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MANUFACTURE AND STUDY OF SELF-HEALING PROPERTIES IN CONCRETE WITH BACILLUS HAYNESSII (RAKAN1508) BACTERIA ISOLATED FROM FARAFRA OASIS, NEW VALLY GOVERNORATE, EGYPT

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ABSTRACT

Concrete failure can be attributed to various factors, including cracks that allow chemical solutions to seep through this may have a substantial effect on Concrete Structures' Mechanical, Physical, and Durability Aspects. The idea that bones and tissues can repair themselves has been exploited, so we needed to make concrete that can self-heal cracks. The ability of a material to identify and mend internal damage without help from outside sources is known as self-healing. Our goal of this work was to identify, isolate, and create a bacterial consortia that could repair tiny concrete cracks and improve the concrete many qualities. Bacillus bacteria that generate endospores were discovered in four different isolates. Of the four isolates, only one was able to grow at 50°C and produce the urease enzyme; 40°C is the optimal temperature for growth. It also demonstrated growth potential at a pH of 12 with optimal growth at 10. Therefore, this isolate was identified using 16s as Bacillus haynesii RAKAN1508 and is stored in Gen-Bank with accession number OR642761. The progression of the healing ratio and the fracture profile were evaluated using both the scanning electron microscope with variable pressureCT scans using X-rays (also known as X-ray mCT). Additionally, this Studies were conducted to determine how bacteria affected properties concrete qualities. VP-SEM results showed that microbial precipitation fully healed a 0.4 mm crack mouth width, which XRD subsequently confirmed to be calcite and vaterite. Therefore, compared to control specimensthe ,bio-concrete specimens' compressive and tensile strengths increased dramatically by 36.3 and 44% following 28 days of curing. Per the sorpativity test results, which showed that after 28 days of healing, In contrast to the control samples, the bio-concrete specimens showed a substantial 23.1% decrease in permeability.The long-term repair of cracks in the concrete skin may be achieved with bacteria-based concrete self-healing.

KEYWORDS: Self-Healing, Concrete, Cracks and Bacillus haynesii.

تصنيع ودراسة خصائص الإلتئام الذاتي في الخرسانة بكتيريا باسيللس هاينسي (راكان 1508) المعزولة من واحة الفرافرة، محافظة الوادي الجديد، مصر

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الملخص

يمكن أن يعزى فشل الخرسانة إلى عوامل مختلفة، بما في ذلك الشقوق التي تسمح للمحاليل الكيميائية بالتسرب من خلالها، وقد يكون لها تأثير كبير على الجوانب الفيزيائية والميكانيكية والمتانة للهياكل الخرسانية. لقد تم استغلال فكرة أن العظام والأنسجة يمكنها إصلاح نفسها بنفسها، لذلك كنا بحاجة إلى صنع خرسانة لديها القدرة على التآم شفاء الشقوق من تلقاء نفسها. تُعرف قدرة المادة على تحديد وإصلاح الضرر الداخلي دون مساعدة من مصادر خارجية بالالتآم الذاتي. كان هدفنا في هذه الدراسة هو عزل وتوصيف وإنشاء اتحادات بكتيرية يمكنها إصلاح الشقوق الخرسانية الصغيرة وتحسين خصائص الخرسانة العديدة. تم اكتشاف البكتيريا العصوية التي تولد الأبواغ الداخلية في أربع عزلات مختلفة. من بين أربع عزلات، كانت Bacillus haynesii RAKASN1508 لديها القدرة على تصنيع إنزيم اليورياز ويمكن أن تنمو عند درجة حرارة 50 درجة مئوية، مع كون 40 درجة مئوية هي درجة الحرارة المثالية للنمو. كما أظهر إمكانات النموعند درجة حموضة 12 مع نمو مثالي عند 10. لذلك، تم تحديد هذه العزلة باستخدام التعريف الجيني وتم إيداعها في البنك الجيني تحت رقم OR642761. تم تقييم تطور نسبة الالتآم وملف الكسر باستخدام كل من المجهر الإلكتروني الماسح ذو الضغط المتغير (VP-SEM) والتصوير المقطعي الدقيق المحوسب بالأشعة السينية (الأشعة السينية mCT). بالإضافة إلى ذلك، تم دراسة تأثير البكتيريا على بعض خصائص الخرسانة. وفقًا لبيانات VP-SEM، تم إصلاح عرض الفم المتشقق بمقدار 0.4 مم بالكامل عن طريق الترسيب الميكروبي، والذي أكد XRD لاحقًا أنه كالسيت وفاتريت. لذلك، بالمقارنة مع عينات الكنترول تحسنت قوة الضغط والشد لعينات الخرسانة الحيوية بشكل ملحوظ بنسبة 36.3، 44٪ بعد 28 يومًا من المعالجة. استنادًا إلى نتائج اختبار المتراكبة، والتي أظهرت أنه بعد 28 يومًا من المعالجة، كانت عينات الخرسانة الحيوية ذات نفاذية أقل بنسبة 23.1٪ من عينات التحكم، فمن الممكن أن تكون الخرسانة ذاتية الالتآم من البكتيريا بمثابة عملية التآم ذاتية طويلة الأمد حل مصطلح لإصلاح الشقوق في الجلد الخرساني.

الكلمات المفتاحية: الإلتآم الذاتي ،الخرسانة ،الشقوق ،باسيللس هاينسي.

1. INTRODUCTION

One of the most used structural materials is concrete, second only to steel. Due to the accessibility of its constituent parts, such as sand, cement, and aggregate, a very common and affordable material used in building [1]. In the construction of infrastructure, concrete is essential. The key characteristics of concrete are its excellent durability, compressive capacity, and availability, compatibility for reinforcement bars, tolerance for fireplaces, ease of production, low cost and ultimately its able to be cast in the required shape. Due to these unique choices, a significant amount of concrete was used in the development of a variety of constructions, including buildings, storage tanks, dams, industrial plants, and transportation infrastructure [2, 3].

Even though concrete has many benefits, cracks in concrete are nearly always present. Because of its perceived brittleness and variety, the concrete matrix [4, 5]. Even in the absence of an external stress, these cracks can be produced by early-age temperature gradients, hemorrhage, shrinkage, costly reactions, and other factors [6 - 8]. In light of this, concrete microcrack formation and persistence particularly For civil engineers around the world, problems with the concrete skin continue to be a concern [9]. This is due to the possibility that the microcracks will allow aggressive material to seep in and eventually destroy the concrete structure [10, 11]. Therefore, it is imperative to design smart and sustainable concrete in order to stop fractures from spreading and to cure them as quickly as possible [12, 13]. Sustainable concrete technology, or self-healing procedures, have advanced recently to the point that they can operate in a variety of settings without the need for human intervention, independent of the defect's location. According to [14]. This particular kind of concrete has additives that have been carefully created to activate promptly upon the formation of a fracture and close it.

Concrete mixes can self-heal using three main techniques:(I) Autogenic healing, a process that treats cracks by hydrating cementitious particles that haven't been moist or converting carbonate that has been insolubilized from soluble calcium hydroxide. Nonetheless, lowering the water to cement (w/c) ratio is a realistic way to advance autogenic healing. ConverselyShrinkage and decreased workability result from raising the cement fraction to a lower w/c ratio, which also requires more cement to be produced. (ii) Encapsulation of polymeric material: When water is present, this technique can fill in fractures by turning the sealing material into foam. While the gaps can be filled by the sealing agents released from the hollow fibers in the concrete mixture, in some cases these

