacterial spores were incorporated during MPC synthesis, which helped their immobilization. The bacterial spore-containing MPC decomposed around 70 (±0.48)% of urea. Further, calcite precipitation was studied. The precipitate formed due to bacterial action in the MPC crack showed the presence of calcium. The calcite precipitation helped to reduce the water absorption by MPC specimens. The spore containing MPC specimens showed around 2.62 (±0.55) % water absorption. These results suggest that it is possible to synthesize bioactive MPC by immobilizing bacterial spores in MPC.

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1. INTRODUCTION

Following the technological revolution and the increase in human population, the construction industry is growing rapidly [1]. Concrete is an inherent part of the construction industry, and it is the Earth's second most widely used material, after water. Besides all its advantages, a concrete structure is prone to deterioration. With time, small cracks appear in concrete structures. The water enters through these cracks, leading to durability issues such as corrosion, which may damage the structure [2]. Therefore, there is an increasing demand for rapid and durable repair material [3]. Magnesium phosphate cement (MPC) has recently emerged as a fast repair material for concrete structures [4]. It differs from ordinary Portland Cement (OPC) but bonds well to the old concrete substrate [5]. It is chemically bonded ceramic. It is a new binder material formed through acid-base reactions between magnesia and the alkali metal or ammonium phosphates [6]. It is a clinker-free binder with a quick-setting, high early compressive strength, good volume stability, strong bonding strength, minor shrinkage, and good abrasion resistance [7, 8].

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With the rapid economic development, the need for basic infrastructure is increasing. This resulted in the construction of new roads. Currently, the roads are built mainly using cement concrete. The traditional maintenance method for roads involves the removal of the damaged part and then rebuilding it. In the case of cement-concrete roads, this method is not only expensive but also time-consuming. Hence, it requires quick repair options. MPC is known for its rapid setting and hardening characteristics. This makes it suitable for quick repairs, allowing faster turnaround times in concrete structure maintenance [9, 10]. It is an effective bonding agent for attaching new concrete to existing surfaces. The cement adheres well to various substrates, promoting a strong bond [11, 12]. Repairing concrete structures is paramount in natural disasters, accidents, and unforeseen situations. There might be situations where the structure needs to be repaired in a submerged or wet environment. The quick-setting nature of MPC makes it suitable for underwater repairs [7]. The corrosion resistance ability of MPC makes it the right choice for repairing structures in aggressive environments, such as those exposed to chemicals or marine conditions. MPC develops high early strength, allowing repaired structures to be returned to service sooner. This can be crucial in minimizing downtime for bridges, highways, and buildings [13, 14]. In cases where the structure's integrity has been compromised, MPC can be used as part of a comprehensive rehabilitation strategy, ensuring that the repaired sections meet the necessary structural requirements [15, 16]. To extend the applicability of MPC in concrete structure repairs, calcite-precipitating microorganisms can be embedded in the MPC matrix to achieve the self-healing phenomenon in the structures. Previously, geopolymers were used to immobilize calcite precipitation bacteria [17]. Producing geopolymer involves combining an alkaline solution that can trigger the geo-polymerization process with an aluminosilicate source, such as coal fly ash or other waste by-products [18]. The geopolymer material utilizes less carbon and is an energy-intensive process. Doctolero et al. [18] produced bio-geopolymers using alkali activation of coal fly ash mixed with self-healing agents using biochar-immobilized B. sphaericus and B. thuringiensis [18]. Ekinci and his colleagues [19] studied the microbial self-healing capacity of geopolymer binders. The Na_2SiO_3 activated GP samples were made from ground blast furnace slag (GBFS). Bacillus subtilis was used as the healing agent to produce GP samples. This resulted in geopolymer composites having self-healing mechanisms with good mechanical qualities and durability. Similarly, Soltmann et al. [20] showed that it is possible to immobilize Saccharomyces cerevisiae and Rhodococcus ruber in MPC. They produced MPC at low temperatures by mixing magnesium phosphate ($Mg_3(PO_4)_2$) powder and ammonium phosphate solution. They found the produced MPC had developed strength rapidly with low shrinkage and very good mechanical and chemical durability. The immobilization of microorganisms in the MPC matrix is promising since it follows an aqueous preparation route. Considering these benefits, the present study was undertaken to immobilize the calcite-precipitating bacteria in MPC. Such bioactive material can be used as cement plaster in sewers. The compatibility of MPC with concrete will open new paradigms for self-healing applications in concrete.

2. MATERIALS AND METHODS

2.1. The Microbial Culture and Chemicals

Paenibacillus sp. NCIM 5410 was procured from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. Magnesium phosphate powder [$Mg_3(PO_4)_2$], urea, diammonium hydrogen phosphate [$(NH_4)_2HPO_4$], ammonium dihydrogen phosphate [$NH_4H_2PO_4$], potassium phosphate dibasic [K_2HPO_4], potassium phosphate monobasic [KH_2PO_4], Nessler's reagent was purchased from Sigma Aldrich. Luria Bertani (LB) was used as a growth medium. The composition of the precipitation medium was – 3 g/L Nutrient broth, 20 g/L urea, 1 g/L ammonium chloride [NH_4Cl], and 5.6 g/L calcium chloride [$CaCl_2$].

2.2. Bacterial Spore Formation

Cells of Paenibacillus sp. were grown in LB media containing $MnSO_4.H_2O$. The media was placed in a water bath at 80 °C for 10 minutes, followed by ice water treatment for 5 minutes. Media was centrifuged at 8000 rpm for 15 minutes, then the pellet was taken, and the supernatant was discarded. Saline wash was given to the pellet, and cells were stored at 4 °C. Endospore staining was performed for confirmation of spores. A clean glass slide was taken. The bacterial sample was spread on the slide and heat-fixed. The slide was flooded with a primary stain of malachite green solution and heated to steaming three or four times. The slide was allowed to cool, and the smear was rinsed with water to remove excess stains. The bacterial cells were counterstained with 0.5% safranin, contrasting the endospores. The glass slide was washed, dried, blotted, and observed under a light microscope. Lyophilization was carried out to obtain the spore powder.

