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Cell Viability Studies on *Bacillus sp.* under Different Storage Conditions for Usage in Improving Concrete Compressive Strength

Sk Rahaman [1,*], Datunaka Sai Srujan [1], Jayati Ray Dutta [2], Arkamitra Kar [1] and Mohna Bandyopadhyay [3]

[1] Department of Civil Engineering, Birla Institute of Technology and Science—Pilani, Hyderabad Campus, Hyderabad 500078, India; saisrujandatunaka@gmail.com (D.S.S.); arkamitra.kar@hyderabad.bits-pilani.ac.in (A.K.)
[2] Biological Sciences Department, Birla Institute of Technology and Science—Pilani, Hyderabad Campus, Hyderabad 500078, India; jayati@hyderabad.bits-pilani.ac.in
[3] Sharp Edge Labs, Pittsburgh, PA 15203-5118, USA; mohnab04@gmail.com
* Correspondence: rahamansk58@gmail.com

Abstract: Bacterial concrete is a possible approach toward sustainability in concrete construction through crack-healing. Including a bacterial culture as an admixture in concrete can enhance the service life of a structure through the self-healing of cracks. Incorporating bacterial cells as an admixture in concrete is a major challenge as bacteria are living organisms with a limited shelf-life. It is essential to evaluate the shelf-life of bacterial cultures to encourage the inclusion of bacteria in concrete applications. Hence, the main focus of this study was to record the cell viability of these microorganisms before addition to cementitious systems. In the first stage, three different bacterial cultures of *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus licheniformis* were stored in Luria Bertani broth under two different conditions of room temperature and refrigeration. These stored bacterial solutions were checked for viability based on cell count after 1 day, 3 days, 7 days, 15 days, and 20 days of storage. In the second stage, the fresh bacterial cultures and the 15-day stock were added to prepare bacterial concrete and cement paste samples to assess their compressive strengths and microstructural changes, respectively. It was observed that the cell viability in terms of cell count of the selected bacterial strains attained up to 15 days when stored at room temperature. It was also observed that the compressive strength of the bacterial concrete prepared with stored bacterial cultures increased by 6% and 11% at 7 and 28 days compared with the control Portland cement concrete mix, respectively. However, the compressive strength decreased by 6% to 12% compared with the bacterial concrete prepared with fresh cultures at the same ages. Additionally, the compressive strength results were validated using microstructural analyses.

Keywords: cell viability; compressive strength; bacterial concrete; *Bacillus sp.*

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1. Introduction

Concrete used in traditional construction activities has some drawbacks due to its viscoelastic nature and cracking tendency. Cracks form due to unexpected loading, the use of cheap-quality material, chemical attacks, and environmental hazards [1,2]. Several conventional methods are available for enhancing the mechanical performance of concrete and concrete crack repairs, which are expensive and harmful to the environment as the materials used are non-biodegradable [3]. For concrete crack repair, mostly epoxy-based materials are used and require skilled labor and continuous monitoring over the lifespan of the structure [4]. Data reported by Statista in 2018 show that the annual maintenance and repair cost of concrete structures in the U.S. accounted for 0.52% of its GDP, India spent approximately 1.08% of its GDP, and China spent a maximum of 5.57% of its GDP [5].

To overcome the shortcomings of these conventional techniques, researchers have proposed using alternative biological methods to improve the mechanical performance and self-healing of cracks in concrete [6]. The inclusion of bacterial cells into concrete is a potential technique to enhance the longevity of concrete by improving the mechanical performance and self-healing of cracks via microbially induced calcite precipitation (MICP) [7]. This technique does not adversely affect the environment as no synthetic processing is required to produce bacteria. The advantages of bacteria-incorporated concrete in crack-healing and enhanced mechanical properties lead to the conservation of natural resources and reduced CO_2 emissions during repair and new construction work [8].

The incorporation of bacteria into concrete was initially adopted to heal cracks in concrete, but researchers observed that it also improved the mechanical performance of concrete [9]. Mortar specimens prepared with *Shewanella sp.* with a concentration of 10^5 cells/mL of water showed a 25% increase in compressive strength with regard to the control specimen after 28 days [10]. *Bacillus sp.* CT-5, isolated from cement, was used to prepare mortar specimens, which showed a 36% increase in compressive strength over 28 days due to MICP [11]. *Bacillus subtilis* spores with varying cell concentrations enhanced compressive strength by 17.97% compared with control mortar specimens [12]. Mortar specimens prepared with *Bacillus subtilis* with a cell concentration of 10^5 cells/mL showed a maximum enhancement in compressive strength of 27% compared with the control specimen, whereas cell concentrations of 10^3 and 10^7 cells/mL showed 15% and 19% increases after 28 days of curing, respectively [13]. The direct addition of a *Bacillus cereus* culture as a 1% replacement of the total water content resulted in 15% to 24% increases in compressive strength after 90 days of curing compared with the control mix [14]. A recent study reported that mortar specimens prepared with *Bacillus subtilis* and *Bacillus cereus* showed significant increases in compressive strength after 7, 14, and 28 days of curing [15]. There are various techniques available to incorporate bacteria into concrete, viz., (i) direct addition [16], (ii) immobilization [17], and (iii) encapsulation [18], to improve mechanical performance. For all these techniques, especially for direct addition during concrete mixing, the number of healthy cells, also known as the cell viability of a bacterial culture, is an important factor. The bacterial cell count can increase or reduce over time as it transitions from the stationary to the death phase [19]. Hence, maintaining a constant cell count is challenging, thereby limiting the practical implementation of this technique for incorporating bacteria into concrete to improve its properties. As the effects of bacterial inclusions are directly connected to the viable cell count during mixing [20], it is essential to know the active period of the bacterium after growth. It is crucial to see the cell viability of a bacterial culture after growth at various ages. In order to propose a bacterial culture as an additive for concrete, it is essential to determine the shelf-life of the culture in terms of cell viability with varying durations of storage. However, there is no systematic report available on the mechanical performance of concrete containing bacterial cultures with varying periods of storage. Additionally, there are no reports yet on the mechanical performance of concrete containing bacterial cultures stored for a period of up to 15 days. The absence of this information is an obstacle to the in situ casting of structural members prepared with bacterial concrete.