agents function differently from concrete structures and can potentially make the cracks worse. Moreover, this technique needs capsules that are able to stay in a concrete mixture and combine easily with concrete. According to [15, 16]. These capsules should maintain the filling factor for an extended length of time without affecting the mechanical properties or workability of concrete. Due to these requirements, it is challenging to commercially use the encapsulation technology in the self-healing concrete [17]. (iii) Microbes causing calcium carbonate to precipitate. It can be accomplished by adding the right kind of bacterial spores to the concrete mixture together with nutrients to encourage the growth of bacteria. Water and oxygen were allowed to enter through the cracks, and the bacterial spores then matured into vegetative cells that broke down the available nutrients and altered the pH and other environmental parameters. When dissolved inorganic carbon (DIC) and Ca^{2+} ions are present,, the bacteria help to expedite the synthesis of calcium carbonate, which often forms as calcite but can also occasionally occur as vaterite within fissures. The utilisation of microorganisms in the production of calcium carbonate is regarded as the optimal technique for various purposes, includes long shelf life, unlimited ability to fix cracks, environmental sustainability, and compatibility using the concrete matrix as a healing agent [18]. Despite the new technology's potential uses, a few issues still need to be worked out before it can be widely used. Maintaining the life of bacteria in the concrete matrix is the main challenge. So the main objectives of the research: To understand the mechanisms behind the self-healing of concrete, knowing the new technology and knowing to full control it, study the effect of age on the healing process and develop/design a cementitious material (or a set of materials) having specific chemical composition that under certain environmental conditions will be capable to self-repair, both internally and externally. where the bio-concrete's performance will be adversely impacted by the high pH and shear force created during mixing. The goal of the current investigation was to identify endospore-forming, thermotolerant, alkalophilic, and bacterial isolates with the ability to manufacture calcium carbonate. Furthermore, combining bacterial isolates to create a synthetic bacterial consortium, figuring out how well they can adhere calcium carbonate to cracks in it on their own., and improving concrete's properties.

2. Materials and Methods

2.1 Bacterial isolation and identification

A specific kind of bacteria was discovered from soil samples that were gathered from the New Valley Governorate in Egypt in the Farafra Oasis: at different depths, latitude 27°3′30 north and longitude 27°58′12 east. All samples were collected to form one sample. Next, we proceeded to isolate and dispose of bacteria that create endospores. 90 milliliters of sterile 0.85% saline solution were used to suspend 10 grams of sediment sample in order to extract endospor-producing bacteria. The bacteria that were present in the soil were allowed to thrive by warming the sediment suspension in a water bath for 30 minutes at 80 degrees Celsius. The individual bacteria were then separated from the others using the streak plate technique [19]. Stated differently, Every single microbe would multiply and grow, eventually establishing a Petri plate colony.. Through several steps, 100 μL of the sediment solution made earlier was spread out on nutrient agar plates after being serially diluted from 1 ml to 105. For two days, Incubation was conducted at

40°C for the inoculated NA plates. with this medium's pH set to 8. On the Petri dish, each individual bacterium would multiply and thrive, forming a colony. It's interesting to observe that every bacterium has a unique form, size, and color. Additionally, as colony form is easy to recognize, the colony was visible to the unaided eye, according to [20, 21].

2.2 Tolerance to alkaline pH and high temperature

Using either NaOH or HCl (1.0mol/l) as an initial pH (7–12) solution0.1 milliliter of a broth culture that was left overnight (OD 620 nm = 0.8) in nutritional broth was added to each bacterial isolate and then it was cultured in an incubator shaker set to 40 °C for 24 hours at 150 rpm. To ascertain their temperature profiles, each bacterial isolate was grown in nutritious pH 8 broth was placed in a shaker incubator set to operate at 150 rpm for 24 hours at 20, 40, and 50 degrees Celsius. The optical density at 610 nm (UNICO 2100 UV Visible Spectrophotometer, Dickinson, Texas, USA) was measured following incubation to assess bacterial growth **[22].** The mean and standard error values were computed after each treatment was carried out in triplicate.

To ascertain whether the recovered bacterium belonged to was related to the Bacillus gene or not, the conventional Gram stain was also taken into consideration. The gram stain test procedure followed **[23]** lead. In the meantime, endospore creation was done in accordance with a study by **[24]** that was carried out to see if the isolated bacteria could be able to produce spores. VP-SEM was also used to examine the size and shape of the isolated bacterium's morphology. The identified bacteria was cultivated overnight as the initial stage. It was then harvested using a centrifuging device. Then, using a tiny centrifuge tube, It was twice cleaned with purified water (dH_2O). Afterwards, in order to aid the bacterial cells' adherence to the glass, the target sample was moved to cover glasses manufactured by Fisher Scientific and coated with Poly-lysine. After thatThe bacteria was fixed by adding 1 milliliter of a 2.5% glutaraldehyde solution. The sample was then submerged in hexamethy ldisilazane for two hours. It was then placed in a desiccator for an overnight period so the bacterial cells could dry up. Urease activity in the isolated bacteria was further investigated using the Nessler method to determine if it belonged to bacteria that were ureolytic or not, as explained in the following section.

2.3 Ureolytic activity

Following an incubation period of three to five days at 37 °C, the selected bacterial isolates were inoculated onto urea agar plates ((g/l); urea, 20.0; NaCl,1.0; glucose, 5.0; peptone, 1.0; KH2PO4, 2.0;agar, 15.0; (pH 6.5)) and phenol red, 0.012 . To check for any signs of urease activity, such as a change from yellow to pink in the medium color, the plates were inspected according to [13, 25].

2.4 Calcium carbonate precipitation in broth culture and acid fizz tests

A $CaCo_3$ precipitation test using broth media was performed on the isolates.In short, 25 milliliters of sterile nutrition broth-urea-calcium (NB-U-Ca) medium were filled with 2% bacterial culture [26]. The final optical density of the inoculum was 0.8 at 620 nm regardless of whether the bacterial culture was pure or mixed (bacterial consortium) at comparable ratios. Both the control media (without bacteria) and the infected flasks were incubated for 7 days while being shaken at a speed of 150 rpm. After incubation, the conical flasks underwent a 30-minute centrifugation process at 5000 rpm. Following that, two drops of 10% HCl were added to the supernatant to test for the production of calcium

carbonate; the emergence of bubbles indicated the generation of CO_2 gas and the subsequent production of $CaCo_3$ [27]. In contrast, a little amount of distilled water was used to resuspend the precipitated $CaCo_3$ from each culture, gathered on filter paper that had been preweighed, dried at 80 degrees Celsius for 24 hours, and weighed. The following equation was used to compute the weight of produced $CaCo_3$ (Wc):

$$Wc = Wfc - Wf,$$

In this case, Wfc stands for the weight of the filter paper that includes precipitant, and Wf for the weight of the empty filter paper. The mean and standard error values for each treatment were computed after being carried out in triplicate.

2.5 The isolated bacteria's DNA identification

Given that DNA identification is a form of genetic fingerprinting, it is crucial to understanding how it works. Using the 16 S rRNA gene from bacteria, it sheds light on the species and strain names [28]. To obtain the pure DNA, Four procedures were considered: extraction, purification, PCR amplification, and sequencing. Following the manufacturer's instructions, the kit for extracting genomic DNA from Gene Jet (Thermo K0721) was used to extract every bacterial isolate's genomic DNA from 50 milliliters of liquid bacterial culture. Using the primers Bact27f (50-GTTTGATCCTGGCTCAG-30) and 1492r (50-CGGCTACCTTGT TACGAC-30), according to [29]. Using the polymerase chain reaction, the selected isolates' 16S ribosomal DNA was amplified (PCR). Next, internal, reverse, and forward primers (27 f and 1492 r) were used in conjunction with the ABI 3730 l DNA sequencer (GATC Biotech, Konstanz, Germany), the PCR products were sequenced in both directions. Each isolate's sequence was collected, and the resulting sequences were compared to the existing sequences in the GenBank database to determine how similar they were.

2.6 Bacterial culture and bacterial spore preparation

The selected bacterial isolates were incubated for 48 hours at 120 rpm on a rotary shaker and 40^{o}C using nutritional broth with an initial pH of 10. The following slight adjustment was made to the method described by [30]. For the bacterial spore preparation: The bacterial culture was centrifuged for 30 minutes at 5000 rpm. After removing the supernatant, the remaining bacterial pellets were once again combined with sterile saline solution (8.5 g/l NaCl). The bacterial suspension was kept at 40°C in an incubator for 7 days, during which time the bacteria were starving. As previously noted in the section on the endospore inquiry, microscopic analysis of these suspensions confirmed that practically all vegetative cells later turned into spores. Then, under aseptic circumstances, In test tubes, 1 milliliter of the bacterial isolate's spore suspension was serially diluted (10-1 to 10-9). To guarantee homogeneity, test tubes were vertexing for two minutes at 200 rpm. Next, each dilution's 0.1 ml was plate, distributed, and incubated overnight at 40^{o}C on nutrient agar plates with a starting pH of 10. These plates' expanding bacterial colonies were measured in CFU/ml after the appropriate incubation period. This count was used to modify the bacterial isolate's spore concentration to 106 CFU/ml and preserve them at 4^{o}C.