2.3. Microbial Urea Hydrolysis

0.5 g spores of Paenibacillus sp. NCIM 5410 was inoculated in 50 ml LB media and incubated at 30 °C for 24 h. 200 µl grown culture was spread on a Luria Bertani agar plate and incubated at 30 °C for 24 h. Isolated colonies were picked and inoculated in a flask with 50 ml LB media and incubated at 30 °C for 24 h. The grown culture is taken and centrifuged at 8000 rpm for 15 minutes. Then, OD was adjusted to 1, and a 500 µl culture was inoculated in urea containing LB media (50 ml). It is incubated at 30 °C for 24 h. After 24 hours, the media is centrifuged at 8000 rpm for 15 minutes. The supernatant (free of bacterial cells) was collected by centrifugation. The urea hydrolysis was determined by NASH assay. NASH reagent method gives an idea about bacterial hydrolysis of urea to ammonia and carbon dioxide. Nessler's reagent and ammonia combine to form a yellow complex, and the complex's absorbance is directly proportional to the ammonia concentration. Consequently, it can be used to calculate urea hydrolysis. First, 0.1 ml supernatant was added to 1.9 ml buffer for the NASH assay. 0.5 ml 500 mM H_2SO_4

was added. Then 0.5 ml of Nessler's reagent was added and incubated at dark conditions for 30 minutes, and absorbance was recorded on a spectrophotometer at 480 nm. Readings were acquired, and ammonium content was determined by comparing them to a standard graph. For a standard graph, known concentrations of ammonium chloride are used [21].

2.4. Optimization of Bacterial Urea Hydrolysis

Various parameters were used to study bacterial urea hydrolysis. The parameters studied were pH (7–11), temperature (30–60 °C), and time duration (24–96 h).

2.5. MPC Synthesis

The MPC was synthesized as described by Soltmann [20] with slight modifications. Various combinations of ammonium phosphate solution (10–14 ml) are mixed with 5 g magnesium phosphate powder in a beaker. The content is mixed vigorously and poured into an ice-making tray with trapezoidal shape wells. The ice-making tray is used as a mold to form the specimen. The tray is kept at 37 °C for 24 h. After 24 h, specimens were removed from the tray. The specimens, 1.8 cm x 1.5 cm x 1.3 cm in size, were formed. They were kept in a hot oven at 60 °C for 36 h. Specimens are taken out from the oven for further analysis. The spore containing MPC was synthesized by taking a mixture of 0.5 g Paenibacillus sp. NCIM 5410 spores and 4.5 g magnesium phosphate powder. An ammonium phosphate solution was added to this mixture.

2.6. Analysis of Spore Leakage from MPC

The analysis of spore leakage was carried out as described by Jadhav et al. [17]. MPC specimens with and without spores were crushed. Both samples' powder (~ 0.15 g) was added into 20 ml sterile saline in two falcon tubes. The content of the falcon was vortexed for 2 minutes and then kept static for 15 minutes at room temperature. From this solution, ten-fold serial dilutions (a total of five dilutions) were prepared. After that, 100 µl suspension was taken from an appropriate dilution and was spread onto a urea-containing agar plate homogeneously. All agar plates were incubated upside down at static conditions at 30 °C for 48 h and monitored for the appearance of colonies.

2.7. Viability of Immobilized Spores Using Urea Hydrolysis

The viability of bacterial spores was determined by studying urea hydrolysis. The MPC specimens with and without spores were sterilized by Tyndallization [22]. The specimens were then immersed in urea containing 50 ml LB media. One flask with only urea-containing media was kept as control. In another flask, free spore powder was added and used as a positive control. All the flasks were incubated at 30 °C for 24 h. After 24 h, the specimens were taken out, and the media was centrifuged to collect the supernatant. The supernatant was used to study urea hydrolysis. NASH assay was performed to check the urea hydrolysis.

2.8. Calcite Precipitation and Self-healing

For crack healing of the specimen, precipitation media was prepared with 0.3 g nutrient broth, 1 g NH_4Cl, 2 g urea, and 0.56 g $CaCl_2$ in 100 ml distilled water. The cracks were generated in spores containing MPC specimens. Calcite precipitation and self-healing were studied by running wet and dry cycles. The specimens were immersed in a 100 ml precipitation medium. The flask was incubated at 30 °C for 24 h at static conditions. After 24 h, specimens were removed from the press and dried at room temperature for 24 h. Then, the specimens were again immersed in precipitation media. Such wet and dry cycles were performed for seven days.

2.9. Effect of Calcite Precipitation on Water Absorption by MPC Specimen

The MPC specimens with and without spores were immersed in precipitation media. The wet and dry cycles were carried out for 7 days. The specimens were dried in an oven at 60 °C. Afterward, these specimens were immersed in the beaker with 100 ml of distilled water. The specimens were kept in water overnight. Specimens were taken outside. The weight of the specimens was determined before (w) and after (w′) immersing them in water. The % water absorption was calculated as shown in the formula:

$$\%Absorption=(W'-W)x100/W$$

2.10. Analytical Techniques Used for Characterization

The functional groups present in magnesium phosphate powder, MPC, etc., were analyzed by Fourier-transform infrared spectroscopy (Jasco FT/IR-6100, Japan). The microstructure was investigated using scanning electron microscopy (SEM) (FEI Nova SEM 450, Netherlands). The thermal behavior of MPC was studied by thermogravimetric analysis.

