Sustainable Repairing and Improvement of Concrete Properties Using Bacterial Consortium Isolated From Egypt

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Research

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Sustainable repairing and improvement of concrete properties using bacterial consortium isolated from Egypt

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Abstract

The small cracks in concrete constructions are inevitable due to deterioration during their service life throughout different load combination factors. In this study, we aimed to isolate, identify, and construct a bacterial consortium able to heal small cracks of concrete and enhance the different properties of concrete. Six isolates of bacillus, endospore-forming bacteria were isolated. There are only three isolates out of the six coded as NW-1, MK and NW-9 were showed the ability to produce urease enzyme and able to grow at 60°C with optimum growth at a temperature of 40°C. These isolates were survived in high pH, where isolate NW-1 was tolerated pH up to 11 with optimum growth at 10 while the isolates NW-9 and MK showed growth at pH 12 with an ideal growth at 10. CaCO₃ production was observed by the three bacterial isolates whether in pure or mixed cultures (bacterial consortium) but the consortium consisting of MK and NW-9 was significantly the highest in productivity among them. Therefore, these two isolates were identified using 16s as *Bacillus flexus* MK-FYT-3 and *Bacillus haynesii* MK-NW-9 and deposited to GenBank under accession numbers MN965692 and MN965693 respectively. The effect of bacteria on some properties of concrete was studied, and the results showed that the compressive and tensile strengths of bio-concrete specimens were significantly increased by 31.29, 29 % after 7 days and 36.3, 39 % after 28 days of curing compared to control specimens. The results of permeability indicated that the bio-concrete specimens significantly showed less permeability than the control

specimens by 21.1, 23.1% after 7 and 28 of curing, respectively. To determine the concrete density, Ultrasonic Pulse Velocity (UPV) test was performed, and the bio-concrete specimens gave higher values than control specimens by 26 and 20% after curing for 7 and 28 days, respectively. Also, surface healing of concrete was observed visually, the bio-concrete showed white precipitates around and inside the cracks after 7 days, which led to almost complete sealing of concrete after 28 days of curing, while the control samples were showed only very slight deposits on the surface and away from the cracks. The micro-analysis of concrete samples using SEM and XRD were done. It was found that the bio-concrete specimens showed crystalline precipitate with different shapes under SEM, while no such deposits appeared in the control specimens. On the other hand, the XRD profile was explained the characteristic peaks of calcium carbonate in both the bio-concrete and the control specimens, but the peak intensity was higher in the bio-concrete than the control specimens. This reflects the effectiveness of bacterial consortium in repairing and preventing the concrete cracks from spreading in addition to improving the various properties of concrete leading to increasing its life and sustainability.

Keywords: Sustainable repairing, bacterial consortium, cracks, concrete, *Bacillus flexus* *Bacillus haynesii*

Introduction

Concrete is a very widespread and inexpensive material utilized in construction because of the availability of its components such as cement, aggregate, and sand (Vashisht et al., 2018). Concrete acts a crucial function in infrastructure development. High compressive capacity, durability, versatility, accessibility, matching with reinforcement bar, fireplace tolerance, low price, simple production, and consequently the capability of casting in the desired shape are amongst the main features of the concrete. These distinctive options led to huge usage of concrete in the construction of different structures like buildings, dams, storing tanks, industrial plants, and transport connectivity (Seifan et al., 2019). Despite the advantages of concrete, it tends to form cracks permitting destructive substances to attack the reinforcing steel or the cementitious matrix itself. Cracks are a major reason for concrete deterioration and hence decrease in its durability (Ganesh et al., 2019). Cracks are often produced in each soft and hard status. Formwork movement, plastic settlement, and plastic shrinkage due to the fast lack of water from the surface of concrete bring about crack creation at some stage in the plastic state. while crack formation in a hardened state may occur due to other factors as climate change, dry up shrinkage, error in style, heat