Hence, this study focused on finding the cell viability of stored bacterial cultures and the effects on the mechanical performance of bacterial concrete prepared with the stored bacterial cultures to encourage its widespread practical application. To achieve the main goal of this study, the specific objectives were as follows: (i) to determine the cell viability of bacterial culture media stored in a refrigerator and at room temperature; (ii) to determine the compressive strength values of bacterial concrete prepared with fresh and stored bacterial cultures; and (iii) to correlate compressive strength results with microstructural changes in hardened paste samples prepared with fresh and stored bacterial cultures. The intended outcome of this study was to provide recommendations about suitable bacterial storage conditions before addition to concrete for widespread practical uses.

2. Materials and Concrete Mix Proportions

2.1. Materials

2.1.1. Materials for Bacterial Culturing and Preparing Luria Bertani Agar (LA) Plates

The *Bacillus subtilis* (MTCC 1305), *Bacillus cereus* (MTCC 430), and *Bacillus licheniformis* (MTCC 429T) strains used in this study were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), India, in lyophilized form. HIMEDIA, M1245, Miller Luria Bertani (LB) broth procured from HYCHEM Laboratories, Hyderabad, India, was used to prepare liquid media to revive these bacterial strains as well as to prepare bacterial cultures.

The composition of the LB broth is given in Table 1. To prepare the liquid LB broth, 25 g of LB broth was mixed with 1000 g of distilled water. An amount of 25 g of LB broth and 15 g of agar were mixed with 1000 g of distilled water to prepare LA plates. The physical properties of the agar are presented in Table 2.

Table 1. Chemical composition of LB broth.

Ingredients	Weight/kg
Tryptone	400 g
Yeast extract	200 g
Sodium chloride	400 g

Table 2. Physical properties of agar.

Characteristics	Agar
Appearance	Cream-colored, homogenous, free-flowing powder
Solubility	Freely soluble in hot water at temperatures above 85 °C; insoluble in cold water
pH	6.50–7.50

2.1.2. Materials for Cementitious System

Portland cement (PC) fulfilling the requirements of grade 53 as per IS 269–2015 [21] (similar to type I of ASTM C150/C150M–19a [22]) was utilized to prepare the concrete and paste samples for this study. The obtained chemical compositions of the cement used for this study using X-ray fluorescence spectroscopy (XRF) analysis are presented in Table 3. Fine aggregate (FA) that complies with Zone II and coarse aggregate (CA) with a nominal maximum size of 20 mm conforming to IS 383–2016 [23] (similar to ASTM C33/C33M-18 [24]) were used. The physical properties of the FA and CA are shown in Table 4.

Table 3. Chemical composition of cement.

Ingredients	Percentage
Lime (CaO)	66.26
Silica (SiO_2)	18.75
Alumina (Al_2O_3)	4.27
Ferric oxide (Fe_2O_3)	4.16
Sulfuric anhydride (SO_3)	3.22
Magnesium oxide (MgO)	1.41
Potassium oxide (K_2O)	1.16
Sodium oxide (Na_2O)	0.77

Table 4. Physical properties of FA and CA.

Aggregate Type	Fineness Modulus	Specific Gravity	Water Absorption (%)
Fine aggregate	3.028	2.65	0.5
Coarse aggregate	6.863	2.72	0.1

2.2. Concrete Mix Proportions

Due to the unavailability of a standard code of practice for bacterial concrete preparation, this study adopted conventional PC concrete mix proportions for the preparation of bacterial concrete. PC concrete complying with grade M30 of IS 10262–2019 [25] was prepared as per the provided recommendations. For the preparation of the bacterial concrete, the required water content was replaced with the necessary quantity of the bacterial culture. This study used a 1% bacterial culture to replace the water content for each type of bacterial concrete mix based on previous findings by the present authors [14].

The mix proportions adopted for preparing the PC concrete and bacterial concrete are presented in Table 5.

Table 5. Concrete mix proportions of PC and bacterial concrete.

