Improvement of mechanical properties of bio-concrete using *Enterococcus faecalis* and *Bacillus cereus*

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ABSTRACT

The present study aimed to investigate the potential of *Enterococcus faecalis* (*E. faecalis*) and *Bacillus cereus* (*B. cereus*) in improving the properties of bio-concrete. *E. faecalis* and *B. cereus* strains were obtained from fresh urine and an acid mine water at cell concentration of 1.16×10^{12} and 1.3×10^{12} cells mL^{-1} , respectively. The bacterial strains were inoculated in a liquid medium into the concrete with 1, 3 and 5% as replacement of water cement ratio (*w/c*). The ability of *E. faecalis* and *B. cereus* cells to accumulate the calcite and the decrement of pores size within bio-concrete was confirmed by SEM and EDX analysis. The results revealed that *E. faecalis* exhibited high efficiency for increasing of compressive and splitting tensile strength than *B. cereus* (23 vs. 14.2%, and 13 vs. 8.5%, respectively). These findings indicated that *E. faecalis* is more applicable in the bio-concrete due to its ability to enhance strength development and reduce water penetration.

Keywords: Bacteria, Compressive strength, EDX, SEM, Splitting tensile strength, Water penetrations

1. Introduction

Due to technological advancement, several types of concrete are in existence such as conventional concrete, fly ash concrete, bio-mass concrete, fibre concrete, geopolymer concrete and bio-concrete. The bio-concrete has received more attention by the researchers in the recent years due to its high quality in terms of self-healing process resulting improvements of bio-concrete mechanical properties specially compressive strength [1, 2]. These properties represent the main criteria in the generation of concrete. The porosities rely on the porous material used in concrete whereas the pore structure of porous material is characterized by pore size, surface roughness, volume fraction and pore connectivity [3]. Other factors included compaction, water cement ratio, type of materials and aggregate size as well as the hydration and chemical, changes which are associated with the aggressive environment [4]. The increasing of pores in the concrete leads to cause an increment of concrete permeability, which may affect

concrete properties specially strength due to ingress of water and gasses [5-7].

The utilization of bacteria to fill up the concrete pores or cracks by self-healing process has been reported by authors in the literature [8, 9]. The technique is used in order to accelerate the precipitation of calcium carbonate (CaCO_3) on the bacterial cell wall to increase the compressive and splitting tensile strength as well as reduction in water penetration [8-13]. The bacteria increase the formation of CaCO_3 layer as a function for diffusing $\text{CO}_{2(g)}$ through the wet concrete and production of HCO_3^- (aq) as an intermediate product. Moreover, the bacterial strain which, has the ability to produce urease enzyme acting as catalyst to produce CO_2 and NH_3 , resulting increase pH in the surrounding bacteria cells and induce the precipitation of Ca^{2+} and CO_3^{2-} as CaCO_3 [14].

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The novel properties of bacterial cell surface possession of different functional group enable the bacteria to adsorb Ca^{2+} ions, which are available with high concentrations in the concrete. The $\text{CO}_3^{2-}(\text{aq})$ is generated from $\text{HCO}_3^-(\text{aq})$ in the presence of water and then react with Ca^{2+} ions adsorbed on the bacterial cell wall to form a complex Cell- CaCO_3 [15, 16]. The mechanism would confidently help to improve concrete durability and contribute to prolong concrete structure life with self-repair technic [17-19]. Over the last decade, the utilization of bio-concrete technology has emerged as viable, sustainable and reproducible solution to address the engineering, geotechnical and environmental issues. However, most of the researchers have been focused on the adaptation of microbial induced CaCO_3 precipitation for modification and improvement of constructional materials such as concrete and mortar. Moreover, concrete is susceptible to formation of cracks which in role contribute on reduce the sustainability performance, serviceability and lifespan of the structure. Therefore, bacteria added to different types of concrete to enhance the concrete properties [10, 20].