pressure, chemicals attack, external load, and regular overload. furthermore, concrete structures suffer from moderately low ductileness and tensile force (Seifan et al., 2016). These finally decrease the lifetime of a building that is made with these materials due to cracks in concrete (Basha et al., 2019). To overcome this deterioration, several methods have been carried out to treat the resulted cracks and adjust the pore structure of concrete. Most of the treatments have been focused on the outside masking of cracks by using different materials such as silanes, acrylics, epoxy, waxes, and chlorinated rubbers (Nain et al., 2018). But the concrete has a weak attachment with these coating agents which are subject to deterioration and distortion with age and limits its efficiency and performance. These methods cannot block the pore networks and fill the interior porosities (Seifan et al., 2019). In addition, they are expensive, reduce aesthetics, are labor-intensive, and affect the environment (Pungrasmi et al., 2019). Unlike passive treatments, the active treatment (self-healing techniques) be able to work through various environments with no human intervention and regardless of the location of the defect. This type of concrete contains specially developed additives that can instantly activate upon crack development and closing the crack (Van Mullem et al., 2020). A self-healing in the concrete mixes may be occurred by three major approaches: (i) Autogenic healing, which is the natural cracks treatment process occurred by hydration of unwetted cementitious particles or transformation of solubilized calcium hydroxide into insolubilized carbonate. But the practical means to progress autogenic healing is to decrease the water to cement (w/c) ratio. On the other hand, rising cement part to lower w/c ratio harms shrinkage and workability and requests more cement production. (ii) Encapsulation of polymeric material, this method can fill the cracks by converting the sealing material to foam in the occurrence of water. Though the release of sealing substances from the hollow fibers embedded within the concrete mixture can seal the cracks, these substances don't act like concrete structures in several circumstances and, in certain situations, lead to crack expansion. Additionally, this technology needs capsules that can be blended along with concrete easily and can persist inside a concrete mixture. These capsules should keep the filling factor for a prolonged period and should not affect the mechanical characters and workability of concrete (Al-Tabbaa et al., 2018; De Belie et al., 2018). These needs make the encapsulation technique difficult to commercially apply in the concrete self-healing (Tan et al., 2020). (iii) Microbial calcium carbonate precipitation. It can be achieved by inserting appropriate bacterial spores inside the mixture of concrete plus nutrients to stimulate bacterial growth. By taking place of the crack, water, and oxygen entered, and at this

point, the bacterial spores develop into active vegetative cells which decompose the available nutrient changing some of the environmental conditions such as pH. By the presence of dissolved inorganic carbon (DIC) and Ca^{2+} ions the bacteria assist and speed up the formation of calcium carbonate, generally as calcite but sometimes as vaterite within cracks. Calcium carbonate production by microorganism is considered an ideal method for processing and present an efficient sealing; compatibility from a healing agent with the concrete matrix; long shelf life; ability to treat cracks repeatedly for an unlimited number of times and eco-friendly viability (Silvab et al., 2020). Although this new technology has possible application value, there are yet some challenges that need to be resolved before large-scale application. The main problem is to maintain the viability of bacteria in the matrix of concrete, where the elevated pH condition and the shear force generated by the mixing will negatively affect the performance of the bio-concrete. Therefore, the present study was aimed to isolate endospore-forming, alkalophilic, and thermotolerant bacterial isolates that can produce calcium carbonate. Moreover, mixing of bacterial isolates spores together to form an artificial bacterial consortium, determination of their ability to deposit calcium carbonate in the cracks of concrete autogenously, and enhance the characteristics of concrete.

Materials and methods

1. Sediment sampling

Sediment samples were collected from Elhamra lake, Wadi Elnatron region, Albohayra governorate, Egypt, at 30°23'22.95" N and 30°19'29.96"E at different depths. All samples were mixed and considered as only one sample.

2. Isolation of endospore-forming bacteria

Ten grams of sediment sample were suspended in 90 ml of sterile 0.85% saline solution. In a water bath, the sediment suspension was warmed at 80°C for 30 minutes. Then, 1 ml of the previously prepared sediment suspension was serially diluted up to 10^5 and the 100- μl suspension was spread on nutrient agar (NA) plates (Sarkar et al., 2015). The pH of this medium was adjusted at 8 and the inoculated NA plates were incubated at 40 °C for 2 days. The bacterial colonies that appeared with different cultural features were selected and restreaked again on the same medium. These bacteria were stained with Gram stain and checked for endospore formation according to (Chahal et al., 2011; Kim et al., 2016)

2.1.Tolerance to alkaline pH and high temperature

Each bacterial isolate was inoculated (0.1 ml of overnight broth culture (OD 620 nm = 0.8) in nutrient broth at different pH (7-12) using NaOH or HCl (1.0 mol/l) and incubated at 40°C in an incubator shaker at 150 rpm for 24h. For determination of the temperature profile of the bacterial isolates, each isolate was cultured in nutrient broth at pH 8 and incubated at 20, 40, and 60 °C in an incubator shaker at 150 rpm for 24h. After incubation, the optical density was measured at wavelength 610 nm (UNICO 2100 UV Visible Spectrophotometer, Dickinson, Texas, USA) to check bacterial growth (Moghannem et al., 2015).

2.2. Qualitative urease assay

Urea agar plates ((g/l); urea, 20.0; NaCl, 5.0; peptone, 1.0; glucose, 1.0; KH₂PO₄, 2.0; phenol red, 0.012 and agar, 15.0; (pH 6.5) were prepared, inoculated with the selected bacterial isolates (OD 620 nm = 0.8) and incubated at 37 °C for 3-5 days. The plates were investigated to observe any change in the medium color from yellow to pink revealing urease positive action (Vashisht et al., 2018).