Mix ID	Cement (kg/m^3)	CA (kg/m^3)	FA (kg/m^3)	Water (kg/m^3)	Bacterial Culture (kg/m^3)
PC concrete	438	2.65	3.028	196	0
Bacterial concrete	438	2.72	6.863	194.04	1.8

In this study, bacterial concrete was prepared with fresh bacterial cultures and stored bacterial cultures of all three strains selected for this study. The bacterial concretes prepared with the fresh culture and stored culture of *Bacillus subtilis* are denoted as FBS and SBS, respectively. Similarly, the bacterial concretes prepared with the fresh culture and stored culture of *Bacillus cereus* are denoted as FBC and SBC, respectively. Also, the bacterial concretes prepared with the fresh culture and stored culture of *Bacillus licheniformis* are denoted as FBL and SBL, respectively.

3. Methodology

The simplified flow of the research approach adopted for this study is presented in Figure 1.

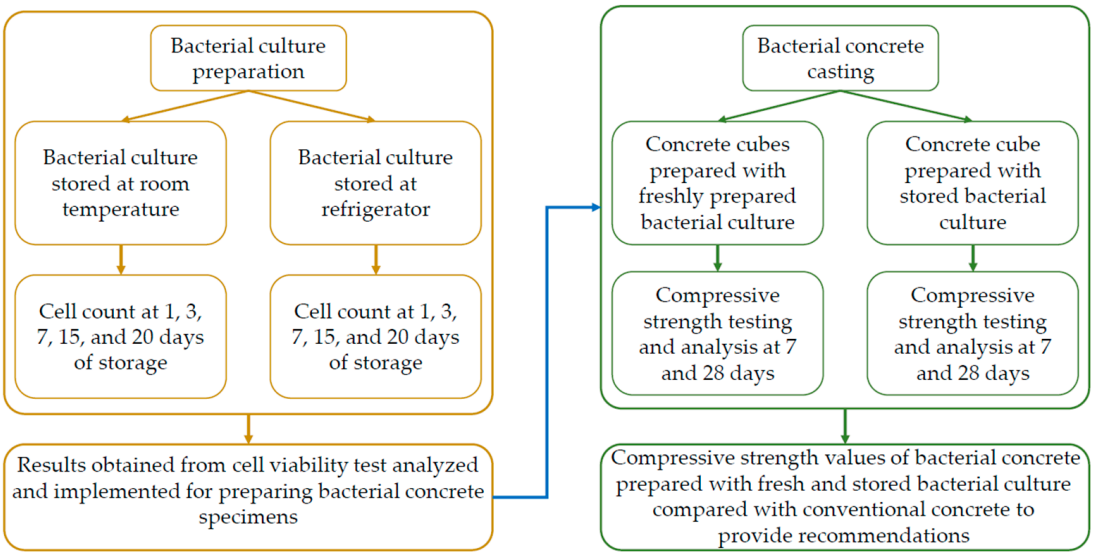


Figure 1. Flow of the present study.

3.1. Revival of Bacterial Strains

The bacterial strains obtained from MTCC in lyophilized form were revived as per the recommendations provided by MTCC. Falcon tubes of 15 mL containing 5 mL of LB broth medium were sterilized and allowed to cool down to room temperature. Then, a loopful amount of each lyophilized bacterial strain was collected using a needle-type inoculum loop and inoculated into a previously prepared falcon tube. The inoculated falcon tubes were

transferred to an incubator at the appropriate temperature and conditions recommended by MTCC for the culture. For the selected bacterial strains for this study, the incubation temperature was maintained at 37 °C, and the shaking was maintained with an RPM (rotation per minute) of 120 for 24 h. Once the incubation period was over, each growth was observed visually and inoculated further into an LA plate using a 10 µL loop from the culture media using the streak plate technique [26]. Then, the inoculated LA plate was transferred into the incubator and incubated for 16 h until the colonies of the bacterial strain grew in the plate. The bacterial colonies formed in LA plates were then tested for contamination using a Gram-staining technique as per the protocol given by Smith and Hussey [27]. Microscopic observations of the selected strains after Gram staining showed a purple-blue color, which confirmed that the selected strains were Gram-positive. These findings are similar to the results of previous studies [28–30].

3.2. Cell Viability Test

A serial dilution technique was used to count the bacterial cells in the culture media, conforming to the conventional Serial Dilution Protocols [31]. Three 250 mL conical flasks containing 100 gm of liquid LB broth were sterilized in an autoclave. Then, these flasks were cooled and inoculated with the optimized IV of each strain from the full-grown primary cultures of the three selected bacterial strains, separately. In the next step, these flasks were kept inside an orbital-shaking incubator at a temperature of 37 °C and an RPM of 120 for 24 h. After 24 h of growth, 100 µL of each bacterial culture was collected in an autoclaved Eppendorf tube to count the cells, considered as a 1st-day count. Then, the remaining full-grown bacterial culture was divided into two parts, with one stored at room temperature (maintained at 25 ± 2 °C) and another in a refrigerator (at 4 °C) to observe the cell viability at 3, 7, 10, 15, and 20 days. Then, 900 µL of sterilized distilled water was poured into the Eppendorf tube and thoroughly mixed with the previously collected 100 µL of full-grown culture. From this diluted culture, 100 µL was obtained and mixed with another 900 µL of distilled water in another tube. The process was repeated for 10 tubes to achieve a 10^{-1} to 10^{-10} dilution factor. An amount of 100 µL of diluted bacterial culture from each tube was then obtained using a micropipette and spread evenly on 10 separate LA plates with an L-shaped spreader. Then, the inoculated plates were kept inside the incubator at 37 °C for 16 h for the bacterial colonies to grow. A schematic diagram of the serial dilution method is presented in Figure 2.