2.7 Preparation of mortar sample

2.7.1 Concrete Materials

Ordinary Portland cement made up a large portion of concrete mixtures, which was done to increase the matrix's compactness. CEM I, 42.5 N Portland cement, mostly composed of clinker and 5% limestone, was used. Sand that complies with Egyptian Standard Specifications (ESS) 1109/2002 zone II and is easily accessible in the area was used. The sieve's pores measure 4.75 mm. As coarse aggregates, dolomite with a maximum size of 20 mm that complied with ESS 1109/2002 was utilized (Code, 2007) [31]. The coarse and fine aggregates' specific gravities were found to be 2.68 and 2.6, respectively.

2.7.2 Mix Design and preparation of concrete Specimens

In accordance with ESS and the American Society for Testing and Materials (ASTM), concrete specimens were created with a 28-day compressive strength of 200 kg/cm^2 For the concrete mix, 185 L/m3 of water (pH value ≒ 7 at zero turbidity) was used to prepare the control sample. In contrast, for the bio-concrete sample, 185 ml/L of the total water volume was replaced with Bact-Cal solution, which is made up of calcium lactate (15 kg/L), yeast extract (185 g/L), and bacterial spore suspension (1×105 CFU/ml). The mix proportions are shown in Table 1. Three mixes were set (There were two control concrete mixes and one with added nutrients), namely three concrete mixes (1) control mix, (2) nutrient-infused mix and (3) Use the bio-based healing agent in a bacterial concrete mix. Six cube-shaped samples of the mixture were cast (with dimensions of 100*100*100 mm) and cylinders (with dimensions of 150*300 mm) of different dimensions, Figure (1) and Table (1). Each of the specimens control concrete (control), control concrete (nutrient), and self-heal concrete (bio-concrete)—was cured in water tanks for a total of 28 days in separately. Experiments were conducted 3 times to ensure the same results.

Table 1. Concrete mixing properties.

Ingredient									
Cement		Water (L)	Coarse aggregate		Fine aggregate	Admixture (L)	Yeast extract (g/m^3)	Calcium lactate (kg/m^3)	Bacterial solution (L)
			1	**2**					
Mass (kg/m^3)	**300**	**185**	**560**	**560**	**730**	**5**	**185**	**15**	**10.45**

2.8 Assessment of the impact of the bacterial consortium on the characteristics of concrete.

2.8.1 Fresh concrete lump

The slump test for concrete is an experimental test performed on fresh concrete. It is employed to confirm the concrete's uniformity and straightness. This is accomplished using a special cone to measure the amount of slump, assess the concrete's workability, strength, and determine the proportion of water that needs to be added to the cement. The test was done according to (ASTM C 143-15) [32] and According to the Egyptian code (laboratory testing guide (sec.6-2)) [31].

2.8.2 Compressive Strength Test

A crucial aspect of concrete qualities is their compressive strength. It refers to concrete's capacity to support loads. While the samples were still wet, testing was done on them, after being removed from the water. We tested its compressive strength using a compression testing machine.. In accordance with (ASTM C39, 2017) [33], it was tested for compressive strength utilizing the compression testing apparatus a constant 13.5 KN/sec increase in the load was applied until the specimen's ability to withstand the increasing load collapsed and no further load could be applied. By calculating the area divided by the maximum load, the cross-section of the sample and the coefficient of modification were taken into account according to the Egyptian Code 2020 [31] (cubes with dimensions of 100*100*100 mm were used). Three samples of control concrete, nutrient concrete, and 28 days after curing, a compressive strength test was conducted on the bio-concrete. Calculate the averages values.

2.8.3 Splitting Tensile Strength Test

Using a density testing machine, the splitting tensile strength of concrete is measured on a cylinder, In accordance with (ASTM C496, 2017) [34]. As shown in Figure (2-B), First, the specimen was placed horizontally between the testing apparatus's load sides. After then, the constant load was delivered without shock at a pace between 70 and 140 (kg/cm^2)/min till the cylinder failed. Tensile failure occurs in the specimen due to stretchy pressures created on the plane supporting the load by the applied force.. To find the splitting tensile strength, divide the maximum usable load by the relevant geometric components. The splitting tensile strength of three control and bio-concrete specimens was measured after 28 days.

2.8.4 Absorption of water test (sorpativity test)

Sorpativity test measures the susceptibility of an unsaturated concrete to the penetration of water through capillary suction. In accordance with ASTM C1585-04 [35] the sorptivity test was performed on cylindrical specimens measuring 100 mm in diameter and 50 mm in height following a 28-day curing period.

The samples were dried inside an oven at 50+ 5C for 3 days. Silicone was used to seal the specimen's sides so that the flow was only one way, then the surfaces of the specimens were exposed to water by immersion in a basin at a depth of 5-10mm.

In time periods (1, 5, 10, 20, 30, 60, 120, 180, 240, 300, 360 minute) the specimens was removed from the water and recording the change in mass per unit area versus square root of time. Figure (3-22) shows the procedure details of the sorpativity test.

The following equation expresses the water absorption of concrete:

$I = mt/ (a/d)$

Where: 1 = the absorption in mm.

mt =the change in specimen mass (gram), at the time (1).

a= the exposed area of the specimens (mm^2).

d = the density of the water (0.001) (g/mm^3).

2.9 Microstructural Analysis

The following techniques were used to evaluate, interpret and analyze the results and materials formed inside the crack.

2.9.1 Particularly, CT and X-rays

A 0.4 mm copper plate was placed to create a groove during the casting process. Interestingly, the insertion was made 20 mm below the surface and was taken out a day later. Submersion in water was used after a 24-hour period to further treat the target specimen and start the healing process. In engineering applications like determining a material's porosity profile, X-ray micro computed tomography, or X-ray μCT, has a good reputation X-rays micro computed tomography (X-rays μ CT) was used in this study to examine the crack.

2.9.2 Visual, VP-SEM-EDS, and XRD

In this work, the healing precipitation on the crack surface was tracked and observed using a variable-pressure scanning electron microscope. Moreover the filling product was identified through EDX analysis using VP-SEM-EDX (Model JEOL JSM-IT300LV). 28 days after curing, several broken fragments of concrete at fractured areas in the control, nutrient-infused concrete, and bio-concrete were collected to be examined under a scanning electron microscope. However, Using X-ray powder diffraction (XRD), the precipitation product that remained on the fracture surface after the healing process was complete was also discovered. The healing product was then subjected to XRD testing (Model Rigoku) after being ground into a fine powder.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of endospores forming bacilli bacteria

As indicated in Figures 1, 4 soil samples were taken from various locations in Farafra, New Valley Governorate, Egypt .To obtain a single colony, 4 isolations were made and subsequent dilutions were performed. Four colonies were selected from each bacteria based on the size, shape, and color of its colonies. Because the other isolates did not match the requirements for the intended bacterial target for the concrete mix, only bacterium isolate number three was taken into consideration. The isolates 1 were disregarded because they do not produce spores and bacteria, the isolate 2 was disregarded because it does not stimulate the urease enzyme, and the isolate 4 was disregarded because it does not belong to the bacillus strain. The Isolate RAKAN 1508 was chosen because, unlike bacillus bacteria, which form a single colony as seen in Figure 3, it may construct a chain of connected cells. In comparison to a single colony, the cell chains will have a wider area for calcium carbonate deposition, and they have the good ability to react to any deformation during growth. The bacteria were gram-positive and had a line-like structure in the concrete medium. They produced spores when subjected to extreme conditions like nutrition deprivation or high concrete ph. The cell's length and width were determined to be 5-4 and 1-1.5 micrometers, respectively, which was previously known. Germs and spores are crucial. The results represented in Table No. 2 showed that among all isolates, Compared to the other isolates, isolate RAKAN 1508 was allowed to develop by PH 12 and shown maximal growth at PH 10 and 12. compared with the anther isolates.Therefore, 4 can be described as an isolated alkaloid. Alkaloids are microbes that can grow best at pH levels higher than 9, typically in the range of pH 10–13 [36]. Alkali bacteria such as Bacillus species can survive in concrete in harsh conditions; hence, it is a highly targeted bacterial genus for self-healing bio concrete [37].