2.11. Statistical Analysis

The data analysis was performed utilizing OriginPro 2020 SR19.7.0.188 statistical analysis software. The statistical significance of the differences between means was assessed using a one-way analysis of variance (ANOVA) followed by Tukey's test. All experiments were conducted thrice; the presented values represent the means ± standard deviation (SD). Symbols with different letters indicate significant differences ($p<0.05$) between the compared groups.

3. RESULTS

3.1. The Optimization of Bacterial Urea Hydrolysis

Paenibacillus sp. NCIM 5410 was used in the present study considering its halophilic nature, urea hydrolyzing, and spore-forming ability. The endospores protect ureolytic bacteria from death under adverse conditions [23]. This makes the application of Paenibacillus sp. NCIM 5410 is suitable for the bio-cementation process. The endospores can help survive the high pH conditions of Portland cement-based concrete. It also helps to overcome desiccation and pore size shrinkage in concrete environments. The urea hydrolysis at different pH, temperature, and incubation times was experimentally investigated. As shown in Figure 1, the urea hydrolysis increased with increasing pH. PH 9 was found to be optimum for urea hydrolysis.

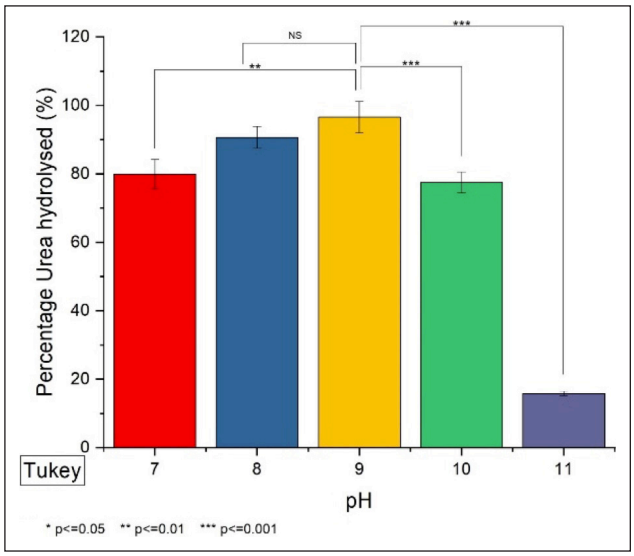

Figure 1. Effect of pH on urea hydrolysis by Paenibacillus sp. NCIM 5410.

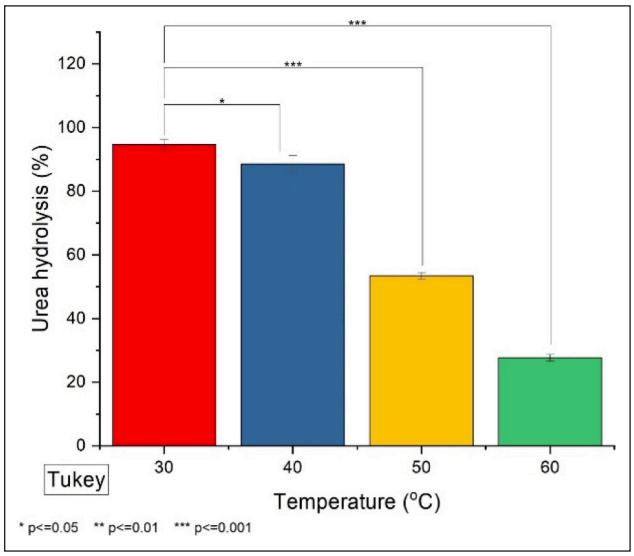

Figure 2. Effect of temperature on urea hydrolysis by Paenibacillus sp. NCIM 5410.

The urea hydrolysis decreased at higher pH values. Several researchers reported that pH 8–9 is ideal for bacterial calcite precipitation [23]. Bacterial hydrolysis of urea is important to achieve calcite precipitation [24]. Hence, the ability of Paenibacillus sp. NCIM 5410 to hydrolyze urea at pH 9 is advantageous. The effects of temperature on urea hydrolysis can be found in Figure 2. The urea hydrolysis steadily decreased and increased in temperature from 30 °C to 60 °C. The optimum temperature in the present study for Paenibacillus sp. 5410 was 30 °C. Some reports showed that 37 °C is the optimum temperature for B. sphaericus [25].

Similarly, the optimum pH in the present study was 9, whereas Singh et al. [25] got 7 for B. sphaericus [25]. Figure 3 shows the effect of incubation time on urea hydrolysis. The hydrolysis increased with incubation time up to 72 h and subsequently decreased. The increasing trend might be because of an increase in bacterial growth and an increase in bacterial production of urease enzyme. The exhaustion of nutrients, cell death, and enzyme degradation might be responsible for the decrease in urea hydrolysis after 72 h. A similar phenomenon of urea hydrolysis has been reported by several researchers [26]. Dhami et al. [27] said the urease activity to be around 650 U/ml for Bacillus megaterium S33, which was investigated at 37 °C for 96 h [27]. In the present study, the spores of Paenibacillus sp. NCIM 5410 was generated and used in further experiments to prepare bioactive MPC.