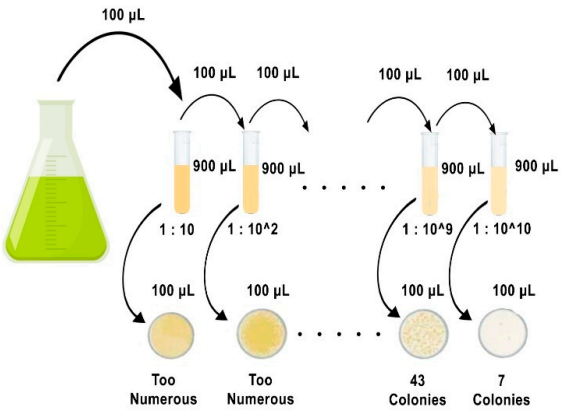


Figure 2. Schematic diagram of serial dilution technique.

After 16 h of incubation, the bacterial colonies formed in the LA plates were observed using a colony counter instrument to count the bacterial cell colonies. Each plate with a particular dilution factor was observed separately, and the number of colonies was recorded. The plates for these three selected bacteria, *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus licheniformis*, with different dilution factors of 10^{-5}, 10^{-6}, and 10^{-7}, are shown in Figure 3. For the cultures stored at room temperature and in the refrigerator, the exact

process was repeated at 3, 7, 10, 15, and 20 days to count the cells. The results obtained from this experiment are presented in Section 4.1.

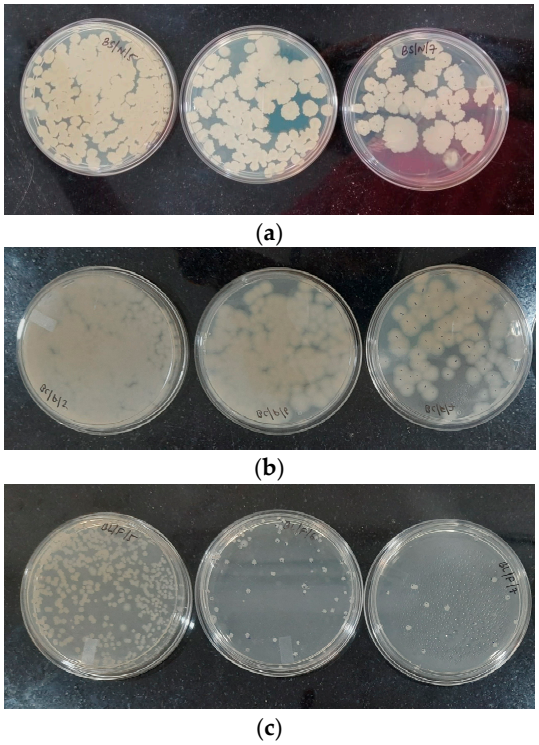


Figure 3. LA plates with bacterial colonies with different dilution factors for three different bacterial strains (10^{-5}, 10^{-6}, and 10^{-7}) of (**a**) *Bacillus subtilis*, (**b**) *Bacillus cereus*, and (**c**) *Bacillus licheniformis*.

3.3. Concrete Mixing Procedure and Sample Preparation

At first, the CA and FA were homogeneously mixed using a pan mixer. Once the dry aggregates were mixed properly, two-thirds of the water content was added for the PC concrete, whereas water containing a bacterial culture was added for the bacterial concrete. After adding water or water containing the bacterial culture, the mixer was rotated for three minutes. Then, the cement was added to the mix, followed by adding the remaining water or water containing the bacterial culture. The mixer was rotated for another two minutes to achieve a uniform workable fresh concrete mix. The schematic representation of the mixing procedure is presented in Figure 4.

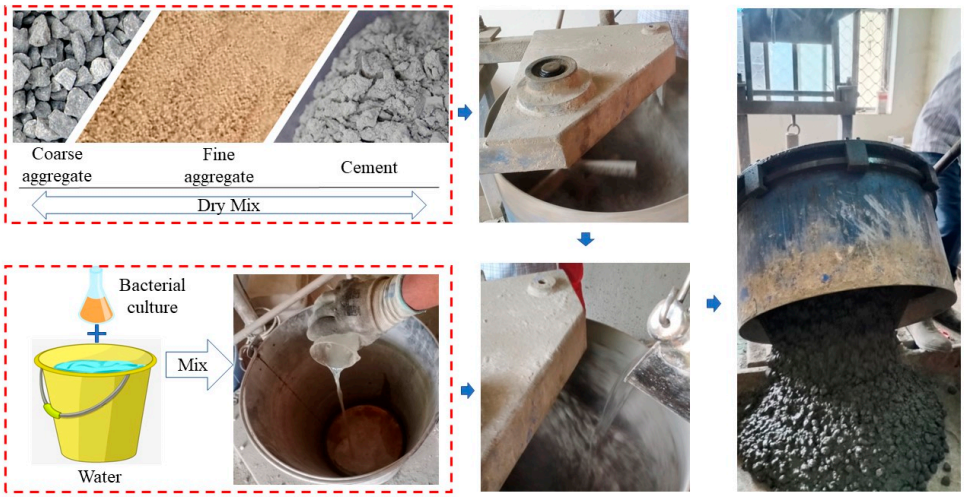


Figure 4. Schematic representation of the concrete mixing procedure.