According to Siddique et al. [17], the addition of *S. pasteurii* bacteria into fly ash concrete enhanced the durability and strength through self-healing process as well as reduced the porosity of concrete, which is associated with the reduction the ingress of moisture. Therefore, the inoculation of bacteria into the concrete might be an alternative technology for acids, chlorides and sulphates, which reduce the strength and sustainability of concrete. However, the selections of bacterial species represent the bottleneck in the bio-concrete potent bacteria. Some of bacteria have been used in the bio-concrete included *Bacillus cereus* (*B. cereus*), *B. pasteurii*, *Pseudomonas aeruginosa*, *Shewanella* sp., which have the ability to precipitate calcium carbonate CaCO_3 [10, 19-22]. However, it has been reported that these bacteria have some deficiency to be applied in the bio-concrete including their potential to survive and grow in concrete environment due to the extreme pH and salts conditions [22]. This gap offers an opportunity for the researcher to find more applicable bacterial strains. In refereeing to the property of concrete, the bacterial strain used in the bio-concrete should have the potential to grow and have high activity within the concrete.

In the present study, the ability of *Enterococcus faecalis* (*E. faecalis*) to increase the compressive strength, splitting tensile strength and reduce the water penetration was investigated in comparison to *B. cereus*, which has been reported in the literature and was used as a control [21]. Moreover, the potential of *E. faecalis* was enhanced by the adaption process. The mechanism in which *E. faecalis* improve the property of bio-concrete was explained based on the analyses of bio-concrete by scanning electron microscope (SEM) and energy dispersive X-ray EDX.

2. Materials and Methods

2.1. Bacterial Strains

Two bacterial strains were investigated in the present work, namely

E. faecalis (KC113205.1) and *B. cereus* (KF788142.1). *E. faecalis* was obtained from fresh urine, while *B. cereus* was recovered from an acid mire water located at Sungai Pelepah Kota Tinggi, Johor Malaysia. *E. faecalis* was isolated by inoculated 1 mL of urine into a conical flask (250 mL) containing 25 mL of autoclaved nutrient broth, 10 mL of urea (40%). The control was conducted without urine. For *B. cereus*, the medium consisted of 25 mL of autoclaved nutrient broth, 10 mL of $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$ and 1 mL of acidic water, the control was carried out without acidic water. pH of the culture medium was adjusted before the sterilization to be more than pH 9 by using 0.1 M of NaOH. The nitrogen gas was pumped from nitrogen gas cylinder throw pipe into the culture medium, which in role help on released oxygen. The dissolved oxygen (DO) was determined every day by oxygen meter to confirm that bacteria could survive in anaerobic condition. The flasks were incubated on the shaker with 125 rpm for 16 d. These conditions were performed to stimulate the environmental conditions in the bio-concrete, and thus examine the bacterial potential to survive in the hard environment [23]. The concentration of bacterial cells were estimated by using spectrophotometry technique, 1 mL of the medium was diluted using sterilized distilled water to 10^{-5} and the optical density was measured by using a Nano-drop 2,000 spectrophotometer (Fisher Scientific). The ability of the bacterial strains to produce of urease enzymes was conducted by urease test according to Benson [24]. The ability to produce urease enzyme is the basic principle for the bio-concrete technique in this study.

In order to purify the bacterial strains, one mL of the culture medium was spread on Nutrient Agar (NA) medium according to Benson [24]. The plates were incubated at 30°C for 24 h. One grown colony on NA was picked up and streaked on a new NA, and then incubated at 30°C for 24 h. Thereafter, one pure colony was used for Gram staining and microscopic observation for identify the bacterial reaction for the gram staining and determine the bacterial cell morphology. Pure culture on NA media was sent to Universiti Sains Malaysia (USM) for molecular identification.

2.2. Experimental Setup

The chemical composition of Ordinary Portland Cement used in the current work is illustrated in Table S1. It was manufactured by Holcim Malaysia with all requirements defined by BS 197-1:2,000 [25]. The River sand used in this study as fine aggregate passing through 5 mm sieve, with the chemical composition presented in Table S2. The crushed stones with bulk density of 2,600 kg/m^3 were used as coarse aggregate in the size range of (12-20 mm). The water used for the concrete mixture was autoclaved to avoid the microbial contamination.