Fig. 1. Location in Farafra, New Valley Governorate, and Egypt which samples were collected.

Fig. 2. 4 soil samples were taken from various locations in Farafra, New Valley Governorate, Egypt.

Fig. 3. Colony shape and characteristics of isolated bacteria.

Table 2. Biochemical characteristics of bacterial isolates.

TEST	RAKAN 1502	RAKAN 1501	RAKAN 1508	RAKAN 1507
SEM shape	Rod	Rod	Rod	Rod
Gram stain	Gram Positive	Gram Positive	Gram Positive	Gram Positive
Spore staining	+	+	+	+
O/F test	F	F	F	F
Catalase test	+	+	+	+
Indole production	-	-	-	-
Methy red	-	-	-	-
Voges Proskaur	+	+	+	+
Citrate utilization	+	+	+	+
Urease test	+	+	+	+
Starch hydrolysis	+	+	+	+
Gelatin hydrolysis	+	+	+	+
Nitrate reduction	+	+	+	+
H2S production	+	+	+	+

Fig. 4. Bacterial shape with gram reaction and spore formation under light microscope.

3.2. Urease enzyme bacterial growth activity

After 48 hours of incubation, the phenol red as a pH indicator added to the medium changed color from yellow color to red color, allowing for a qualitative assessment of urease production. This depends on the amount of ammonia generated by the bacterial urease's breakdown of urea. In this assay, the four isolates exhibiting spore-forming, thermotolerance, alkaliphilia, and Gram-positive characteristics demonstrated positive urease activity. The bacterial isolates for bio concrete would be ideally be able to degrade urea. Ammonia and carbonate are both produced during the urea's breakdown by the urease enzyme. The environment's pH rises as a result of the ammonia production. This circumstance causes the formation of $CaCO_3$ precipitates by increasing the binding of carbonate with environmental calcium. **[38, 39, 40]**.

Table 3. Urease activity for isolated bacteria.

Bacterial isolates	Urease activity
RAKAN 1502	+
RAKAN 1501	+
RAKAN 1508	+ +
RAKAN 1507	+

3.3 The ability of isolated bacteria to withstand high temperatures and an alkaline pH

The findings, which are graphically displayed in Table (4) and Fig. 5, showed that neither of the isolates of RAKAN 1502 and RAKAN 1501 were assumed to be neutrophilic bacteria incapable of surviving in an alkaline environment. The RAKAN 1507 isolate was able to withstand alkalinity, growing moderately by PH 10 and weakly by PH 11, but not at PH 12. When compared to other isolates, RAKAN 1508 was the only one that could grow at pH 12, with a maximum growth of pH 10. RAKAN 1508 could therefore be classified as an alkaliphilic isolate. Alkaliphiles are microorganisms that can grow optimally at pH levels higher than 9, typically in the 10–13 range [36]. Because they can survive in the harsh conditions of concrete, alkaliphilic bacteria such as The most sought-after genus of bacteria for bio-self-healing concrete is Bacillus species [37].

The bacteria isolated from concrete must be able to withstand a wide range of temperatures, especially high ones, in order to be used in the biological treatment process. Our isolates of bacteria, which developed fully at 20 °C and reached their maximal growth at 40 °C, are the subject of the results. However, at 50 °C, only two isolates (RAKAN 1502 and RAKAN 1507) demonstrated a moderate ability to grow (Table 5) and Fig. 5. Temperatures of up to 70°C can be reached by harden concrete, which often has a pH of 10 to 13 [41]. As a result, the chosen bacteria must demonstrate strong resistance to high temperatures and pH levels. Based on the findings of tthe earlier screening, which included resistance to high alkalinity and high temperature, Gram staining, and spore staining, only the isolate RAKAN 1508 was selected forthe following next studies.

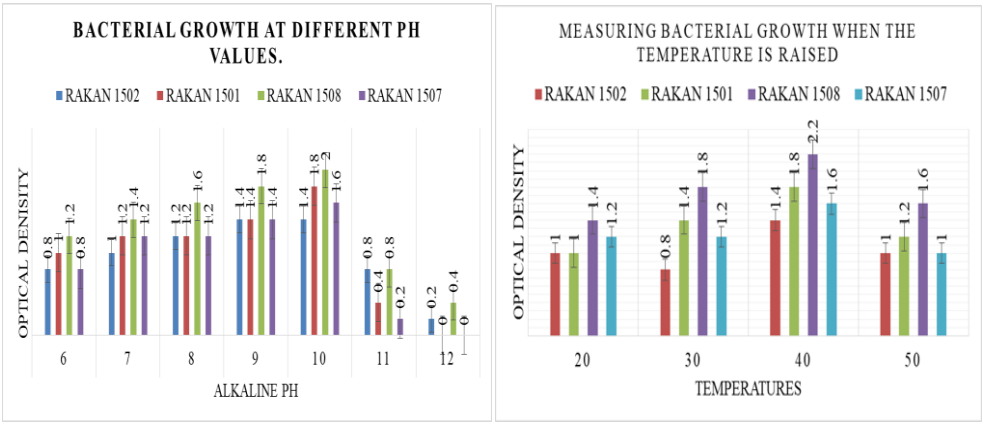


Fig. 5. Charts the ability of isolated bacteria to withstand different temperature and pH changes.

Table 4. Bacterial growth at different pH values.

PH	Optical Density			
	RAKAN 1502	**RAKAN 1501**	**RAKAN 1508**	**RAKAN 1507**
6	0.8	1.0	1.2	0.8
7	1.0	1.2	1.4	1.2
8	1.2	1.2	1.6	1.2
9	1.4	1.4	1.8	1.4
10	1.4	1.8	2.0	1.6
11	0.8	0.4	0.8	0.2
12	0.2	0	0.4	0

Table 5. Bacterial growth at different temperature values.

Temp.	**Optical Density**			
	RAKAN 1502	**RAKAN 1501**	**RAKAN 1508**	**RAKAN 1507**
20	1.0	0.8	1.4	1.0
30	1.0	1.4	1.8	1.2
40	1.4	1.8	2.2	1.6
50	1.2	1.2	1.6	1.0

3.3 Precipitation of calcium carbonate in broth culture and observation of acid fizz

In contrast to the control medium,pure calcium carbonate precipitation and acid fizz generation and mixed cultures was investigated in three potential isolates in this experiment. By using an acid-fizz reaction, the precipitate's calcium carbonate content was confirmed. Gas bubbles are produced when calcium carbonate and 10% hydrochloric acid react due to the release of carbon dioxide [42]. All of the tested cases had positive reactions from this reaction, proving that the bacterial cultures generate calcium carbonate. The adding of two drops of 10% HCl to the control medium, on the other hand, did not cause it to fizz, indicating that the control sample was devoid of calcium carbonate. The fact that calcium carbonate developed in every instance—whether as pure or mixed cultures—was stressed while talking about the outcomes of calcium carbonate precipitation. Notably, the least amount of calcium carbonate by weight was produced by isolation RAKAN 1501. The weight of calcium carbonate produced by isolates RAKAN 1502 and RAKAN 1507 in pure culture did not differ significantly. Farm RAKAN 1507 is thought to have produced the most calcium carbonate compared to the other 3 isolates. As shown in Table (3.3) and Fig. 6, the most crucial consideration Based on their capacity to produce calcium carbonate, the best bacteria to use for self-healing concrete are selected. Consequently, isolate RAKAN 1508 was discovered and used as a culture in the study that followed.