2.3. Antagonistic activity of the tested isolates

The antagonistic activity of the bacterial isolates was tested by the agar diffusion method. It was grown on nutrient agar media and the plates were incubated at 40 °C for 3 days. After incubation, agar plugs (6mm) were cut from these cultures with a sterile cork borer. The agar plugs of each two bacterial isolates were transferred to the surface of nutrient agar plates previously inoculated with the third bacterial isolate. The plates were then kept in a refrigerator at 4°C for 2h. to allow the diffusion of the metabolites into the medium then, all plates were incubated at 40°C for 24h. After incubation, the antagonistic activities of the tested plugs were determined by observation of the inhibition zones around the agar plug (Messaoudi et al., 2015).

3. Calcium carbonate precipitation in broth culture and acid fizz tests

The isolates were tested for CaCO₃ precipitation in broth media. Briefly, in conical flasks, 30 ml of sterile nutrient broth -urea- calcium (NB-U-Ca) medium (Dhami et al., 2016) were inoculated with 2% inoculum (Whether the isolates are pure or mixed culture (bacterial consortium) at equal ratios, the final OD of inoculum = 0.8 at 620 nm). The inoculated flasks as well as the control medium (without bacteria) was incubated under shaking conditions for 7 days at 150 rpm and 40 °C. After incubation time, the conical flasks were centrifuged at 5000 rpm for half-hour. Then the supernatant was collected, and the calcium carbonate formation was tested by adding 2 drops of 10% HCl; the formation of bubbles indicated CO₂ gas generation and subsequently CaCO₃

production (Basha et al., 2018). On the other part, the precipitated CaCO_3 of each culture was resuspended in a little amount of distilled water and collected on preweighed filter paper and dried at 80 °C for 24 h. and subsequently weighed. The weight of formed CaCO_3 (W_c) was calculated from the following equation:

$$W_c = W_{fc} - W_f$$

where (W_{fc}) is the weight of filter paper containing precipitant, and (W_f) is the weight of empty filter paper.

4. Molecular characterization of potential bacterial isolates

Genomic DNA of each bacterial isolate was extracted from 50 ml of liquid bacterial culture using the Gene Jet genomic DNA extraction kit (Thermo K0721) according to the manufacturer's instructions. Amplification of the 16S ribosomal DNA of the selected isolates was carried out using the primers Bact27f (5'-GTTTGATCCTGGCTCAG-3') and 1492r (5'- CGGCTA CCTTGT TACGAC-3') by polymerase chain reaction (PCR) according to (Altschul et al.,1990)After that, the products of PCR were sequenced bi-directionally by using forward, reverse, and internal primers (27 f and 1492 r) with ABI 3730 1x DNA sequencer (GATC Biotech, Konstanz, Germany). The obtained sequence of each isolate was blasted on the GenBank to detect and compare the similarity of the isolates with the available sequences on the GenBank database.

Cultivation of bacteria and preparation of bacterial spores

The selected bacterial isolates for this study were cultivated on nutrient broth with initial pH10 and incubated for 48h. on a rotary shaker at 120 rpm and 40 °C. Preparation of bacterial spores was performed according to (Xu et al., 2019) with small modification as follows: at 5000 rpm, the bacterial culture of each isolate was centrifuged for 30 min. The supernatant was discarded, and the residual bacterial pellets were re-mixed in sterile saline solution (NaCl, 8.5 g/L). The bacterial suspension was left in an incubator for 7 days at 40 °C, meanwhile, the bacteria are in a state of starvation. Later, almost all vegetative cells became spores, and this was checked by microscopic examination of these suspensions as previously mentioned in the part of the endospore investigation. After that, 1ml of the spore suspension of each bacterial isolate was serially diluted (10^{-1} - 10^{-9}) in test tubes under aseptic conditions. To achieve homogeneity, test tubes were subjected to vortexing at 200 rpm for two minutes. Then 0.1 ml of each dilution was plated and spread onto alkaline (pH10) nutrient agar plates and incubated overnight at 40°C. After incubation time, the growing bacterial colonies in these plates were counted as CFU/ml. According to this

count, the concentration of spores of each bacterial isolate was adjusted to be $\sim 10^6$ CFU/ml and stored at 4 °C.

6. Preparation of mortar sample

6.1. Concrete Materials

The composition of concrete mixes was characterized by the high content of Ordinary Portland cement aiming at the high compactness of the matrix. Portland cement CEM I, 52.5 N, containing 95% of clinker and 5% limestone was used. Locally available sand that passes via a sieve with pores 4.75 mm and corresponding to zone II of Egyptian standard specifications (ESS) 1109/2002 was used. Dolomite of maximum size 20 mm conforming to ESS 1109/2002 was used as coarse aggregates (Code, 2007). The specific gravity of the coarse and fine aggregates was determined to be 2.7 and 2.6 respectively.