Factorial Complete Randomized Design (CRD) ($2 \times 7 \times 3 \times 3$) in triplicate was used in this study to investigate the effects of selected bacterial strains in the improvement on the properties of concrete. Where: two (2) bacterial strains (*E. faecalis* and *B. cereus*) were isolated from urine and acid mire water from Sg Pelepah Kota Tinggi, Johor Malaysia, respectively. Each type was added to concrete mixture at the end to be the last step of mixing concrete as described by Juki et al [23]. Three (3) replacements (1, 3, 5%) for water contents was carried out with *E. faecalis*

Table 1. Mix Proportions of Bio-concrete with Different Percentages of *E. faecalis* and *B. cereus* Liquid Culture

Mixture no.	M-1	M-2	M-3	M-4	M-5	M-6	M-7
Cement (kg/m ³)	420	420	420	420	420	420	420
Water (L/m ³)	210	207.9	203.7	199.5	207.9	203.7	199.5
<i>E. faecalis</i> (L/m ³)	0	2.1	6.3	10.5	0	0	0
<i>E. faecalis</i> (%)	0	1	3	5	0	0	0
<i>B. cereus</i> (L/m ³)	0	0	0	0	2.1	6.3	10.5
<i>B. cereus</i> (%)	0	0	0	0	1	3	5
Coarse aggregate (kg/m ³)	1,115	1,115	1,115	1,115	1,115	1,115	1,115
Fine aggregate (kg/m ³)	685	685	685	685	685	685	685
W/C ratio	0.54	0.54	0.54	0.54	0.54	0.54	0.54

Note: M: denote mix number and percentage (%) represent the replacement ratio of water by *E. faecalis* and *B. cereus* liquid culture by weight.

and three (3) replacements (1, 3, 5%) was performed with *B. cereus*, while one concrete mixture (concrete without bacteria inoculation) was used as control making a total of (7) groups (Table 1). Three (3) tests were performed for each concrete including compressive strength, splitting tensile strength and water penetration were tested on three (3) curing periods (7, 14 and 28 d). Two different shapes of specimens were prepared namely, cube and cylinder following requirement of the tests. Cube specimens with size of (150 mm × 150 mm × 150 mm) were prepared for examining compressive strength and water penetration, while cylinders specimens with size (Φ150 mm × 300 mm length) were prepared for examining the splitting tensile strength. The control concrete mixture was designed according to Department of Environment (DoE) to achieve 30 MPa of compressive strength at 28 d [26]. The water cement ratio (w/c) used was 0.54 for all mix proportion with and without bacteria.

2.3. Testing of Specimens

The compressive strength, splitting tensile strength and water penetration were tested on 7, 14 and 28 d. The compressive strength test was performed according to BS 12390-3:2002 with 0.15 MPa/s loading rate of compressive strength test machine to obtain constant result. Water penetration test was conducted according to BS 1239-8:2000. In the water penetration test, the specimens were put under constant pressure of 5 kPa for 72 h. Then, the specimens were cut into half and measured the maximum depth of water penetration under the test area and the results were recorded in millimetre. Splitting tensile strength test was conducted according to BS 12390-6:2000.

The microstructure of bio-concrete was determined after 28 d of the curing period by the analyses using SEM and Energy Dispersive X-ray (EDX). Both SEM and EDX used DD ISO/TS 10798:2011: Nanotechnologies-Characterization as a guideline. Concrete samples for both SEM and EDX with length (2 cm) were collected and dried at 60°C in oven for 24 h until constant conditions then send the samples to the lab to start analysis process. The analyses were performed in order to determine the bacteria precipitation of calcium and reduction in pores diameter in bio-concrete specimens compared to control.

3. Results and Discussion

3.1. Bacterial Growth

E. faecalis and *B. cereus* as identified based on 16S rRNA sequences (Table S3 and S4) exhibited high potential to grow in the culture media with conditions similar to that available in the concrete environment. The growth curve of both strains is depicted in Fig. S1. The explanation here was performed based on the bacterial growth curve reported in previous study, where four phases for the bacterial growth included lag, log, stationary and death phase. As presented in the Fig. S1, the growth rate during the log phase was high, whereas the growth rate in the stationary phase was slow. Many of the studies have been reported that the growth rate of the bacterial cells during the stationary phase are less than the in the log phase [27]. The lag phase occurred in the first two days, where the optical density (OD) of the growth was increased with slow motion. The log phase started from 3rd to 4th days and the stationary phase started from 4th to 12th days, where the growth density increased from 0.03 to 0.61 and 0.02 to 0.56 nm for *E. faecalis* and *B. cereus*, respectively. The results revealed that *E. faecalis* exhibited more growth than *B. cereus* during the growth curve except for lag phase. The death phase started on 13th day, the bacterial growth curve dropped from 0.61 to 0.58 and 0.57 to 0.55 NTU for *E. faecalis* and *B. cereus*, respectively. These findings are in line with those reported by Conlon [28] who indicated that the bacterial growth has four growth phases. In the lag phase the bacterial cells started to acclimatize on the new medium and then accelerate the growth in the exponential phase until reach the peak growth rate. The last phase is the death where the growth curve going down due to the deficiency in the nutrients and accumulation of secondary metabolic products, which affect negatively the bacterial growth.