Table 6. Production of calcium carbonate isolates

Bacteria	$CaCo_3$ mg/ml
RAKAN 1502	2.6
RAKAN 1501	2.4
RAKAN 1508	3.4
RAKAN 1507	2.6

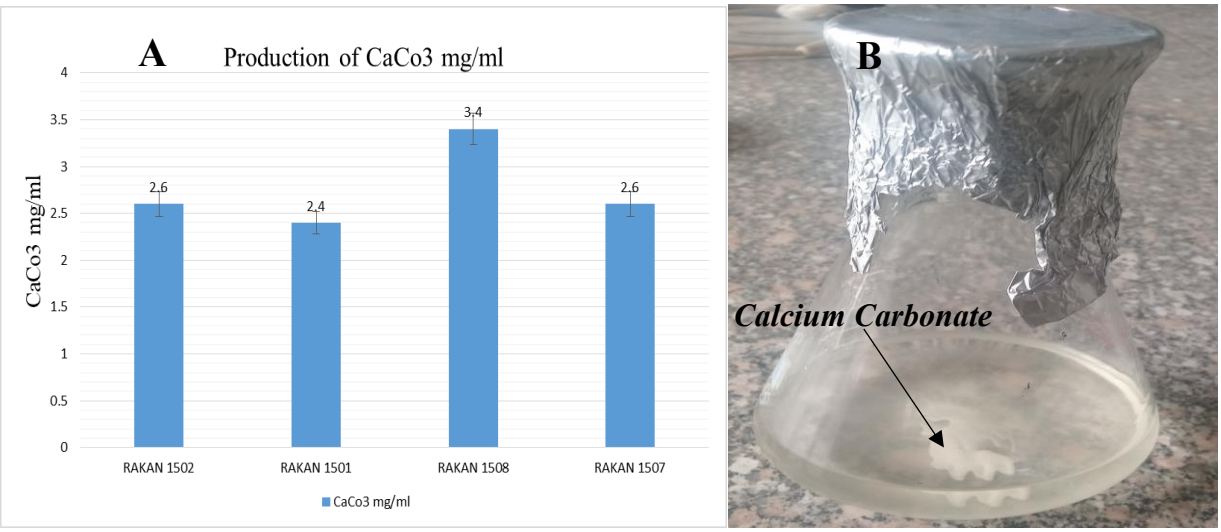


Fig. 6. A: Production of $CaCO_3$ by different isolated bacteria, B: Calcium in media product by bacteria reaction isolated.

3. 3 16 S rRNA gene sequence for most potent bacterial isolate

By comparing the obtained 16 S rRNA gene sequence with known bacterial sequences stored in Egypt's Gen-Bank, the sequence was examined. It was discovered that the isolated bacteria's gene sequence was strikingly similar to Bacillus haynesii's, as shown in Fig. 7. In the current study, a new species was presented and a concrete crack restoration technique was suggested. The aforementioned findings can be attributed to the frequent use of S. pasteurii [35], Bacillus sphaericus [43], Bacillus subtilis [44], Bacillus megaterium [45], and Spore-forming alkali-resistant bacteria [46] in previous studies. The isolated bacteria's (Bacillus haynesii) gene sequence was then added to the Egypt's Gen-Bank. Databases with accession number RAKAN1508.

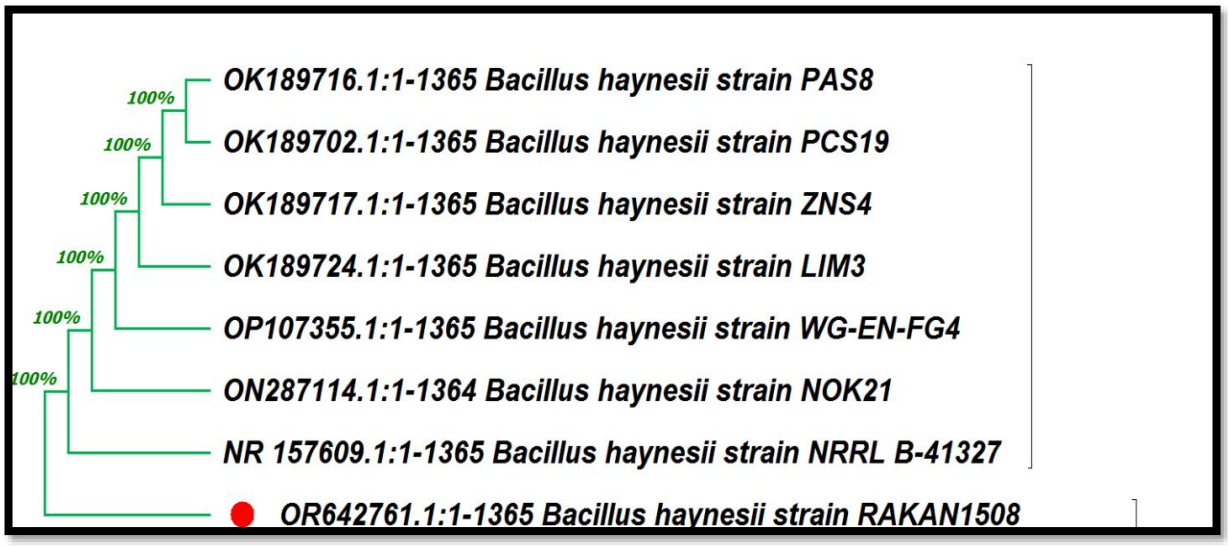


Fig. 7. Phylogenetic tree of the isolated bacteria.

3.4 Analyzing the impact of bacterial consortiums on concrete's properties

The idea of crack biosealing relies on the ability of the bacterial species to convert organic substances that are soluble into insoluble precipitates of $CaCO_3$ that fill the crevices. Thus, the bacteria (*Bacillus haynesii*) were carefully chosen, and they were added as soon as the concrete was mixed, in addition to lactate of calcium and yeast extract, It provides the bacteria with nourishment.

3.4.1 Slump of fresh concrete

To find out how nutrients and bacteria affected the workability of concrete, a slump test was performed. The target average slump for the control concrete (without the bacterial solution) was 40 mm. The data are represented in Fig. 8, Fig. 9 and fig. 10 The results of the overall comparison between the bio concrete sample, the nutrient concrete sample and the control showed that the slump of the bio concrete sample and the nutrient concrete was higher by 25% and 13%, respectively. (Note 1: There was an error in Trial 1 calculating the required admixture ratio and it exceeded the required limit, and this affected the results of the slump.so, the amount of admixture must be calculated carefully) This is because calcium ions and carbonate combine to form calcium carbonate and water, as in the following equations:

$$Cao + H_2O \rightarrow Ca(OH)_2 \quad (1)$$

$$Ca(OH)_2 + CO_2 \rightarrow CaCo_3 + H_2O \quad (2)$$

Fig. 8. Some pictures of three mixtures for concrete Slump of fresh concrete.

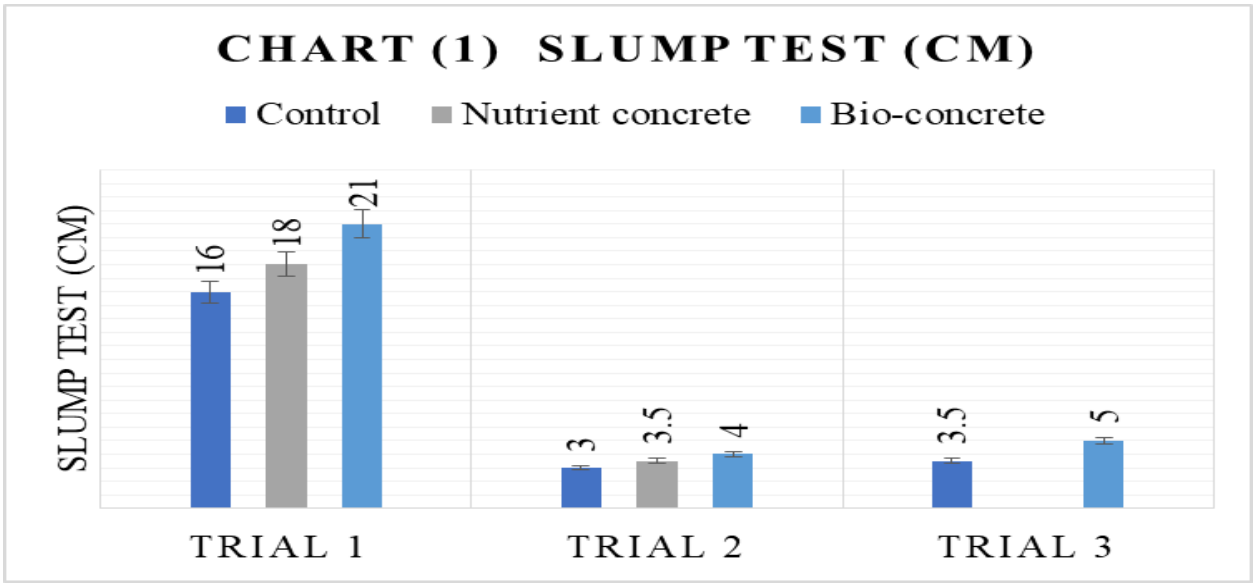


Fig. 9. Charts the slump test results for fresh concrete in three trial mix.