6.2. Mix Design and preparation of concrete Specimens

Concrete specimens were designed according to ESS and the American Society for Testing and Materials (ASTM) to construct 28-day compressive strength of 40 MPa and to give a slump of 12–38 mm. For concrete mix, the water volume (pH value ~ 7 at zero turbidity) used for the preparation of the control sample was 200 L/m³ while in the case of the bio-concrete sample, 200 ml/L from total water volume was replaced with Bact-Cal solution which composed of bacterial spore suspension (1×10^5 CFU/ml) and calcium lactate (252 g/L), the mix proportions are listed in Table 1. Control and bio-concrete specimens were cast in the form of cubes and cylinders with different dimensions Figure (1) and Table (2). All control and bio-concrete specimens were cured for 28 days separately in water tanks.

6.3. Evaluation of bacterial consortium effect on the properties of concrete.

To evaluate the effect of bacteria on the properties of concrete, the prepared samples of both control and bio-concrete were subjected to different tests as follows:

6.3.1. Compressive Strength Test

Compressive strength is a very important feature in concrete properties. It means the ability of concrete to bear loads. The specimens were taken out of the water and tested as soon as they were still wet. It was subjected to compressive strength testing using a compression testing machine as in Figure (2-A), according to (ASTM C39, 2017). The load was applied and increased continuously by about 13.5 KN/sec till the resistance of the specimen to the increased load collapses until no larger load can be handled. The final load was noted, and the compressive

strength of the specimen was calculated by dividing the maximum load by the cross-sectional area of the specimen. The compressive strength experiment was performed on three specimens of control and bio-concrete at curing ages 7 and 28 days and the average values were taken.

6.3.2. Splitting Tensile Strength Test

The concrete splitting tensile strength is checked on a cylinder employing a density testing machine, the procedure was started on the (ASTM C496, 2017). Initially, the specimen was placed between the load sides of the testing machine horizontally as represented in Figure (2-B), the continuous load was used without shock at a rate in the range of 0.7 to 1.4 MPa/min until the cylinder failure. This used load induces stretchable pressures on the plane which contain the applied load, producing tensile failure of the specimen. The splitting tensile strength will be measured by dividing the maximal used load by the suitable geometrical factors. A splitting tensile strength test was performed on three specimens of control and bio-concrete at different curing ages: 7 and 28 days.

6.3.3. Durability Properties (Permeability Test).

The German Water Permeation Test (GWT) was used to calculate the quantity of water that enters the substrate in the presence of conditions with controlled pressure as in Figure (2-C), the cubes of control and bio-concrete specimens were subjected to a constant water pressure of 100 KPa on curing periods of 7 and 28 days. Then, the gauge readings were documented on time in millimeters and utilized to calculate the water penetration characteristics of the surface tested. Usually, a single test takes about 5-10 minutes, according to the quality of concrete (Andrzej and Marta, 2016).

6.3.4. Non-Destructive Testing (Ultrasonic Pulse Velocity (UPV) Test)

The UPV test was done as had been performed by (ASTM C 597, 2016) UPV testing of concrete is constructed on the pulse velocity procedure mainly to give data on the consistency of concrete, occurrence of holes, cracks, and deficiencies. The pulse speed varies according to its compactness and elastic features. Direct transmission measurements on cylinders were implemented using the portable digital indicator tester known as PUNDIT. This test was conducted by passing a pulse through concrete specimens, then UPV was measured for three specimens of control and bio-concrete cylinders which cured in water for 7 and 28 days. The average velocity was recorded. Higher velocities indicate a good internal healing rate of cracks, while slower velocities indicate poor crack healing.

6.3.5. Visual and SEM microscopy evaluation

Cracks were generated in the specimens by loading specimens with axial load approximately 10% of the target load after one day of curing of both control and bio-concrete specimens in water separately. After curing for 28 days, the cracked specimens underwent a visual inspection and photographed at different time intervals (1, 7, 14, and 28 days) to observe the surface healing. For scanning electron microscope (SEM), after 28-day of curing some broken pieces of concrete near cracked surfaces in both control and bio-concrete were collected to be visualized by SEM. Samples were dried for 24h. at 60 ° (Basha et al., 2018). The sample surface was micrographed using Quanta FEG 250 scanning electron microscope (FEI Company, USA) presented at Desert Research Center (DRC), Cairo. Samples were installed on the stubs of SEM. The conditions of applied SEM were a 10.1 mm working distance, with an in-lens sensor and an excitement voltage of 20 kV.