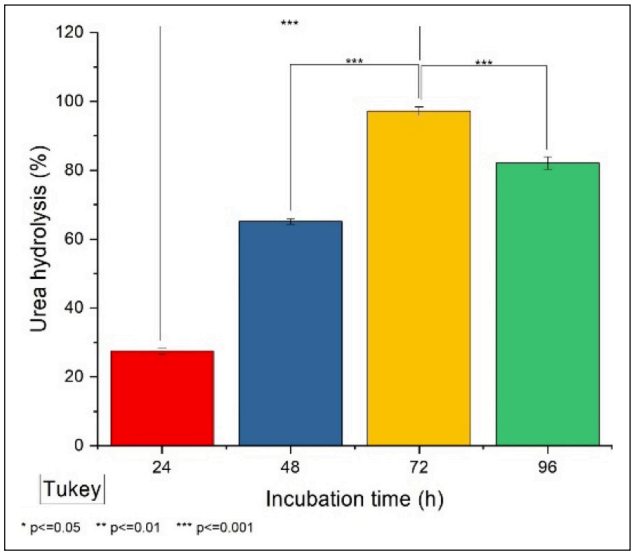

Figure 3. Effect of incubation time on urea hydrolysis by Paenibacillus sp. NCIM 5410.

3.2. Preparation and Characterization of Cements with Embedded Microorganisms

The MPC without bacterial spores was prepared as described by Soltmann et al. [20] with slight modifications using magnesium phosphate powder and ammonium phosphate solution. Adding 13 ml ammonium phosphate solution to 5 g magnesium phosphate powder gave a better consistency; hence, this combination was used in further experiments. The slurry system showed a fast setting caused by the formation of ammonium phosphate hexahydrate (Fig. 4). Soltmann et al. [20] also reported similar observations.

The reaction was carried out at neutral pH. This type of binder material is suitable for the immobilization of calcite-precipitating bacteria. MPC specimens with and without spores were not breaking after overnight treatment with water (data not shown). The MPC with bacterial spores is prepared by adding 0.5 g of spore powder to the reaction mixture. It is reported that ammonium magnesium phosphate hexahydrate [$(NH_4)MgPO_4.6H_2O$, Struvite] is formed as a primary reaction product when ammonium phosphate solution and magnesium phosphate powder are mixed [20, 28]. It is further confirmed by FTIR analysis. The functional groups are observed in the region of OH stretching vibrations of H_2O molecules at 3728, 3604, and 3532 cm^{-1}. A band at 2382 cm^{-1} corresponds to the stretching vibrations of the PO–H bond. The bands at 1643 and 1535 cm^{-1} correspond to in-plane H–O–H bending vibrations of at least two different types of water molecules (Fig. 5). The other

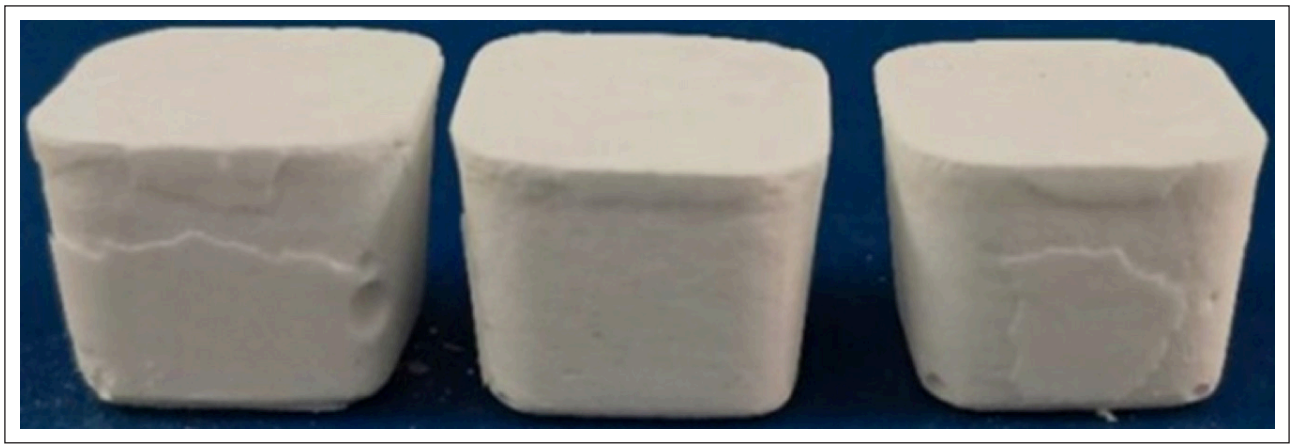

Figure 4. Magnesium phosphate cement specimen.

