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Isolation and Identification of Ureolytic Bacteria Isolated from Livestock Soil to Improve the Strength of Cement Mortar

Abstract- The aim of the present study is to isolate and recognize calcium carbonate-producing bacteria and to check these bacterial strains for use in cement mortar to improve its properties such as strength and water absorption. Bacteria were isolated from livestock soils were examined for urease producing activity, the possibility to precipitate calcite and its activity to improve the compressive strength. Based on the results, four isolates were selected and identified. They were characterized as *B. atrophaeus*, *B. subtilis*, *B. aryabhatai* and *B. amyloliquefaciens*. Experimental work was performed to evaluate the effect of bacterial concentration in term of optical density (OD) on the compressive strength. Bacterial cement mortar samples revealed improvement in compressive strength and water sorptivity. The efficiency of bacterial strains towards crack remediation was also investigated. Considerable increase in compressive strength and complete cracks remediation was detected in cement mortar samples cured with all bacteria isolate (using OD=1). This specifies the suitability of these bacteria for use in cement mortar. The precipitate of calcium carbonate inside the cracks of cement mortar by bacterial isolate was analyzed under a scanning electron microscope (SEM).

Keywords- Ureolytic bacteria, Cement mortar; Compressive strength, Crack remediation

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1. Introduction

Nowadays, the usage of Portland cement concrete is growing significantly for the construction and the requirements of more robust and durable structures are increasing [1]. Because of the cumulative requirements for environment-friendly concrete conservation, self-healing cement technology using bacteria has become a research point in the field of civil and environmental engineering in recent periods [2]. Currently, microbially induced mineral precipitation causing metabolic actions of specific urease-producing bacteria in concrete to increase the strength of concrete has become a significant area of research. Mineral precipitation processes are active in nearly all environments on the earth [2,3]. Naturally, calcium carbonate precipitation is complemented biologically. Urease-producing bacteria in the soils and water have been commonly stated to mediate the calcium carbonate (calcite) [4]. Microbially induced calcite precipitation (MICP) contains urea hydrolysis by urease enzyme which produced by specific bacteria to form finally carbonate ions. Carbonate ions then precipitate as

calcium carbonate in the existence of calcium ions [5,6]. MICP has been considered widely in several applications for example biomineralization, bioconsolidation [7,8], and improve the strength of cement mortar and bricks [9,10]. Lately, cement based biomaterial has been advanced to treat the cracks in the concrete structure [11]. Many previous researches have been revealed that the using of ureolytic bacteria, in the presence of urea and calcium, to cement-sand mortar or concrete precipitate calcite in the porous structure, which can be utilized as a binding materials to repair the cracks inside the structure and therefore, increase the compressive strength [12-16]. The biologically remediated cement consequently also showed better durability and crack repairing properties compared with ordinary concrete material [17]. For crack repairing, an assortment of conventional methods is suggested but these repairing systems have many of disadvantageous features such as changed thermal expansion coefficient associated with concrete matrix and environmental threats. Consequently, MICP has been suggested as a substitute and environmentally-friendly crack

repairing method [18]. Crack remediation microbiologically utilizes calcite has revealed a varied range of applications as a sealant [13]. Previous studies on aerobic microorganism showed that calcite precipitation induced by different urease producing bacteria such as *Bacillus pasteruii*, *Pseudomonas aeruginosa* and *Bacillus sphaericus* was suggested to be operative for healing the cracks in concrete and therefore improved its strength up to 18% of cement mortar [19,20] also found that types of bacterial strains and medium structure had a great effect on the calcite morphology where clean culture of bacterial strains resulted in a more noticeable actions. Recent researches have shown that only some specific bacterial species can be beneficial to improve the durability and strength of cement mortar constructions; those that can outrun in the alkaline environments [19]. *Bacillus* species are common Gram positive mesophilic, aerobic heterotrophs that produce heat-resistant endospores is a common soil bacterium which thrives in the alkaline soil environment and is expected to survive in concrete and induce the precipitation of strong and durable calcite in concrete since other bacteria has been used successfully for bacteria concrete [13,21]. Therefore, the present study aims to determine the impact of calcite activity of bacillus bacterial isolates on the strength and durability of cement mortar samples. Four *Bacillus* species induced calcite precipitation were selected in this study to improve the strength and durability properties of cement mortar and crack remediation.

2. Materials and Methods

I. Isolation and identification of bacteria

Urease-producing bacterial strains were isolated from livestock barn soil. Soil samples were collected from different sites in Al-Jadriya horsemanship club, Baghdad University, Baghdad, Iraq. 1 g of each soil samples were suspended in 100 ml sterile water and spread onto autoclaved nutrient broth (NB) plates. The NB media (HiMedia Labs Pvt. Ltd. India) contains the following constituents: 5 g/L Peptic digest of animal tissue, 5 g/L sodium chloride, 1.5 g/L beef extract and 1.5 g/L yeast extract. The plates were incubated at 30 °C for 1 day. The separate positive colonies were carefully chosen based on their visual crystal creation and purified by frequent streaking. Later, the isolates were tested for urease production. The bacterial isolates were plated on urea test agar. The urea test agar supplied from Sigma-Aldrich and consists of 20 g/L urea, 5 g/L sodium chloride, 2 g/L potassium phosphate, 1 g/L

peptone, 1 g/L dextrose, 0.012 g/L phenol red and 15 g/L agar. The plates were incubated at 30 °C for 2 days and the isolated colonies were nominated according to color change to pink. The positive isolates were purified by repetitive streaking and moved to a liquid NB media.