Concrete specimens with dimensions of 150 mm^3 were cast for all the mixes for the compressive strength test. The casted specimens were de-molded after 24 ± 1 h and kept for water curing for 28 days. The relative humidity and the average lab temperature maintained during the curing period of these specimens were 60% ± 5% and 31 ± 2 °C, respectively. The corresponding results obtained are presented in Section 4.2.

3.4. Preparation of Paste Samples for SEM

For the SEM analysis, paste samples were prepared in a controlled environment for all the mixes selected for this study. An amount of 100 gm of PC was blended with water or water containing a bacterial culture as per the mix proportions and placed inside a silicone mold to prepare the paste sample. After 24 ± 1 h, the hardened paste sample was de-molded and stored in a small container for water curing, maintaining the curing regime mentioned in Section 3.3. SEM analysis was performed for each sample at 7 and 28 days.

3.5. Compressive Strength Test

The specimens prepared for the compressive strength test discussed in Section 3.3 were tested at 7 and 28 days. The tests were performed using a Heico 3000 kN digital compressive testing machine as per the recommendations of ASTM C39/C39-21 [32] and IS 516 (Part 1/Sec 1): 2021 [33].

3.6. Scanning Electron Microscopy (SEM)

To identify the morphological changes and the presence of bacterial cells inside the hardened paste samples for each mix, SEM analysis was performed after 7 days and 28 days of curing. An FEI Apreo LoVac Field Emission SEM was used to capture high-resolution micrographs at a magnification of 10,000×. A 10 mm working distance was maintained throughout the SEM analysis. An Everhart Thornley detector at an operating voltage of 10 kV was used to capture the micrographs. Crushed particles of hardened cement paste samples with approximate dimensions of 2 mm^2 with flat surfaces were derived from the cores of the samples to perform the SEM analysis. The samples were then oven-dried overnight at 50 °C to remove moisture before the SEM analysis. The samples were coated with a gold layer of 10 nm thickness using a LEICA EM ACE200 sputter coater to make the samples conductive. The coated samples were then loaded into the SEM, and high-resolution micrographs were captured under vacuum at a pressure of 9.9 × 10^{-5} Pa. The observations from the SEM analysis are presented in Section 4.3.

4. Results

4.1. Cell Viability of Selected Bacterial Strains

Figure 5 shows increased bacterial colony (cfu/mL) formation for all three strains stored at room temperature. Taking the 1st-day value as the reference, BS, BC, and BL showed an increase in bacterial cell count after 15 days of storage at room temperature, whereas at 20 days of storage, the bacterial cell counts were reduced. BS showed increases in cell count of 13%, 25%, and 3% compared with the 1st-day cell count after 3, 7, and 15 days of storage, whereas there was a 92% cell count reduction after 20 days of storage. Similarly, BC showed increases of 52%, 30%, and 16% compared with the 1st-day count after 3, 7, and 15 days of storage, respectively. After 20 days of storage, BC's cell counts were reduced by 21% compared with the 1st-day count. Also, BL showed increases in cell count of 39%, 21%, and 9% compared with the 1st-day cell count after 3, 7, and 15 days of storage, whereas there was a reduction of 28% in the cell count after 20 days of storage. Figure 5 shows that the increase in the cell count in the case of BS was at a maximum after a 7-day storage period, whereas in the cases of BC and BL, the maximum cell count was obtained after a 3-day storage period. These observations conclude that the bacterial culture of BS experienced a growth phase until 7 days. In contrast, for BC and BL, the growth phase lasted for 3 days.

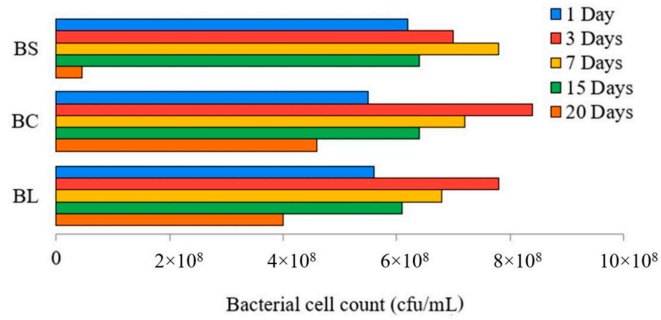


Figure 5. Cell counts of *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus licheniformis* after different storage periods at room temperature.

Figure 6 presents the cell viability of BS, BC, and BL under refrigerated conditions. From Figure 6, it is observed that there was a drastic reduction in the cell count after the storage of this bacterial culture inside the refrigerator. BS showed a significant decrease of 91% in cell count after 3 days of storage. BS showed a reduction of almost 99% in cell count after 7 days of storage inside the refrigerator. A similar observation was noticed in the case of BL, where there was a reduction of 87% in cell count after 3 days of storage and almost a 99% reduction after 7 days of storage. Though BS and BL showed similar trends, BC showed a slight increase in cell count after 3 days of storage, that is, 5% compared with the 1st-day cell count. After 7 days of storage, cell counts decreased to a large extent, that is, 93% compared with the 1st-day cell count. Fifteen days of BC culture storage showed an almost 99% reduction in the cell count compared with the first-day cell count.