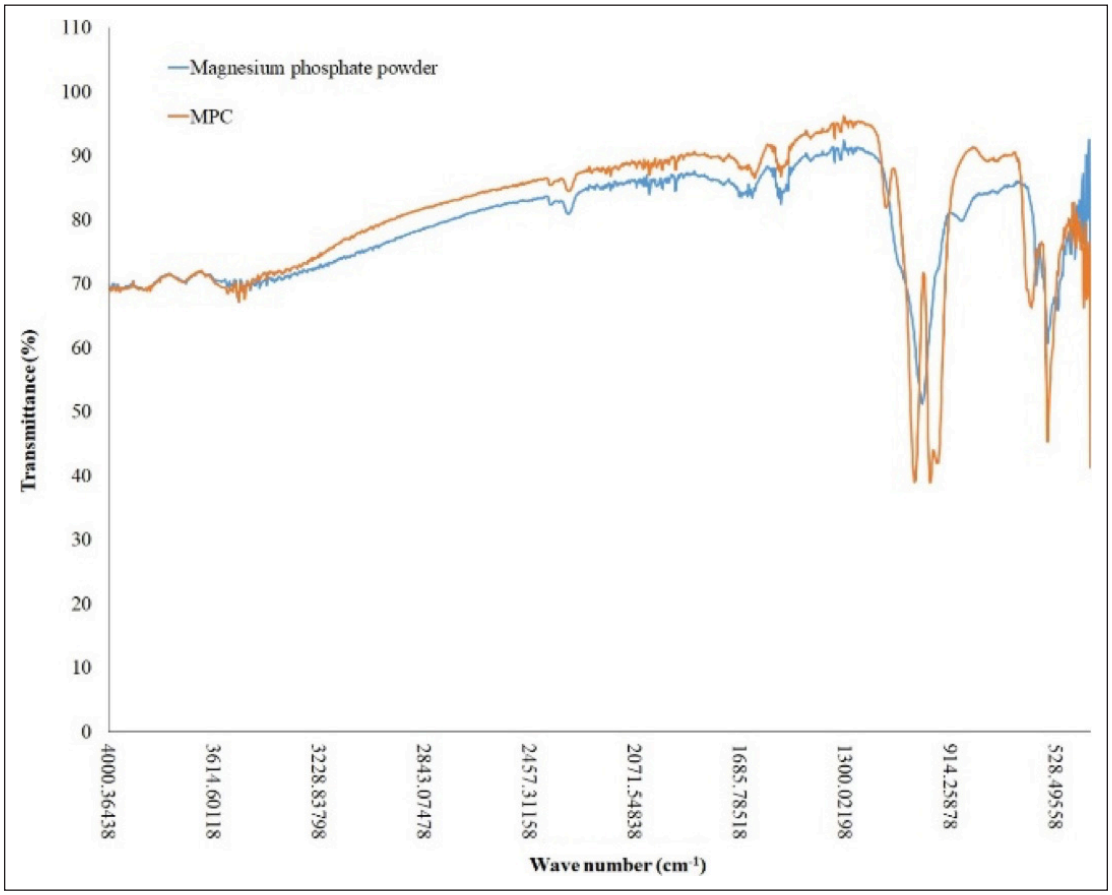

Figure 5. FTIR Spectrum of (a) $Mg_3(PO_4)_2$ (b) MPC.

bands are assigned to 1138 and 1012 cm^{-1} stretching vibrations of $(PO_4)^{3-}$ and bending vibrations at 680 cm^{-1} [29]. Meanwhile, Figure 4 shows structural vibrations of water occurring at 3847, 3604, and 3525 cm^{-1}. Also, at 1641 and 1535 cm^{-1}, bending vibrations of water occurred. The bands appeared at 752, 680, and 999 cm^{-1}, assigned to different modes of vibrations of $(PO_4)^3$. The band at 1427 cm^{-1} is attributed to NH_3. These results confirm the presence of the struvite [30]. The abrasion resistance of the as-prepared MPC specimen was tested by shaking the MPC specimen in water for 24 h. A minimal abrasion was observed (data not shown). The samples of spores, $Mg_3(PO_4)_2$, and MPC with and without spores were analyzed using a photography camera and SEM. Figure 6a–d in the supporting document shows the appearance of spores, $Mg_3(PO_4)_2$.

MPC without spores, and MPC with spores in images captured by the photographic camera. Figure 7a–d show images of the same samples analyzed by SEM. Small circular structures in Figure 7a are spores of Paenibacillus sp. whose particle size ranges from 2–20 µm. Figure 7b shows irregular morphologies present in $Mg_3(PO_4)_2$ powder, for which the particles have a size between 0.05–0.75 µm. Figure 7c