3.2. Compressive Strength

Compressive strength of the concrete inoculated with different percentage of *E. faecalis* and *B. cereus* and in comparison with the control is depicted in Fig. 1. The results revealed that the

Fig. 1. Compressive strength for bio-concrete specimens with *E. faecalis* and *B. cereus* compare to control.

compressive strength of the concrete mixed with bacterial strains were more than in the control (No bacteria). The maximum compressive strength was recorded at 28 d of the curing period (> 30 MPa). The compressive strength of bio-concrete has no significant differences after 7 d of the curing period. However, the maximum compressive strength (31.43 and 36.77%) was recorded with 3% of *E. faecalis* on 14th and 28th day, respectively (Fig. 1). In contrast, the highest compressive strength (27.67 and 33.8%) of the bio-concrete inoculated with *B. cereus* was observed with 5% on 7th and 28th days, respectively of the curing period. The increments of compressive strength of the specimens contained bacteria species were due to self-healing process of concrete pores via precipitation high amount of available Ca in cement on the bacteria cell wall, which in role react with bacteria cell resulting precipitate ions Ca^{2+} and CO_3^{2-} as $CaCO_3$. However, increasing of *E. faecalis* ratio up to 5% in concrete caused a decrement on compressive strength compared to 3%, whereas, the reduction on compressive strength may occurred due to the excessive bacteria activity with high concentration [17].

In a comparison between *E. faecalis* and *B. cereus*, it can be noted that *E. faecalis* was more efficient than *B. cereus* for the curing days considered. These findings might be due to the characteristics of *E. faecalis* which have more advantages compared to *B. cereus*. *E. faecalis* has the ability to grow at high pH ($> pH 9$) and with 6.5% of NaCl, as well as the bacteria have high potential to produce urease enzyme which play an important role in precipitating high amount of calcium in the bio-concrete structure.

3.3. Splitting Tensile Strength

The influence of *E. faecalis* and *B. cereus* on splitting tensile strength in the bio-concrete is presented in Fig. 2. The results indicated that the inoculation of bacterial cell into the concrete enhanced the tensile strength compared to the control. The tensile strength increased significantly with the curing period up to 28

d. Among three inoculations of *E. faecalis* (1, 3 and 5%), the maximum tensile strength recorded was at 3% (3.21 MPa). The increasing of bacterial replacement percentage to 5% affected, negatively, the tensile strength, which dropped to 3.04 MPa after 28 d. *B. cereus* exhibited less efficiency in the increasing of bio-concrete tensile strength in comparison to *E. faecalis*. It has enhanced the concrete tensile strength by 6.1%, 9.6% and 10.7% with 1, 3 and 5%, respectively compared to control specimens, respectively. The highest tensile strength (3.11 MPa) was noted with 5% of the *E. faecalis* replacement percentage and after 28 d. The results indicated that *E. faecalis* and *B. cereus* play an important role in increase the strength of bio-concrete whether compressive or splitting tensile strength. However, splitting tensile strength considers as sensitive test of mechanical properties of concrete compared to compressive strength therefore, the increments of strength percentage using bio-concrete technology between both tests were different [29].

3.4. Water Penetration

The effect of bacterial inoculation of the water penetration of bio-concrete is depicted in Fig. 3. The results revealed that the water penetration of bio-concrete specimens reduced in comparison with the control. It can be noted that the water penetration decreased at all percentages of both types of bacteria. The maximum reduction in the water penetration was recorded with 3% of *E. faecalis* (10.34%, 106.56 mm) and 5% of *B. cereus* (8.5%, 109.73 mm) at 28 d compared to control (119.99 mm). These findings reflect role of bacterial inoculation in the reduction of water penetration in the bio-concrete, which represent one of the major problems affecting concrete sustainability. In addition, water penetration results confirmed the ability of that both bacterial strains used in the present study to reduce the water penetration is related to precipitation of the calcite on the surface of bacteria cells as a result of the production of urease enzyme, which in role make bio-concrete more sustain-

Fig. 2. Splitting tensile strength for bio-concrete specimens with *E. faecalis* and *B. cereus* compare to control.