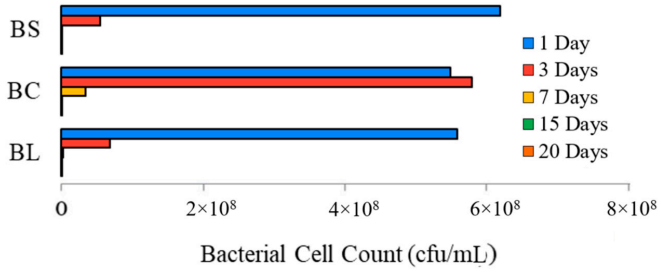


Figure 6. Cell counts of *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus licheniformis* after different storage periods in the refrigerator.

From the above observations, it can be seen that storing the bacterial cultures at room temperature was suitable until 15 days, as the cell count is nearly the same compared with the 1st-day count. An increase in bacterial dosages in concrete leads to a decrease in the strength gain, as reported in a previous study by the present authors [14] and previously reported studies [6,8,34]. Hence, the stored bacterial cultures for 15 days at room temperature were used to prepare bacterial concrete of each bacterial strain to compare with concrete prepared with freshly prepared bacterial cultures. A similar approach was used to prepare paste samples for SEM analysis.

4.2. Compressive Strength Comparison

The efficiency of the fresh bacterial cultures and the bacterial cultures stored at room temperature for 15 days was obtained by comparing the compressive strength of the PC concrete and the bacterial concrete prepared with the selected bacterial strains for this study. The compressive strengths of FBS, SBS, FBC, and SBC increased after 7 days of curing. Similarly, FBL showed reductions in compressive strength, whereas SBL obtained a similar strength value to PC concrete after 7 days of curing. From Figure 7, it can be seen that the compressive strength gain of FBS was 21%, whereas SBS showed only a 6% increase compared with PC concrete after 7 days of curing. In the cases of FBC and SBC, the increases in compressive strength were 19% and 11%, respectively. Subsequently,

FBL's compressive strength was reduced by 8%, whereas SBL showed the same strength compared with PC concrete after 7 days of curing.

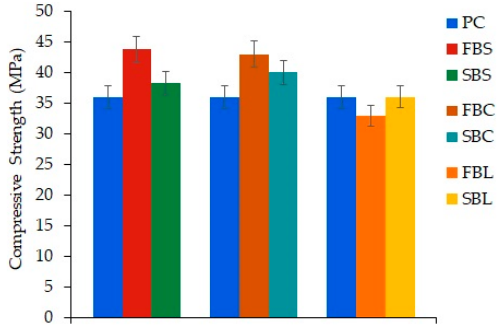


Figure 7. Comparison of compressive strength values of PC, FBS, SBS, FBC, SBC, FBL, and SBL after 7 days of curing.

After 28 days of curing, bacterial concrete prepared with fresh cultures exhibited a significant increase in compressive strength, whereas bacterial concrete prepared with stored cultures showed a gain in strength but not on par with that using the fresh cultures. Figure 8 shows that the compressive strengths of FBS and SBS after 28 days of curing showed increases of approximately 20% and 10% compared with the PC concrete strength, respectively. Similarly, in Figure 8, FBC and SBC show values of approximately 26% and 11% compared with PC concrete after 28 days of curing. Furthermore, in Figure 8, FBL shows a nearly 11% increase in compressive strength compared with PC, whereas SBL obtains a negligible strength gain of approximately 2%.

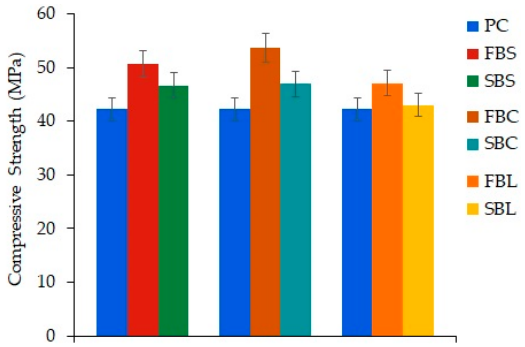


Figure 8. Comparison of compressive strength values of PC, FBS, SBS, FBC, SBC, FBL, and SBL after 28 days of curing.

The cell count of the 15-day stored sample of each bacterial culture was almost the same compared with the 1st-day count. However, the corresponding compressive strength gain in the bacterial concrete was not to the same extent as the concrete prepared with the fresh bacterial culture. This phenomenon is attributed to bacterial aging or bacterial senescence, denoting the gradual decrease in certain metabolic activities of the bacteria with the increase in age [35]. Due to aging, the bacteria can enter into a long-term stationary phase that leads to the modulation of metabolic activity [36].

These observations are correlated with the morphological changes demonstrated in the following section.

4.3. SEM Analysis

Figure 9 shows the micrographs produced using SEM analysis of the hardened paste samples of PC and bacterial concrete prepared with fresh and stored cultures of the selected bacterial strains for this study at the ages of 7 and 28 days. The micrographs were obtained with magnifications of 10,000×.