3.4.2 Concrete strength

The bacterial concrete's compressive strength was value after 7 and 28 days. The control concrete (without the bacterial solution) reached its target mean strength of 200 kg/cm^2 after 28 days. The data represented in Fig. 10 and Fig. 11. According to the results that were achieved, the three types of concrete's increases in compressive strength are time-dependent, meaning that they get stronger over time. After 7 and 28 days of curing, the bio-concrete specimen's compressive strength was generally much higher than the control. In the trial mix (1), there was an error in calculating the required admixture ratio and it exceeded the required limit, and this affected the results of the cubes, as the control concrete produced better results than both the bio concrete and the nutrient concrete, and the average results after 7 days were: 263, 228, and 145 (kg/cm^2), respectively. Age 28 days: 278, 241, 226 (kg/cm^2), respectively, and this is shown in Fig. 10. As for the second trial mix (2), after verifying and reviewing the correct mixing ratios, the results of the bio concrete were better than the nutrient concrete and better than the control concrete, and

the results were for 7 days.: 227, 223, 207 (kg/cm²), respectively, as well as at 28 days age: 281, 241, 229 (kg/cm²), respectively, and this is shown in Fig. 10. The trial mix (3) was conducted on the bio concrete and the control concrete to confirm the results and to be the last trial mix. In comparison to the control concrete, the bio concrete produced higher results. Which were at the age of 7 days 189 and 140 (kg/cm²), respectively, as well as at 28 days age, 260 and 240 (kg/cm²), respectively, and this is shown in Fig. 1because the concrete's pores and micro cracks would be filled with the microbial product, This was described as the microbiological calcium carbonate inside the concrete matrix precipitating.

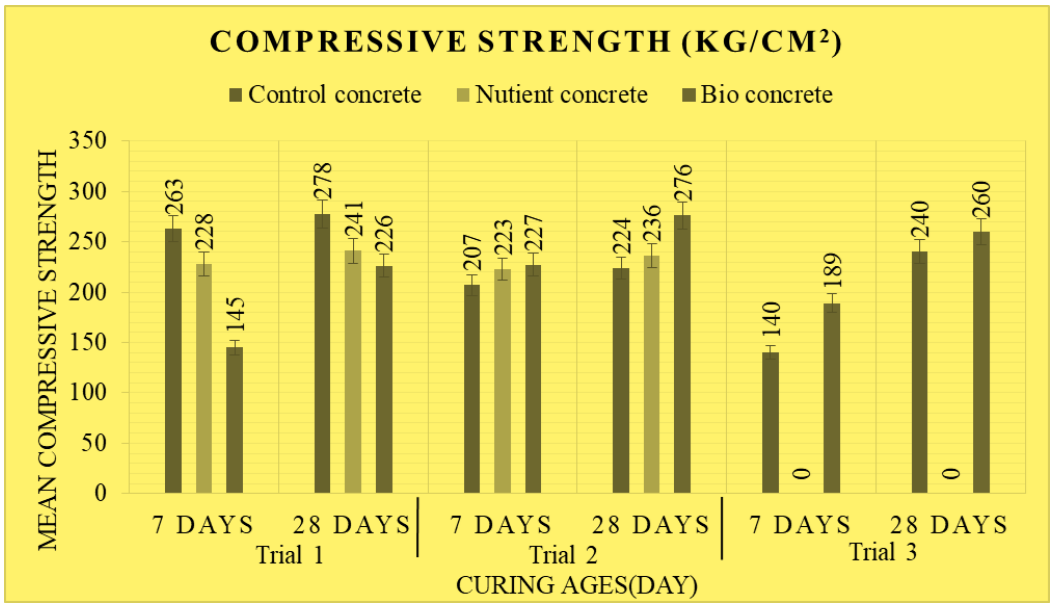


Fig. 10. Charts the compressive strength test results for hardened concrete in three trial mix.

Fig. 11. Some pictures of three mixtures for concrete Slump of the compressive strength test.

3.4.3 ***Tensile strength splitting***

At 28 days old, bio concrete, nutritious concrete, and control samples were found to differ significantly from one another when the concrete's split tensile strength was assessed. as shown in Fig. 12, In the trial mix (1)), there was an error occurred in calculating the required admixture ratio and increased than the required limit and its impact on the results of the concrete's split tensile strength, where the control concrete performed better than the nutrient concrete and the bio concrete, and the average results over a 28-day age were 25, 23, and 20 (kg/cm^2) respectively, and this is shown in Fig. 12. As for the trial mix (2), after verifying and reviewing the correct mixing ratios, the results of the bio concrete were better than the nutrient concrete and better than the control concrete. The findings at 28 days of age were, 45, 35, and 25 (kg/cm^2)respectively, and this is shown in Fig. 12. And the trial mix (3) was conducted on the bio concrete and the control concrete to confirm the results and to be the last mixture in the experiments. The results of the bio concrete were better than the results of the control concrete and were at an age of 28 days 47 and 26 (kg/cm^2) respectively, and this is shown in Fig. 12. It was discovered that there were notable variations in the concrete's split tensile strength in three three trial mix between the bio concrete, nutrient concrete, and control samples. Additionally, the bio tensile strength of the bio concrete and nutrient concrete was found to be higher. At a curing age of 28 days, concrete samples increased by 44% and 28%, respectively, in contrast to the control. This is brought on by bacteria that produce calcium carbonate in the concrete mold, increasing the concrete's capacity to withstand loads. Comparing the outcome to normal concrete, [47] observed a noteworthy difference. The split tensile strength increased by over 10% following a 28-day curing period. Conversely, [48] found that adding Bacillus sphaericus to concrete at a concentration of 107 cells/ml increased the material's split-tensile strength by about 29% compared to the control. This happened as a result of bacteria producing calcium carbonate in the concrete matrix, which increased the concrete's ability to withstand loads. The kinds and concentrations of bacteria used account for the variations between our results and the other. Overall, the bio-concrete's increased tensile strength is advantageous because it shows that there is a low chance of cracks forming, meaning the concrete will last for a long time.

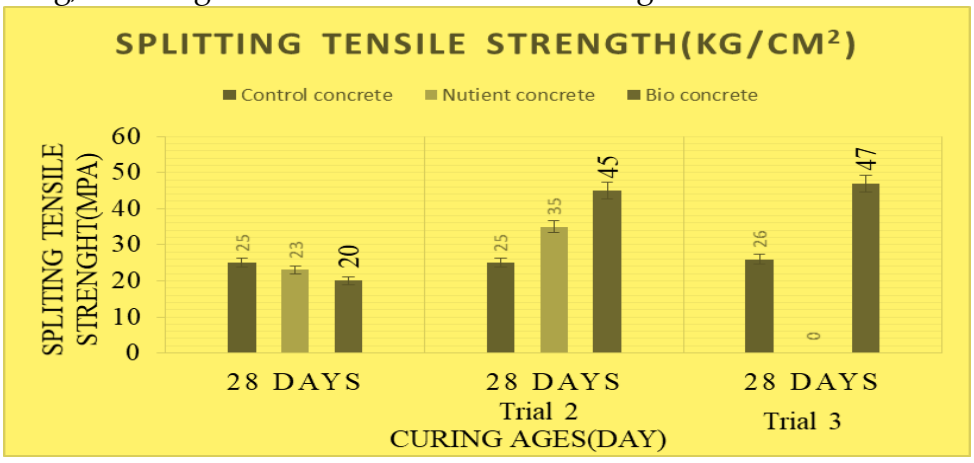


Fig. 12. Charts the Tensile strength splitting testing results for hardened concrete in three trial mix.