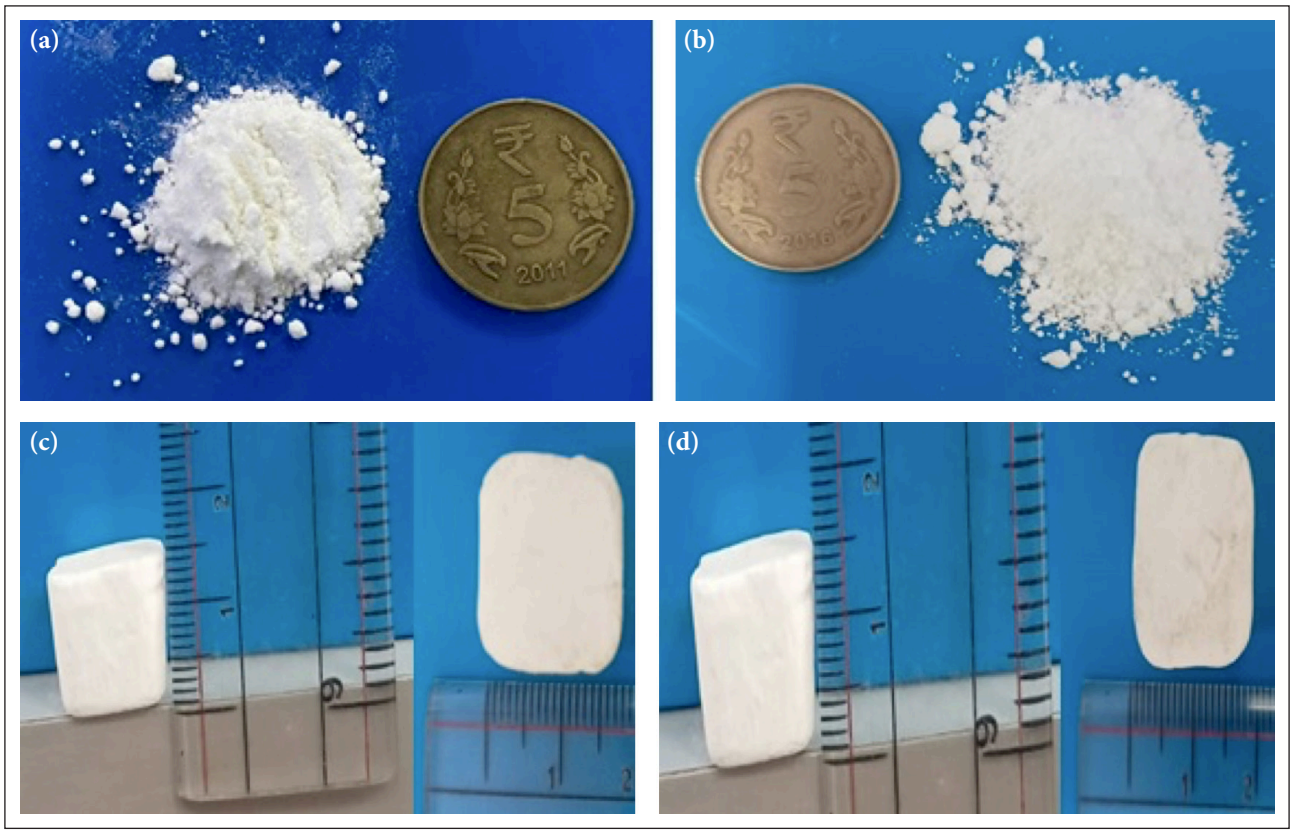

Figure 6. Appearance of various samples **(a)** photographic image of spore powder **(b)** photographic image of $Mg_3(PO_4)_2$ powder **(c)** photographic image of MPC cube without spores **(d)** photographic image of MPC cube with spores.

shows the presence of rod-shaped struvite in MPC having 1.5–9.5 µm particle size. Similar needle or rod-shaped morphologies were reported previously for MPC [31, 32]. MPC is formed due to a continuous dissolution and precipitation reaction, resulting in a stable ceramic product. It comprises equimolar concentrations of Mg, ammonium (NH^{4+}), and phosphate (PO_4^{3-}) combined with six water molecules. $Mg_3(PO_4)_2$ reacts with ammonium phosphate solution to form struvite ($MgNH_4PO_4 \bullet 6H_2O$). The most general reaction equation assumes that three kinds of free ions bind with 6 moles of water to form $MgNH_4PO_4 \bullet 6H_2O$ (Eq. 1) [28, 33, 34] Figure 7d shows that struvite particles of 11 µm size cover the spores.

$$Mg^{2+} + NH^{4+} + PO_4^{3-} + 6H_2O \quad MgNH_4PO_4 \bullet 6H_2O \quad (1)$$

Thermogravimetric analysis (TGA) and derivative thermogravimetry (DTG) were also conducted on the MPC samples; the results are shown in Figure 8. Only two prominent peaks were observed in the DTG of the sample, both below 200 °C. The peak at ~150 °C corresponds to a loss of physically and chemically bound water, while the peak at ~100 °C could correspond to the decomposition of struvite according to previous research [30, 35, 36].

3.3. Study of Leaching of Spores from MPC Matrix, Viability, and Crack Healing Phenomenon

A study was conducted to find the immobilization of spores in the MPC matrix. The results show no bacterial colonies on the plates for MPC with and without spores (Fig. 9).

4. DISCUSSION

It is evident from these results that the MPC matrix holds and protects the spores from external mechanical force. The viability of bacterial spores in the MPC matrix was determined by immersing the spore-containing MPC specimen in a containing medium. The amount of urea decomposed was studied. The control, urea-containing medium without spores, and the urea-containing medium containing MPC (without spores) showed negligible hydrolysis of urea. Free bacterial spores showed the highest urea hydrolysis. The bacteria-containing MPC decomposed around 70 (±0.48) % of urea (Fig. 10). These results suggest that the cementation process did not cause any damage to the bacterial spores.

The MPC provided a microenvironment for the germination and growth of bacterial spores, thus protecting the bacteria from the external environment. The crack was created in an MPC cube, and the self-healing of the crack was studied. The crack healing was observed in 7 days (data not shown). The samples were collected from the healed area and analyzed using SEM-EDS. The EDS analysis confirmed the presence of Ca in the healed product (Fig. 11). This shows the calcite-precipitating ability of Paenibacillus sp. NCIM 5410.

Wang et al. [37] found that the amount of urea decomposed by the free bacterial cells in the high pH cement slurry was significantly decreased, from 95% to less than 5%. However, they observed that the immobilization of bacterial cells increased the urea hydrolysis. In another study, Jad-