The bacterial isolates were examined for their capability to create endospores by Gram stain procedure based on the physical properties of their cell walls under a microscope (100×) the endospores can be shown. The bacterial isolates were checked periodically for pollution from other bacterial species by streaking in the plates containing nutrient agar. The microbial isolates were identified rapidly and accurately using VITEK-2 Compact device. Bacteria suspension was prepared in 2.5 ml volume of saline and modified to McFarland standard of 1.8–2.2 by VITEK-2 Denis-Chek (France). BCL cards were occupied routinely in the vacuum compartment, closed, protected at 35.5°C and read periodically every 15-min for 15 h. The data were automatically analyzed using the VITEK-2 database ver. (3.01) [22].

II. Biomass and urease activity analysis

For determination, the biomass concentration of the cultured isolates, the optical density (OD) method was used measured at a wavelength of 600 nm using UV-Spectrophotometer (GENESYS 10, Thermo Electron Scientific Instruments LLC, USA). To increase the OD of the isolates, bacterial isolates were cultivated under batch conditions in NB media, supplemented with 25.0 mM of calcium chloride and 20.0 g/l urea. Medium pH was set to 6.5 before sterilization by 2 N HCl. Urea and calcium chloride was supplemented post autoclaving by 0.22 filter sterilization to avoid decomposition under high temperature and pressure condition.

Urease activity was defined as the quantity of enzyme urease that catalyzed the hydrolysis of 1 mM/min of urea. In this work, urease activity was estimated based on the conductivity process. The method contains mixing 1 ml suspension of bacteria with 9 ml of urea with a concentration of 1.11 M [6]. The change in relative conductivity was recorded over five min at 22 °C using electric conductivity meter (WTW inoLab Cond 7110, Germany). Then, the urease activity was computed considering the dilution. The measurements of conductivity ($\text{mS cm}^{-1} \text{min}^{-1}$) correlated with a hydrolysis activity of 11.1 mM urea min^{-1} [23]. According to the high urease activity, four bacteria (coded as BU1, BU2, BU3 and BU4) were selected for use in cement mortar and crack remediation.

III. CaCO₃ precipitation experiments

The selected bacteria were examined for their ability to precipitate of calcium carbonate (CaCO₃). For measurement the precipitation of calcium carbonate in the broth, nutrient broth (NB) complemented with 20g/l of each urea and calcium chloride. This system was designed as (NB-U/Ca). 50 ml of NB-U/Ca was mixed with 2% inoculum then incubated under shaking at 30°C for 1 week using a shaking incubator. Three duplicates were examined. The precipitated calcium carbonate was filtered using filter paper, washed several times with distilled water, dried at 60°C in the oven for 2 h and weighed. Calcium carbonate precipitate weight (W_p) was calculated as follows:

$$W_p = W_{ep} - W_e \quad (1)$$

Where: (W_{ep}) is the weight of filtration paper plus calcium carbonate precipitant; and (W_e) is the weight of clean filtration paper.

IV. Cement and sand

Ordinary Portland Cement (OPC) provided by MAS cement factory was used in this work. Chemical compositions of cement are specified in Table (1). Locally available sand of particle size less than 4.75 mm with the specific gravity of 2.69 was used in this study.

V. Microbial cement mortar preparation

The cement mortar was prepared by using a cement/sand ratio of 1:3 by weight. Cement and sand were carefully mixed, adding along with grown bacteria, at water to cement ratio maintained at 0.45. Water to cement ratio corresponding to bacteria concentration OD₆₀₀ was 0.5, 1.0 and 1.5 for each bacterial strain. The fresh mortar pastes were cast into the cubic molds (dimensions of 50mm×50mm×50mm). After demolding the control specimen was cured with water and BU1, BU2, BU3 and BU4 specimens were cured with water containing 20 g urea/L and 20 mM CaCl₂. After curing, the cubes with water were tested for compressive test on the 28th days of curing.

Table 1: Physical and chemical compositions of cement

Physical properties	
Color	Gray
Gravity	3.15
Chemical constituents (%)	
CaO	62.29
SiO ₂	20.08
Al ₂ O ₃	5.74
Fe ₂ O ₃	3.13
MgO	2.32
Na ₂ O	0.18
SO ₃	2.06
L.O.I	1.91

VI. Water absorption analysis

To define the improvement in resistance to water penetration, water absorption and sorptivity tests were properties used to characterize the propensity of porous materials to absorb and diffuse water by capillarity [24]. Therefore, the water absorption can be determined as a degree of the durability of cement mortar. The water absorption can be tested by measuring the increase in the mass of a specimen resulting from water absorption with time [25]. The mortar samples were coated at all edges neighboring to the treated face, to check unidirectional absorption only by the treated face [26].