6.3.6. X-ray Diffraction (XRD)

The XRD analysis was done using precipitates formed at the surface of the crack after 28 days of curing to determine the crystal structure of the investigated specimens. For this purpose, the precipitates formed inside the cracks were scratched and examined by X-ray Diffractometer at a spectrum ranging from 2 Theta =0° to 90°. This analysis was done using Shimadzu apparatus with nickel filter and Cu-Ka target, Shimadzu Scientific Instruments (SSI), Kyoto, Japan, at National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

7. Statistical analysis

The data were examined by analysis of variance (ANOVA), and the average comparison was done by Tukey's test and 95% Confidence. All the analyses were performed by using Minitab 18.1. and graphed by Microsoft office 365.

Result and discussion

1. Isolation of endospore-forming bacteria.

In the current study, potential isolates with the required characteristics were searched in samples originating from the locality with extreme conditions such as alkaline, hot, and saline habitats. According to the variation in morphological characteristics of bacterial colonies, only six bacterial isolates were picked up after cultivation of a pretreated sediment sample on NA medium. These isolates were coded as MK, NW-1, NW-3, NW-5, NW-7, and NW-9. The micrographs captured under the light microscope (Olympus (2000 x) appeared the tested bacteria as blue, violet, or purple bacterial cells, indicating that all isolates were Gram-positive bacteria Figure (3-A). Also, the

endospores designated by green rods within red or pink cells were visible in the results obtained from endospore staining for all investigated cultures Figure (3-B). This means the bacterial isolates can form endospores. Endospores are a special resistant dormant stage of bacteria with strong cell walls that is capable of surviving in harsh conditions for a long time (Sagripanti and Bonifacino, 1996). The development of endospores makes the bacteria able to resist extreme environmental, mechanical, and chemical stresses through the mixing of concrete (Jonkers and Schlangen, 2007). In good environments, endospores can grow and converted to vegetative bacterial cells in 90 minutes. These vegetative cells can repair and fill the cracks with CaCO_3 in case of entry of water and air into concrete (Krishnapriya et al., 2015).

2. Tolerance of bacterial isolates to alkaline pH and high temperature

The results represented graphically in Figure (4-A) revealed that NW-3, NW-5, and NW-7 isolates were considered neutrophilic bacteria that were not able to survive well in alkaline conditions. The NW-1 isolate was tolerated alkalinity with moderate growth at pH 10 and weak growth at pH 11 then it could not grow at pH 12. Among all isolates, MK and NW-9 were able to grow at pH 12 exhibiting maximum growth at pH 10 as compared to other isolates. So, MK and NW-9 may characterize as alkaliphilic isolates. The microbes that able to grow perfectly at pH values above pH 9, often in the 10-13 range of pH called alkaliphiles (Preiss et al., 2015). Alkaliphilic bacteria like *Bacillus* species can live in concrete with its extreme conditions; consequently, they are the highly targeted genus of bacteria for bio self-sealing concrete (Seifan et al., 2016). To use the bacteria in the biological treatment of concrete, the bacterial isolates should have the ability to live at very variable degrees of temperature, particularly elevated temperatures. The results refer to our bacterial isolates were grown well at 20 °C and displayed maximum growth at 40 °C but at 60 °C, only MK, NW-1, and NW-9 isolates were showed an ability to grow at 60 °C moderately Figure (4-B). The pH of fresh concrete is usually between 10 to 13 and its temperature can go up to 70°C (Talaiekhazan et al., 2014). Therefore, the selected bacteria need to exhibit high resistance against high pH and temperature. According to the previous screening which includes Gram staining, spore staining, and tolerance to high alkalinity and high temperature, the isolates named MK, NW-1 and NW-9 were only selected for the next studies.

3. Qualitative urease assay

Qualitative determination of urease production was performed by observation the color change of the pH indicator (phenol red) amended in the media from yellow to red after incubation for 48 h.

This occurs according to the quantity of ammonia produced from the hydrolysis of urea by the bacterial urease. In this test, the three Gram-positive spore-forming alkaliphilic thermotolerant isolates were exhibited positive urease activity Figure (4-C). The bacterial isolates involved in bio concrete preferably can break down urea. The urease enzyme breaks down urea, producing both ammonia and carbonate. The produced ammonia increases the pH of the environment. This situation increases the binding of carbonate with calcium ions found in the environment causing the development of CaCO_3 precipitates (Lee and Park 2018; Chaparro-Acuña et al., 2018; Syarif et al., 2019).

4. Antagonistic activity of the tested isolates

The results revealed that there is no bacterial isolate that inhibited the growth of the other two bacterial, meaning that, these bacterial isolates can be used for the preparation of synthetic bacterial co-culture (bacterial consortium). Artificial microbial consortia mean a simple microbial population designed with a well-defined makeup of two or above (normally 2-3) species/strains (Che and Men, Y2019). Artificial consortia of microbes have been offered for various functions in ecological biotechnology, biomedicine, and industry (Mauri et al., 2020).