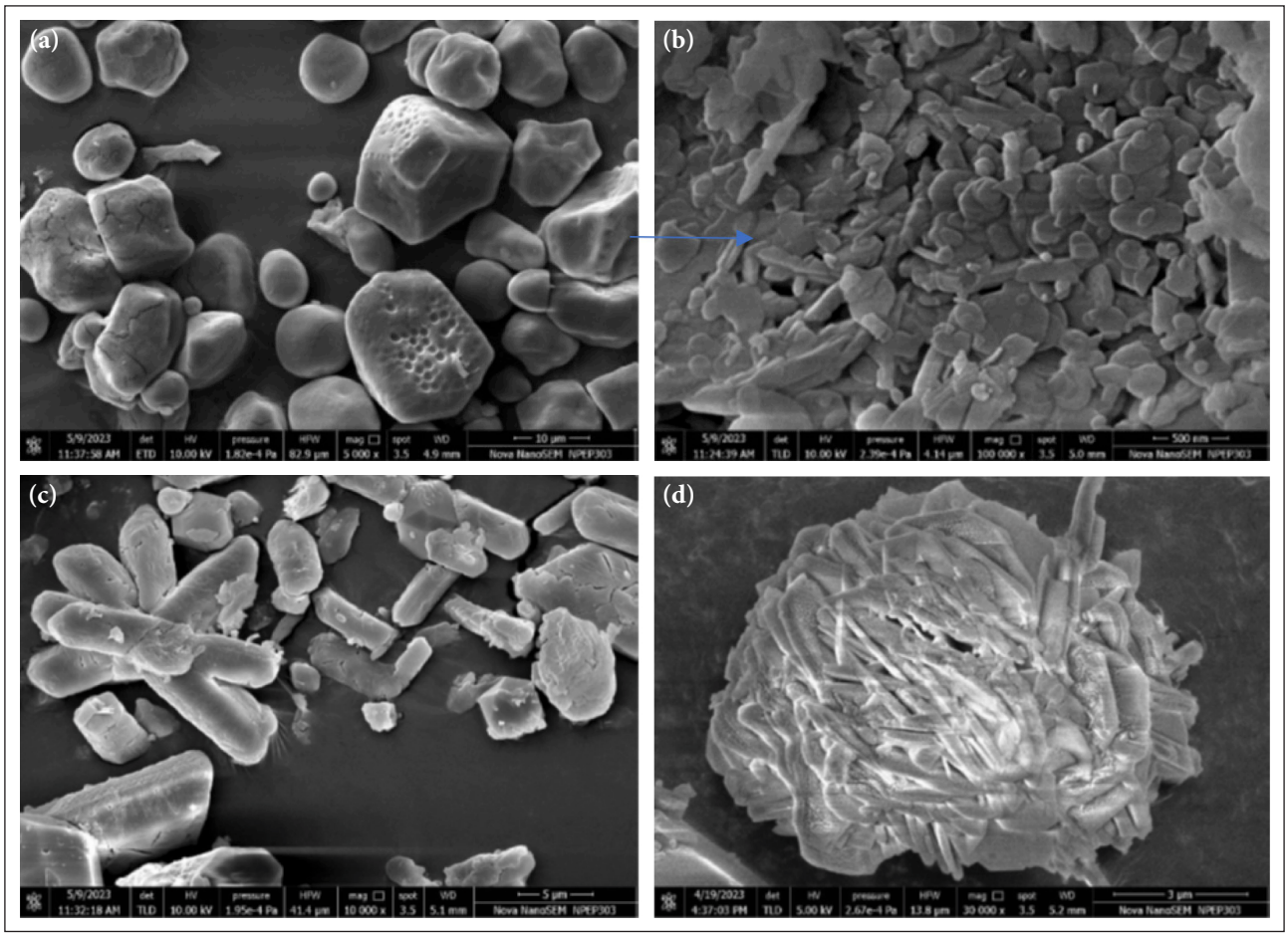

Figure 7. Appearance of various samples **(a)** SEM image of spore powder **(b)** SEM image of $Mg_3(PO_4)_2$ powder **(c)** SEM image of MPC cube without spore powder **(d)** SEM image of MPC cube with spore powder.

hav et al. [17] synthesized geopolymer to immobilize the bacterial cells. They found that the geopolymer reaction did not cause any damage to the bacterial spores, and the spores got activated when the bacteria-containing geopolymer specimen was immersed in a urea-based medium. These activated cells decomposed 98 (±0.20)% of urea was decomposed. The geopolymer provides interconnected pores and sufficient space to accommodate the bacterial spores and reactivated bacterial cells. A similar phenomenon happens with MPC synthesized in the present study. The viability of the spores after being immobilized into microcapsules was also reported by Wang et al. [38].

A study was conducted to examine the effect of calcite precipitation on the water absorption behavior of MPC cubes. The results show reduced water absorption for the specimens containing spores. The specimens without spores showed 5% water absorption, while those with spores showed 2.6% (Fig. 12). These specimens were immersed in a precipitation medium before the water absorption test. The precipitation medium might have provided the nutrients to spores, which helped them germinate. The germinated spores transformed into vegetative bacterial cells, and these cells might have produced calcite. The as-built calcite might have filled the pores of the MPC specimen, thereby reducing the water absorption.

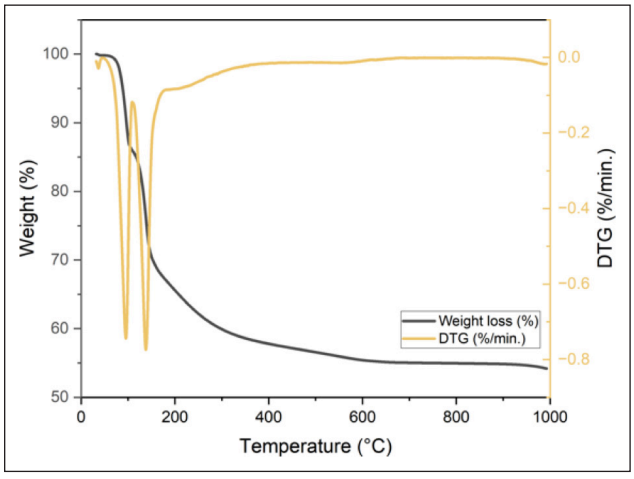

Figure 8. Thermogravimetric analysis of MPC.

5. CONCLUSION

This study successfully achieved the synthesis of MPC via an aqueous route. As a clinker-free binder characterized by rapid setting, high early compressive strength, excellent volume stability, robust bonding strength, minimal shrinkage, and superior abrasion resistance, MPC becomes an ideal material for immobilizing bacterial

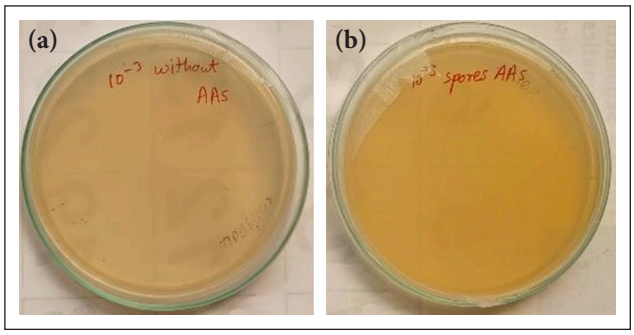

Figure 9. Study of leakage of spores from MPC **(a)** sample without spore **(b)** sample with spore.