Fig. 3. Water penetration for bio-concrete specimens with *E. faecalis* and *B. cereus* compare to control.

able [30-33]. Whereas, concrete with high level of penetration allows gasses and water to permeate into the concrete, which causes deterioration in concrete materials and corrosion on steel bars. Increasing of concrete permeability leads to micro-cracks, which may progress to serious cracks when it's exposed to the load [33].

3.5. Microstructure of Bio-Concrete

The microstructure analysis was conducted to determine the pore size characteristic of the concrete [34] and investigate *E. faecalis* and *B. cereus* effects in healing the pores of bio-concrete specimens. The calcite precipitation and the pores in bio-concrete and control specimens are illustrated in Fig. 4. The SEM analysis of bio-concrete specimens inoculated with *E. faecalis* and *B. cereus* revealed

presence of distinct calcite crystal and decrease in pores size (Fig. 4(b), (c)). Three different sizes of pores (maximum, average and minimum) were measured in each sample test of SEM to analysis the enhancement of healing process in concrete specimens. The results revealed that, at 28 d the average diameter of the pores size in bio-concrete specimens contain *E. faecalis* and *B. cereus* were 6.326 μm and 6.532 μm , respectively in comparison to 13.153 μm of the control specimens. The high decrement of the pores size in bio-concrete specimens confirmed high calcium carbonate formed by the bacterial cells. The presence of calcite makes increased the strength and reduced the permeability of concrete. The improvement in bio-concrete properties incorporating *E. faecalis* and *B. cereus* can be easily understood from their distinct microstructure healing, which is in line with previous studies by [8, 35].

Fig. 4. Scanning electron microscope (SEM) of UB in concrete specimens with scale bar 10 µm. (a) For control specimen normal concrete (b) For bio-concrete specimen incorporated with *E. faecalis* (c) For bio-concrete specimen incorporated with *B. cereus*.

3.6. Energy Dispersive X-ray (EDX)

The chemical constituents of concrete specimens inoculated with *E. faecalis* and *B. cereus* were analysed by EDX. Based on the results presented in Fig. 5(a), it can be seen that the minerals present in control specimen included silica, calcium, aluminium and oxides. Fig. 5(b) and (c) showed the test results of bio-concrete samples with *E. faecalis* and *B. cereus*, respectively. The presence of high percentage calcium in bio-concrete samples is due to the role of bacterial cells in the precipitation of CaCO_3 . These observations are in agreement with decrement of pores size that discussed and showed in microstructure analysis (SEM) results as elucidate in Fig. 6. The results on SEM and EDX further confirmed that addition of *E. faecalis* and *B. cereus* in concrete resulted in significant improvement in concrete properties namely compressive strength, split tensile strength and water penetration.

Fig. 5. Energy dispersive X-ray EDX for concrete specimens. (a) For control specimens (b) For bio-concrete specimen incorporated with *E. faecalis* (c) For bio-concrete specimen incorporated with *B. cereus*.

Fig. 6. Prevents the process of concrete pores self-healing via precipitation of calcium carbonate (CaCO_3) on bacteria cells wall. (a) Calcium (Ca) from cement attracts bacteria cell wall, (b) Precipitation of calcium carbonate (CaCO_3) on the surface of bacteria, (c) Healing of concrete pore due to high precipitation of (CaCO_3).

4. Conclusions

It can be concluded that *E. faecalis* and *B. cereus* has the potential to be used in the bio-concrete in order to improve the concrete properties such as compressive strength, tensile strength and water penetration. The incorporation of both bacteria into concrete produces a higher amount of calcite in the samples of all curing periods and resulted self-healing of concrete pores up to 28 d. Moreover, *E. faecalis* was more efficient than *B. cereus*. The characteristics of bio-concrete have improved with 3% of *E. faecalis* and 5% of *B. cereus*. Microstructure analysis using SEM and EDX confirmed that *E. faecalis* and *B. cereus* are capable precipitating calcium, which leads to increase in CaCO_3 .

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