5. Calcium carbonate precipitation in broth culture and acid fizz observation

In this experiment, we have tested three potential isolates as a pure and mixed culture for acid fizz formation and calcium carbonate precipitation in comparison with the control medium. To make sure the produced precipitate is calcium carbonate; an acid fizz reaction was performed. calcium carbonate produces gas bubbles when reacting with hydrochloric acid (10%) because of carbon dioxide release (Abudoleh et al., 2019). This reaction was given positive results with all tested cases confirming the formation of calcium carbonate by these bacterial cultures while the control medium (without bacteria) did not show any effervescence after the addition of 2 drops of 10% HCl indicating the absence of calcium carbonate in the control sample. Referring to the results of calcium carbonate precipitation, Figure (4-D) emphasized the formation of calcium carbonate in all cases whether in form of pure or mixed culture. Significantly, NW-1 has produced the lowest weight of calcium carbonate. There was not a significant difference between the weight of calcium carbonate formed by NW-9, MK isolates as a pure culture. In all cases of bacterial consortia including NW-1+ NW-9, NW-1+MK, and NW-1+NW-9 +MK, calcium carbonate precipitation significantly more increased than all treatments of pure bacterial cultures. Surprisingly, the mixed cultures of MK+NW-9 have produced the highest weight of calcium carbonate compared to other

cases. The selection of the most suitable bacteria for self-healing concrete is mostly based on their calcium carbonate yield. So, MK and NW-9 isolates were characterized and used as mixed cultures (artificial small bacterial consortium) in the next studies. The creation of artificial microbial consortia has created a new way in synthetic biology, focusing on the system's complexity and functionality. Hence, the consortia generate a different condition for microbial species, causing stimulation of silent gene expression, which does not happen in “normal” culturing conditions (Qian et al., 2020).

6. Molecular characterization of potential bacterial isolates

Analysis of 16 S rDNA partial sequences (804 and 1152 bp) of MK and NW-9 isolates revealed its homology (99%) with *Bacillus flexus* strains IFO15715 and *Bacillus haynesii* strain NRRL B-41327 which found in GenBank databases respectively Figure (5). So, MK and NW-9 isolates were designated as *Bacillus flexus* MK-FYT-3 and *Bacillus haynesii* MK-NW-9. The 16S rDNA sequences of *Bacillus flexus* MK-FYT-3 and *Bacillus haynesii* MK-NW-9 were deposited to GenBank under accession numbers MN965692 and MN965693 respectively. Bacteria belonging to the genus *Bacillus* are used as strong and reliable self-healing agents. Several studies reported that most of the calcite-forming capacity was mainly detected in species belonged to the genus *Bacillus* and then used to increase the durability of concrete (Zhang et al., 2016; Abdulkareem et al., 2019; Vashisht and Shukla, 2020).

7. Evaluation of bacterial consortium effect on the properties of concrete

The principle of the crack bio sealing depends on that the bacterial species should have the capacity of soluble organic nutrients transformation into insoluble inorganic CaCO_3 precipitates that fill the cracks. So, bacterial consortiums which consisted of *Bacillus flexus* MK-FYT-3 and *Bacillus haynesii* MK-NW-9 at an equal ratio in addition to calcium lactate as a nutrient for bacteria were incorporated immediately when mixing of concrete begins.

7.1. Compressive Strength

The data represented in Figure (6-a) explain that the increase of compressive strength in both types of concrete is time-dependent which means, it was significantly increased with time increasing. In general comparison with control, the compressive strength of the bio-concrete specimen was significantly more than control at 7 and 28 days of curing. It was increased by 31.29 and 36.3 % at the age of 7 and 28 days, respectively. The improvement in compressive strength by bacterial consortium may be due to the bio-deposition of calcium carbonate around the cell and inside the

holes of the concrete structure. Similarly, the results of compressive strength in agreement with the results of the research carried out by (Nguyen et al., 2020) who noted that *Bacillus subtilis* could lead the compressive strength to 40 % higher than the controls when used as a crack healing agent in the concrete samples.