3.4.5 ***Absorption of water test (sorpativity test)***

The capacity of concrete to transfer and absorb water through capillary suction is measured using sorbativity. A decrease in sorpativity is a reliable sign of improved durability. According to other research, sorbativity is typically influenced by the concrete's pore structure and curing time. Sorpativity is a concrete durability property. For a material to be more durable, its sorpativity needs to be as low as possible.

It was discovered by examining the concrete's sorpativity test that there were notable variations between bio concrete, nutritious concrete, and the control samples at 28 days old, as shown in Fig. 13 , In the trial mix (1)), there was an error occurred in calculating the required admixture ratio and increased than the required limit and its impact on the results of the sorpativity test of concrete, where the average results over a 28-day age showed that the control concrete performed better than the nutrient concrete and the bio concrete 265 , 301 and 316 *(10^{-4} mm/sec1/2) respectively, and this is shown in Fig. 13. As for the trial mix (2), after verifying and reviewing the correct mixing ratios, the results of the bio concrete were better than the nutrient concrete and better than the control concrete. At 28 days of age, the results were: 260,223 and 200 *(10^{-4} mm/sec1/2) respectively, and this is shown in Fig. 14. And the trial mix (3) was conducted on the bio concrete and the control concrete to confirm the results and to be the last mixture in the experiments. The results of the bio concrete were higher than the results of the control concrete and where the average results over a 28-day age 260 and 195 *(10^{-4} mm/sec1/2) respectively, and this is shown in Fig. 13. By studying the sorpativity test of concrete in 3 trial mix, the sorpativity test results for bio concrete and nutrient concrete indicated an increase, and it was discovered that The control samples and the three types of concrete differed significantly from one another. Concrete samples increased by 23.1% and 14.2%, respectively, at a curing age of 28 days more than the control. In general, less permeable concrete has a lower capacity to absorb waterBecause calcium carbonate precipitates had accumulated on the surface, there was decreased water permeability. A decrease in water absorption slows down the consequences of climate change. Therefore, the surface of bio-concrete may have a healing layer of calcium carbonate that functions as a natural calcite skin [49]. As a response to one of the primary issues with concrete durability, the bacterial consortium's activity is reflected in the bio-concrete specimens' observed decrease in water permeability [50]. Several papers [51-55] concur with our findings.

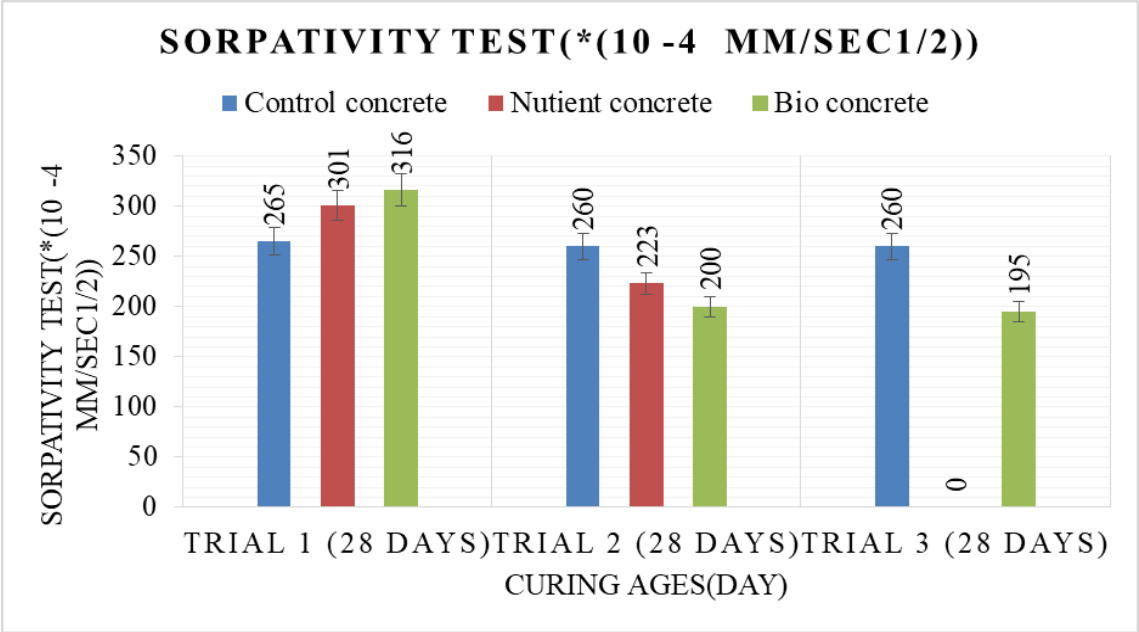


Fig. 13. Charts the sorpativity test results for hardened concrete in three trial mix.

3.7 Particularly, CT and X-rays

Regarding Fig. 15, the 0.4 mm fractured mortar specimen under control showed no healing at all, and the surface of the mortar only showed a very small amount of white deposition. These outcomes were ascribed to a chemical reaction between the carbon dioxide in the air and the $Ca(OH)_2$ that seeped out of the concrete matrix. On the other hand, as demonstrated in Fig. 8b, the artificially cracked Microbial precipitation was used to fully repair 0.4 mm-wide bacterial mortar. It was later discovered that the microbial precipitation contained calcite and vaterite ($CaCO_3$).

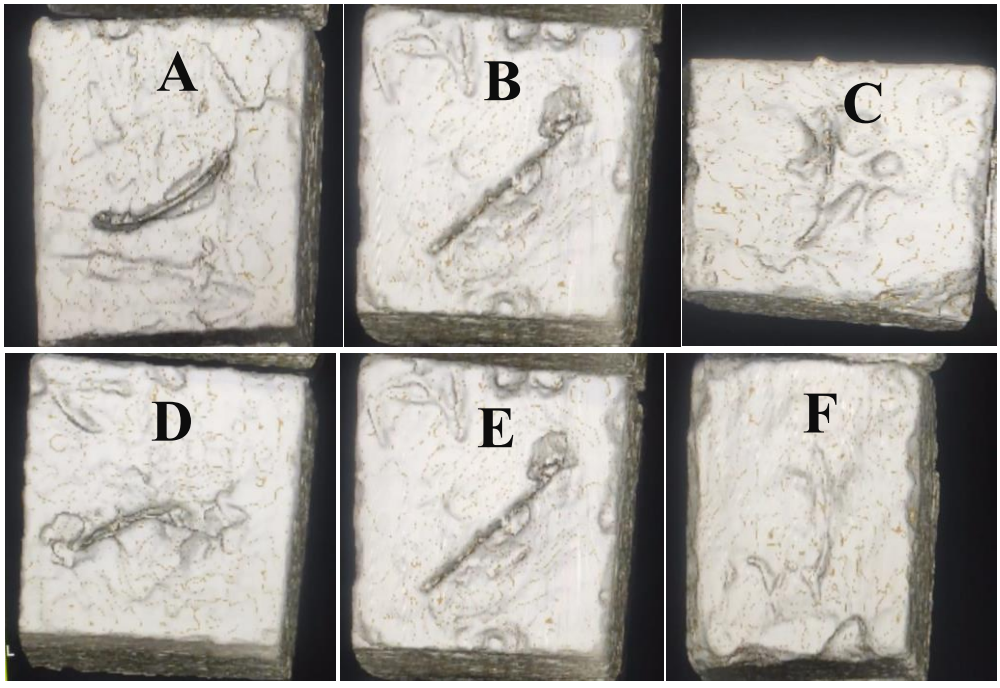


Fig. 14. X rays CT test for Concrete sample from 2 trial mix where: (A and D) Control concrete, (B and E) Nutient concrete and (C and F) Bio concrete.