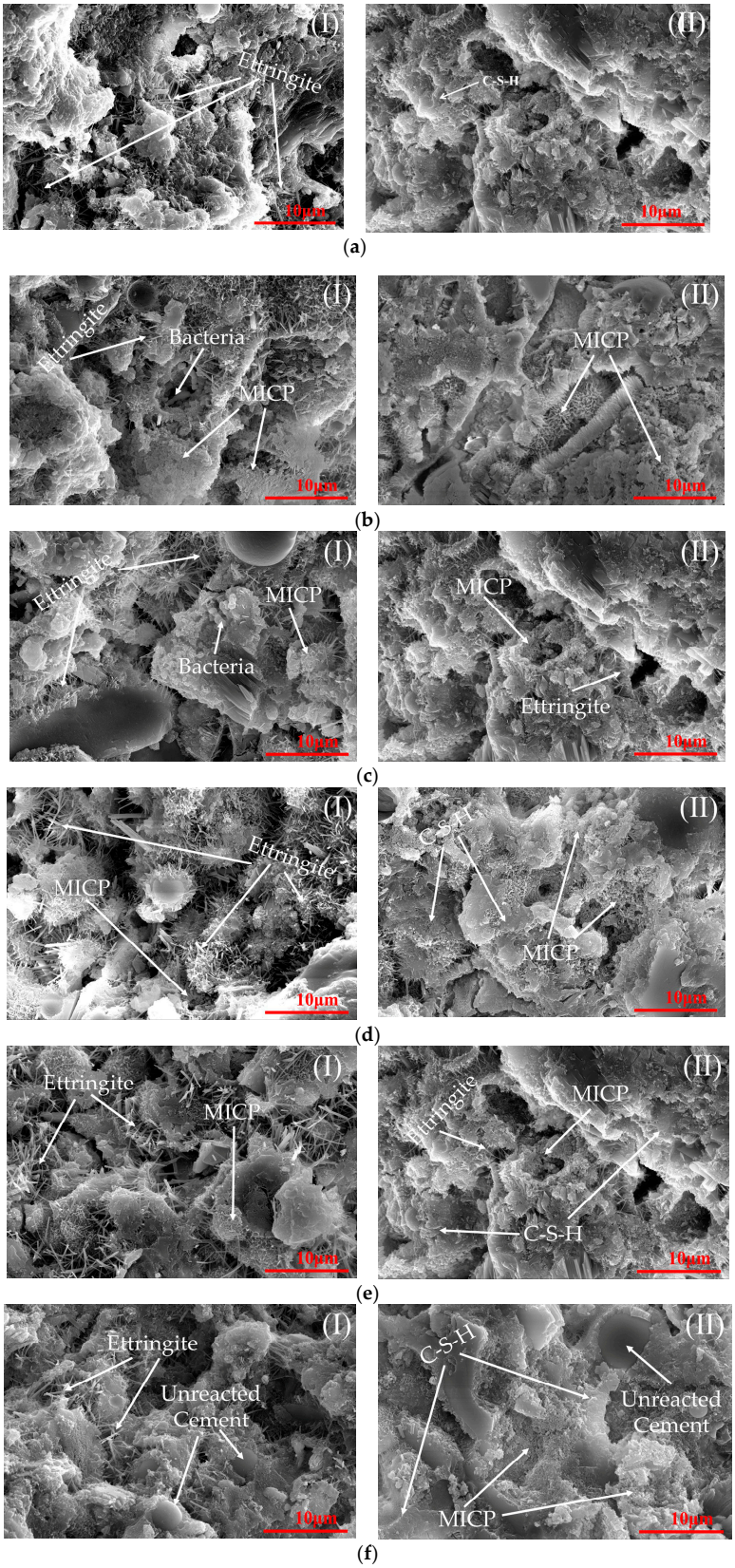
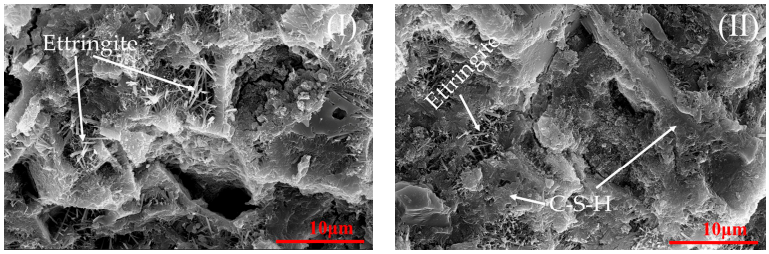


Figure 9. *Cont.*



(**g**)

Figure 9. SEM micrographs of (**a**) PC, (**b**) FBS, (**c**) SBS, (**d**) FBC, (**e**) SBC, (**f**) FBL, and (**g**) SBL after 28 days of curing (I—7 days; II—28 days).

Figure 9 shows the eminent presence of long, slender, prismatic needles of ettringite in 7-day hardened PC paste samples and the predominant reticular network of C-S-H after 28 days. The inclusions of bacterial cultures resulted in starkly different morphologies after 7 days and 28 days. The formation of MICP was observed in the hardened pastes containing fresh and stored bacterial cultures, as validated through comparison with observations from previous research [14]. However, there was a difference in the morphologies between the pastes prepared with the fresh and the stored cultures for each type of bacterial strain, especially in the case of *Bacillus licheniformis*.