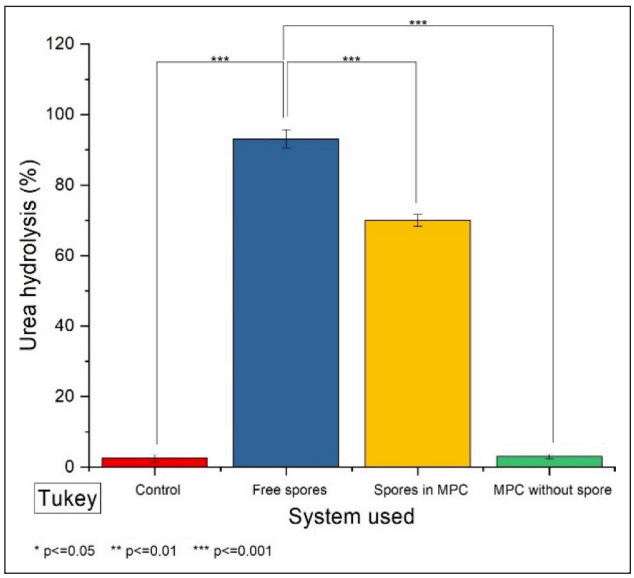

Figure 10. Viability testing by urea hydrolysis test.

spores. It prevents the compression of spores, a phenomenon commonly observed in concrete, conferring a durable self-healing attribute to MPC.

The aqueous route facilitated the effective immobilization of bacterial spores within MPC. Notably, MPC demonstrated its ability to retain bacterial spores, which is evident through urea hydrolysis and calcite precipitation. These findings indicate that MPC can safeguard spores for extended storage durations. However, it is essential to note that the present study did not assess spore viability over periods exceeding five months. The amalgamation of MPC matrix with bacteria holds promise in creating a bioactive material.

Considering the future applications of MPC-containing bacteria, particularly in scenarios requiring rapid repairs, is crucial. The harsh environmental conditions of concrete can be effectively countered by MPC, safeguarding bacteria from adverse conditions. MPC is already employed as a repair material for diverse concrete structures, and incorporating bacteria into MPC provides an additional advantage by imparting self-healing capabilities.

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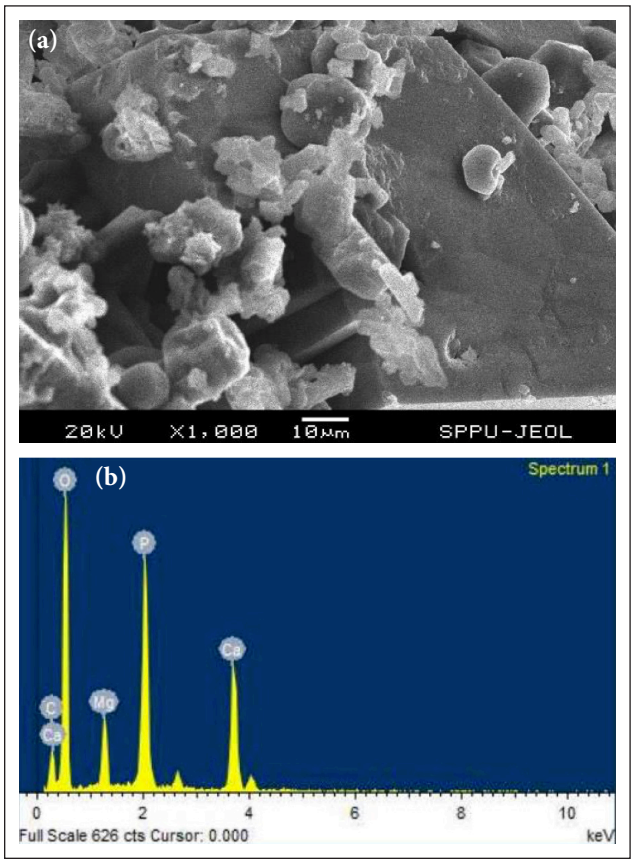

Figure 11. **(a)** SEM and **(b)** EDS analysis of the healed product.

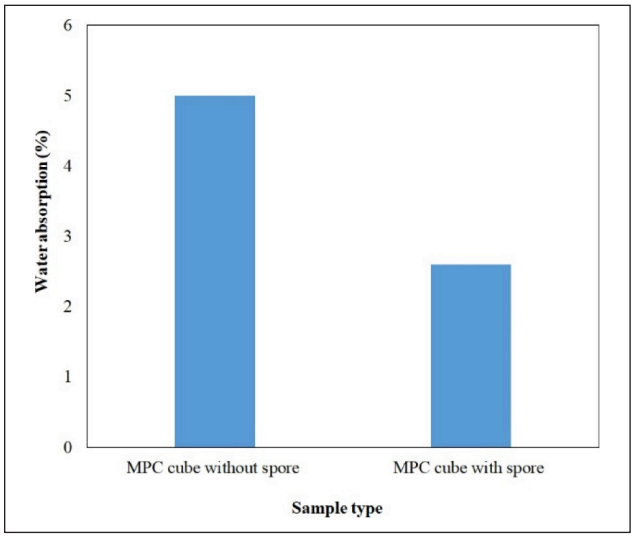

Figure 12. Water absorption **(a)** without spore and **(b)** with spore.

ETHICS

There are no ethical issues with the publication of this manuscript.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FINANCIAL DISCLOSURE

The authors declared that this study has received no financial support.

USE OF AI FOR WRITING ASSISTANCE

Not declared.

PEER-REVIEW

Externally peer-reviewed.

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