3.8 Visual, VP-SEM-EDS

A sample of concrete cubes was taken and tested using SEM. In general bacterial consortium-inoculated bio-concrete specimens subjected to SEM analysis showed that the control specimen (control-concrete) was unable to display distinct crystalline structures. The bio-concrete sample in Figure 16. At 1300X has deposits that resemble rough sponges, which are not present from the control sample with the same level of magnification. The growth and an unequal distribution of precipitated calcium carbonate ($CaCO_3$) on the cracks surfaces, were visible in SEM images at 5000X. Small spherical crystals arranged in clusters and crystals with irregular shapes were the appearance of these precipitates. The bio-concrete sample's SEM scan at 10000Xshows a sealed fracture filled with a substance that looks like biopolymers, together with tiny calcium carbonate crystals that resemble needles or spheres. This material could be the product of bacterial cells creating a network by sticking to one another on a surface. Several shapes also emerged at 20000X, but cubic crystals predominate along with both rod- and irregularly-shaped crystals. The control specimen did not exhibit all of these notes at the same scales. The surface differences between the bio-concrete and control samples are consistent with the findings of earlier research. The surfaces of the untreated concrete seemed to be amorphous and lacking in any observable $CaCO_3$ crystals. On the other hand, samples of bio-concrete showed crystalline surfaces with distinct $CaCO_3$**.** [56, - 58].According to [59] of the bacterial isolates that can create biofilm, Bacillus flexus is the most effective. It was also reported by [60] that Bacillus haynesii produces extracellular biopolymer that is soluble in acid.

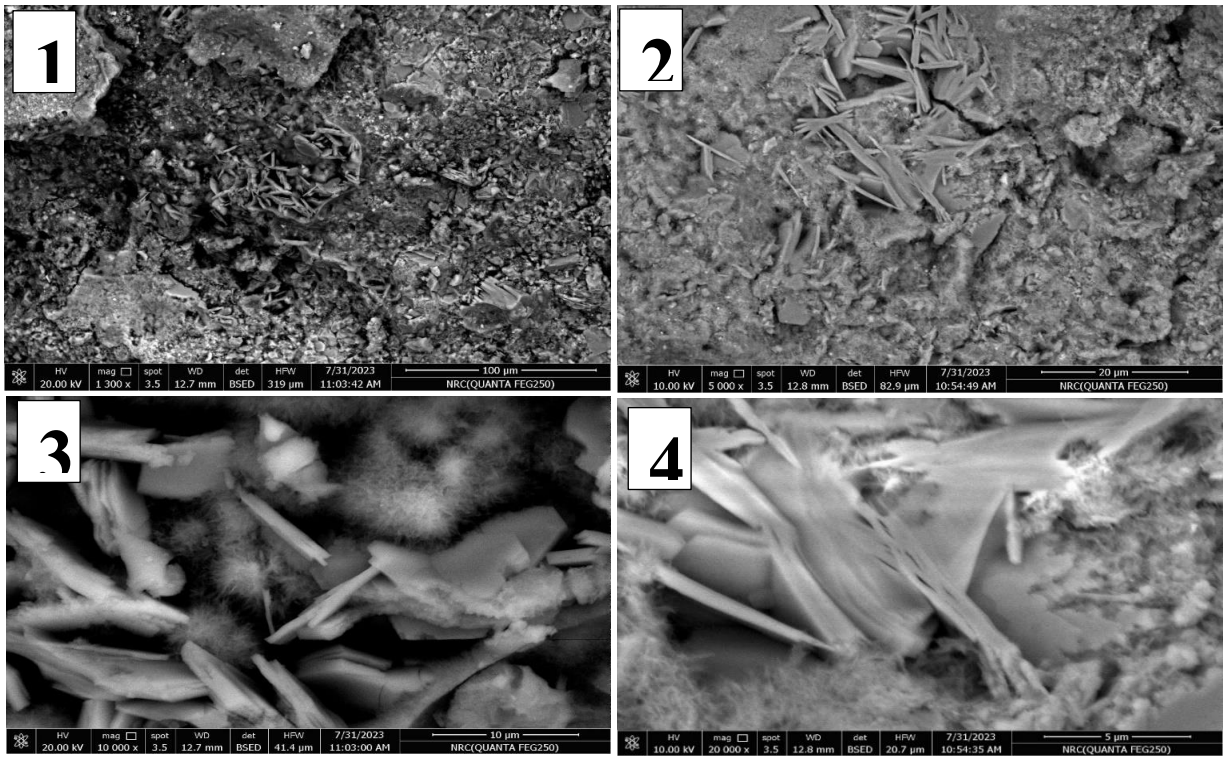
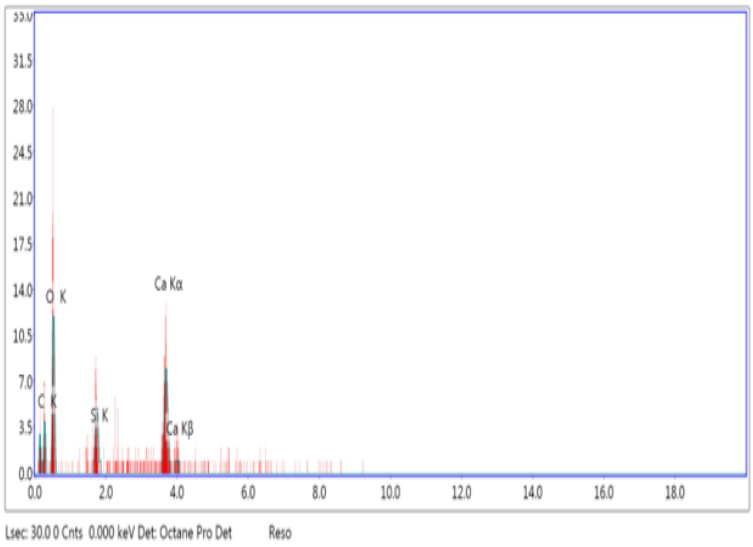


Fig. 15. Scanning electron micrographs of bio-concrete and control specimens (1 at 1300), (2at 5000), (3 at 10000) and (4 at 20000X).

Using SEM analysis, Fig. 17 shows the observed shape of the calcium carbonate precipitation on the fracture surface. It was observed that most of the precipitate belonged to vaterite and was spherical in shape. Their findings indicated a relationship between vaterite and the sphere-shaped bio-mineralization. Notably, the precipitated crystals formed larger aggregates, also referred to as bacterial aggregates, by bonding with one another. Furthermore, SEM-EDS (Fig.16) verified that the precipitate's primary constituents were carbon (C), oxygen (O), and calcium (Ca), showing that it is a carbonate of calcium ($CaCO_3$). Previous research on bacterial concrete has found calcite and vaterite as the two main crystal forms [61]. The results clearly indicated that Bacillus haynesii (RAKAN1508), a bacterium, can encourage the precipitation of calcium carbonate. According to other studies [62 - 65], these results are consistent with each other.



Element	Weight %	Atomic %	Net Int.	Error %
C K	20.86	29.02	1.87	25.13
O K	59.14	61.77	6.54	18.68
Si K	4.87	2.9	3.34	27.03
Ca K	15.13	6.31	6.66	17.98

Fig. 16. SEM-EDS analysis.

Conclusion

Out of four isolates, Only one was able to produce the urease enzyme and could grow at 50°C, with 40°C being the ideal temperature for growth. It also demonstrated growth potential at a pH of 12 with optimal growth at 10. Therefore, this isolate was identified using 16s as Bacillus haynesii RAKAN1508 and has been placed with accession number OR642761 in Gen-Bank. The progression of the healing ratio and the fracture profile were evaluated using both the SEM (variable-pressure scanning electron microscope) with X-ray computed microtomography (X-ray mCT). Additionally, Research was done on how bacteria affected specific concrete properties. According to VP-SEM data, a 0.4 mm crack mouth width was completely repaired by microbial precipitation, which XRD subsequently confirmed to be calcite and vaterite. Therefore, compared to control sample, 28 days after the curing, the bio-concrete specimens' compressive and tensile strengths increased noticeably by 36.3 and 44%. Based on the sorpativity test results, which showed that after 28 days of curing, the bio-concrete specimens had a significant 23.1% lower permeability than the control specimens.

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