Figure 9b(I) shows the presence of hexagonal crystals of $CaCO_3$, indicating MICP and ettringite, in the 7-day sample of FBS. The 28-day sample of FBS presented in Figure 9b(II) shows a denser formation of MICP in two different forms: flower-like bouquet structures and hexagonal crystals along with C-S-H, without the presence of ettringite. This observation matches with previous studies [37,38]. The SBS sample presented in Figure 9c, shows a slightly different morphology in both the 7- and 28-day samples compared with FBS. It is observed that the presence of ettringite is evident in both the 7- and 28-day samples, and the formation of MICP is not to the same extent as for the FBS sample. This ettringite formation at 28 days resulted in a lower strength gain due to the delayed reaction. These observations can be correlated with the compressive strength results of FBS and SBS, where the increase in the strength gain of FBS was almost twice that of the SBS specimens compared with PC concrete. A similar observation is seen in Figure 9d,e of SBC and FBC, where SBS shows the formation of ettringite only in the 7-day sample, whereas the FBC samples show the presence of ettringite at both 7 and 28 days. A completely different morphology is observed in the FBL sample of 7 and 28 days compared with FBS and FBC. Figure 9f shows that the precipitation due to *Bacillus licheniformis* after 7 days of curing resulted in a MICP layer of hexagonal crystals outside the cement particle along with ettringite. This layer of MICP inhibited the cement particle's reaction, leading to a lower strength gain. This observation correlates with the compressive strength results of FBS after 7 days of curing, where the strength gain was reduced compared with PC concrete, as presented in Figure 7. But after 28 days of curing, the presence of ettringite was not visible, the formation of MICP in the form of hexagonal crystals was evident, and a denser microstructure was observed. However, the negligible presence of unreacted cement particles was visible. The morphology of SBL shown in Figure 9g is similar to that of PC, where the presence of MICP is barely visible in both the 7- and 28-day samples, concluding that the stored culture of *Bacillus licheniformis* did not affect the reaction. These observations can be validated with the compressive strength results presented in Figures 7 and 8, where the compressive strengths of the FBL and PC concrete after 7 and 28 days are almost the same.

From the findings of the experiments conducted for this study, it can be observed that the cell count of *Bacillus subtilis* increased after 7 days. In contrast, the *Bacillus cereus* and *Bacillus licheniformis* cell counts increased after 3 days and decreased after 7 days at room temperature. For the bacterial cultures stored in the refrigerator, only *Bacillus cereus* showed a slight increase in cell count after 3 days, but *Bacillus subtilis* and *Bacillus licheniformis* showed drastic reductions from 3 days onwards. After 15 days, the bacterial cell count was reduced to the level of the 1-day count as the bacterial culture reached the death phase;

this can also be seen after 20 days of storage. These observations show that the bacterial strains selected for this study are more viable when stored at room temperature than in refrigerators.

Bacterial concrete prepared with fresh and stored cultures showed significant variations in compressive strength results and morphologies. It can be seen that there was almost a 50% reduction in the strength gain when bacterial concrete was prepared with stored cultures compared with bacterial concrete prepared with fresh cultures in the cases of *Bacillus subtilis* and *Bacillus cereus.* At the same time, bacterial concrete prepared with the stored culture of *Bacillus licheniformis* showed nearly zero strength gain compared with PC concrete. These observations demonstrate that the cell viability of the selected strains was attained after storing them at room temperature. Still, these stored bacterial cultures could not improve the compressive strength due to senescence.

It is also observed from the SEM analysis that the hardened cement paste samples of the stored bacterial cultures showed reduced MICP formation compared with the hardened paste samples prepared with fresh bacterial cultures.

The observations from this study show that cell viability could be attained with a similar cell count compared with the 1-day count after storing the bacterial cultures at room temperature for up to 15 days. Further investigations are required to alter the dosages of stored bacterial cultures to use in concrete to achieve a similar strength to bacterial concrete prepared with fresh bacterial cultures.

5. Conclusions

Based on the findings from the present study, the following conclusions can be drawn.

1. The bacterial cultures of *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus licheniformis* showed more cell viability when stored at room temperature (maintained at 25 $\pm$ 2 °C).
2. The bacterial culture of BS, stored at room temperature, showed an increased cell count at 7 days and a decreased cell count at 15 days compared with the 7-day count. Meanwhile, the BC and BL cell counts increased after 3 days and decreased after 7 days compared with their 3-day counts.
3. It was observed that the compressive strength gain decreased when bacterial concrete prepared with the 15-day stored bacterial cultures of BS and BC was compared with bacterial concrete prepared with the fresh bacterial cultures of BS and BC. However, the cell count was almost the same. The stored culture of BL did not gain any strength, whereas the fresh culture of BL showed an 11% increase in compressive strength compared with PC concrete.
4. The SEM analysis provides conclusive evidence of MICP formation in the hardened cement paste samples prepared with fresh and stored bacterial cultures of BS, BC, and BL. It was observed that the formation of MICP was dense in samples prepared with fresh cultures. In contrast, samples prepared with stored cultures showed reduced MICP formation, which increased the compressive strength of the bacterial concrete.
5. Based on the key findings, this study strongly recommends using bacterial cultures in a fresh state to achieve the maximum benefit. Also, if there is a delay in concrete work, bacterial cultures of BS, BC, and BL can be stored at room temperature (maintained at 25 $\pm$ 2 °C) for up to 15 days. But to use the stored bacteria, additional dosage optimization needs to be conducted to achieve a similar strength to concrete prepared with fresh cultures, as the cell count increases or decreases.

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