7.2 Splitting Tensile Strength

By studying the split tensile strength of concrete, it was found that there are significant differences between bio-concrete and control specimens at 7 and 28 days as shown in Figure (6-b), the results show an increase in splitting tensile strength of bio-concrete specimens by 29% and 39% respectively at curing ages 7 and 28 days more than the control. Regardless of age, there is no significant difference between the control specimens after 28 days and the bio-concrete specimens after only 7 days of curing. Rao *et al.*, (2015) noted a significant result as compared to normal concrete. It showed an increase of over 10% in the split tensile strength after their curing for 28 days. On the other hand, Shashank *et al.*, (2018) when added *Bacillus sphaericus* with a concentration of 10^7 cells/ml in the concrete, detected a ~29% rising in the split-tensile strength of concrete than control. This occurred by the action of the bacteria that formed calcium carbonate in the concrete matrix, which lead to rising in the load resisting strength of the concrete. The variation between our result and the other may be attributed to the bacterial types and concentrations used, the overall enhanced tensile strength in bio-concrete is beneficial as it indicates that the probability of cracks formation is few to happen, and therefore concrete will sustain for a long time.

7.3. Permeability

The water permeability of concrete depends on the porosity and connection of the pores together. Therefore, the higher the porosity of concrete, the more likely it is to deteriorate. Low permeability is essential to prevent destructive compounds from entering the concrete, allowing it to endure longer (Molyneux, 2021). As illustrated in Figure (6-c), the results of the water permeability test showed a significant reduction in bio-concrete specimens by 21.1 and 23.1 % compared with control specimens after curing for 7 and 28 days respectively. We also detected that the permeability of both control and bio-concrete specimens after 7 days of curing was significantly higher than that observed after 28 days. Commonly, less permeable concrete means that concrete has less water absorption capacity. The accumulation of a layer of calcium carbonate precipitates on the surface caused a reduction in the water permeability. A reduction in water absorption leads to a slowing down of the effect of climate change. So, a repairing coating of calcium carbonate

can behave as a natural calcite skin on the surface of bio-concrete (Achal et al., 2013). The observed reduction of water permeability in bio-concrete specimens reflects the action of the bacterial consortium representing a solution to one of the main problems concerning concrete durability (Alshalif et al., 2019). our results in agreement with several reports (Luo et al., 2015; Tziviloglou et al., 2016; Balam et al 2017; Choi Sun-Gyu et al., 2017; Shashank and Nagaraja, 2021).

7.4 Ultrasonic Pulse Velocity (UPV)

An ultrasonic pulse is delivered across the tested concrete and its travel time is determined. When concrete is characterized with decent quality from the view of compactness, uniformity, and homogeneity, greater velocity has to be gained. The data represented in Figure (6-d) referred to the pulse velocity of bio-concrete specimens was significantly increased by 26% and 20% than control specimens after their curing for 7 and 28 days respectively. It is also clear that pulse velocity was increased significantly with increasing curing time in both control and bio-concrete. Bio-concrete specimens showed a compact and dense structure due to calcium precipitation which reduced the internal cracks volume which was confirmed by the ultrasonic pulse velocity test results. In this investigation, it was discovered that adding bacteria to concrete improves its mechanical qualities whereas the development of calcite in the nanopore of concrete causes reduction of water permeation and the increase in compactness and then their strength.

7.5 Visual Observation of self-healing

Figure (7) shows diverse widths of cracks in specimens of control and bio-concrete after different healing stages. Bio-healing was observed in the specimens containing bacterial consortium in the shape of white crystals or powder through curing intervals. The time required for the complete healing of cracks with various widths was different. The crack with a width of 0.1 mm was partially repaired through 7 days and filled after 28 days of curing with tap water. The crack with a width of 2 mm was nearly fully healed after 28 days of curing in water in addition to the filling of some holes which naturally present in bio-concrete specimens. For control specimens, cracks with different widths of 0.10 and 2 mm did not close even after 28 days. There is some surface precipitation with no effect after 28 days of curing. According to Nguye et al., (2020) (0.10 mm or smaller cracks in specimens of control can be appeared smooth and closed by late hydration and the reaction of Ca(OH)_2 with CO_2 in the natural self-healing process.

The SEM analysis of bio-concrete specimens inoculated with bacterial consortium generally revealed that different crystalline structures cannot be seen in the control specimen (control-concrete). In Figure (8), at 400X, there are deposits in the bio-concrete sample resembling a rough sponge which was not seen in the control specimen at the same magnification power. At 3000X, an SEM scan of the bio-concrete sample shows a sealed crack with biopolymers-like material, which could be the consequence of bacterial cells attaching to one other on a surface to form a network, as well as little calcium carbonate crystals that appear as needles or spheres. At 5000X, SEM images showed the growth and distribution of precipitated CaCO_3 that was not uniformly distributed on the crack surfaces. These precipitates appeared as irregular shapes crystals and cluster-like precipitates composed of small spherical crystals. At 6000X, Multiple shapes also appeared, but the predominant crystals are cubic, in addition to both the rod and irregularly shaped crystals. All these notes were not observed in the control specimen at the same scales. These results agree with the previous studies which also reported the difference between the surfaces of bio-concrete and control samples. The surfaces of untreated concrete seemed to be amorphous, without obvious CaCO_3 crystals appearance. In contrast, bio-concrete samples demonstrated crystalline surfaces where distinct CaCO_3 crystals may recognize (Krishnapriya et al, 2015; Siddique et al, 2016; Alshalif et al, 2019). Reda *et al.*, (2020) were reported that *Bacillus flexus* is the most potent one amongst the bacterial isolates that can form biofilm. Also, Rajitha *et al.*, (2020) was noted that *Bacillus haynesii* produce acid-soluble extracellular biopolymer. The ability of both bacterial isolates to form biopolymer may be the cause of the network appearing on the crack surface of the bio-concrete sample

7.6. X-ray Diffraction (XRD)

Figure (9) shows X-ray diffraction (XRD) analysis of control-concrete and bio-concrete. The sample constituents were recognized through their comparison with standards of the International Centre for Diffraction Data. Even though the main typical calcite peaks in bio-concrete and control samples emerged at $2\theta=29.38^\circ$ and 29.51° , respectively, which match to crystal planes (104), but the peak intensity appears to be higher in the bio-concrete than in the control specimen. This implies an increase in the concentration of precipitated calcium carbonate with increasing peak heights in the bio-concrete sample. The XRD pattern indicates the presence of three polymorphic calcium carbonate crystals (calcite, vaterite, and aragonite) in both the bio-concrete and the control specimen. However, calcite is predominant in the bio-concrete specimen, unlike vaterite that is

dominant in the control specimen. XRD is a fast-investigating approach for identifying diverse compounds derived from microbes. Calcite and vaterite have previously been identified as major crystal forms in bacterial concrete (Merve et al, 2019). The data strongly suggested that bacterial consortium which consisted of *Bacillus flexus* MK-FYT-3 and *Bacillus haynesii* MK-NW-9 can promote calcium carbonate precipitation. These findings agree with the results reported in several studies (Kalhori and Bagherpour, 2017; Seifan et al, 2016; Chen et al, 2016; Chaerun et al, 2020).

Conclusion

The bacterial consortium composed of *Bacillus flexus* MK-FYT-3 and *Bacillus haynesii* MK-NW-9 has not the ability to seal cracks only but also improve the properties of bacterial concrete. The compressive and tensile strengths of bacterial concrete are significantly increased than control samples, and the high density of their material makes it more durable. The permeability is decreased, lowering the entry of damaging materials which could harm the structure of the concrete. This research will give a technologically enhanced, environmentally friendly, pollution-free, and non-hazardous approach for achieving the most environmentally clean self-healing of concrete.

Authors Contributions

H.H. A. M.: Preparation of concrete samples and measuring their mechanical properties. M. H. A. K.: Collection of sediment samples, isolation, screening, and identification of CaCO₃ producing bacterial strains; co-wrote the manuscript. G. M. E. S.: Participation in the development of the research plan, interpretation of the results of the microbiological aspects, and revision of the manuscript. A. E. A. M.: Conceiving the presented idea and supervision of the research; co-wrote the paper. M. E. A. O.: Contribution to the interpretation of the findings of concrete mechanical property testing and manuscript review. W. M. F. T.: Characterization of all concrete samples and investigation of its microstructure; co-wrote the manuscript. The manuscript was reviewed and approved by all authors prior to submission for peer review. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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Figures

Figure 1

Control and bio-concrete specimens

Figure 2

Compressive strength test machine (A), splitting tensile specimen shape(B), and permeability device with concrete specimen (C).

Figure 3

A; photographs of Gram staining of bacterial isolates. B; photographs of endospore staining of bacteria under the light microscope (2000X).

Figure 4

(A) Effects of pH on the growth of the bacterial isolates (B) growth of the bacterial isolates at different temperatures (C) urease activity of the bacterial isolates after 2 days of incubation (D) weight of CaCO₃ precipitates after 7 days of incubation in NB-U-Ca medium

Figure 5

Phylogenetic trees of *Bacillus flexus* MK-FYT-3 and *Bacillus haynesii* MK-NW-9

Figure 6

(a) compressive strength of both control and bio-concrete specimens, (b) split tensile strength of concrete and bio-concrete specimens, (c) permeability test of conventional concrete and bioconcrete specimens, (d) ultrasonic pulse velocity test of conventional concrete and bioconcrete specimens.

Figure 7

Visual Observation of self-healing of cracks with 0.5 and 2 mm in width in both control (C) and bio-concrete specimens (Bio) at different curing times (1, 7, and 28) days

Figure 8

Scanning electron micrographs of bio-concrete and control specimens at 400, 3000, 5000, and 6000X.

Figure 9

XRD patterns of bio-concrete and control-concrete specimens.

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