In this work, the mass method was adopted to determine the sorptivity of the mortar. The collective water absorption per unit area of the inflow surface (I) increases correspondingly with the square root of intervened time (t):

$$I = S t^{0.5} \quad (2)$$

Where: S is the sorptivity coefficient (mm.min^{-0.5}). The cumulative water absorption (I) of the inflow surface can be calculated as follows [24, 27]:

$$I = \Delta w / A x d \quad (3)$$

Where: Δw signifies the increase in weight of the cube after the time, Δw is calculated as the difference between the weight of dry cube and the cube after capillary sucking of water during a time period, A represents the surface area (mm²) of the cube through which water enters, and d represents the water density (g/cm³). The accumulated water absorption (I) can be plotted against the square root of time, then S was computed from the slope of the linear relation between I and the square root of time. The specimen's control and bacterial mortar (of OD =1) were cured for 28 days and then dried at 100°C in a ventilated oven for 24 h. After drying, the flow from the outlying surface of the cube was avoided by sealing it appropriately with nonabsorbent covering (paraffin). The cubes were submerged in water with a level of not more than 4 mm from the bottom of the cubes after the filler dehydrated. The amount of water absorbed in time intervals (5-60 min; 2-342 h) was determined after removing the specimens from the water and weighed. Surface water on the specimens was cleaned then immediately the specimens were submerged again.

VII. Compressive strength

Compressive strength is the most common test for cement mortar and concrete. This test was performed to four samples following the technique designated by ASTM [28]. Compressive strength analyses took place using five tones machine (TC-B002, Turkey).

The specimens were analyzed directly after moving from water and though they were still in the wet state. Each tested sample was placed in the compression testing device and exposed to load using loading rate of 100 kg/min till the resistance of the sample to the cumulative load breakdowns and then no additional load is continued. The compressive strength test was performed on three similar specimens and the average value was recorded. The higher load was fixed and the compressive strength of the sample was determined as follows.

$$\text{Compressive strength} = \text{total failure load} / \text{cube area} \quad (4)$$

VIII. Preparation of cracks in cement mortar

Cement mortar specimens dimensions of 50mm×50mm×50mm were cast based on the ASTM [28] specifications. The test samples were cast directly after mixing. A thin copper plate of 0.3 mm thickness was introduced in the fresh mortar paste up to 10 mm depth and 30 mm length. The plates of the mold were moved before the final condition of mortar such that the cracks were clear. The mortar samples were taken away from the molds after one day and then cured in water. The sand was inserted inside the cracks of mortar specimens after 28 days curing at 30 °C. Throughout the next 24 h, 5 ml of bacterial culture of OD=1 and 20 g/l of urea and calcium chloride were added into cracks at the 12 h intervals for each bacterial mortar specimens BU1, BU2, BU3 and BU4. Photographs were documented to imagine the cracks in both control and bacterial mortar samples. The cracks in both control and bacterial mortar samples were reviewed for the existence of calcite precipitate and for the healing of artificial cracks. The compressive strength test was carried out after 10 days of treatment for treated, untreated and control specimens. The analysis was performed on three samples and the average value was recorded.

IX. Scanning electron microscopy (SEM)

The deposition of calcium carbonate inside the artificial cracks of recently fractured mortar specimens by bacteria was analyzed using a scanning electron microscope, SEM (FEI Company Inspect S50, Holland) equipped with an energy dispersive X-ray spectroscopy analyzer EDX (Bruker Company/ Germany XFlash 6110) at the voltage accelerate the rate of 0.2 - 30 kV. Before analysis, all samples were dried at 110 °C for three days.

3. Results and discussion

I. Isolation and identification of ureolytic bacteria

Between the different bacteria isolated from the livestock soil samples, only 20 efficient isolates (about 50% of the all isolated bacterial colonies) offered high positive urease activity based on color change produced on urea agar. Only four unique bacterial isolates coded as BU1, BU2, BU3 and BU4 were chosen based on higher urease activity BU2 revealed highest urease activity (12 U/ml), followed by BU3 (10.2 U/ml), BU1 (9.4 U/ml), and BU4 (7 U/ml). The cell concentrations of all four isolates were expressed in term OD₆₀₀ and their collection was convergent (1.6-2.21). The biomass concentration (OD₆₀₀ nm) and urease activity of the final candidate isolates were shown in Figure 1.

Final candidate four bacterial isolates were recognized by Viteck 2 system. The results revealed that all cultured isolates were *Bacillus* species. Bacterial isolate BU1 was identified as *B. atrophaeus* with 94% probability, bacterial isolate BU2 was identified as *B. subtilis* with 96% probability, bacterial isolate BU3 was identified as *B. aryabhatai* with 94% probability and BU4 was identified as *B. amyloliquefaciens* with 95% probability

The endospores designated by white rods through red or pink bacterial cells were obviously noticeable in the results found from Gram staining experiment. Figure 2 shown the bacillus isolated species are observable as purple colored rods. This specifies that all isolated isolates are Gram-positive bacteria and capable of forming endospores.

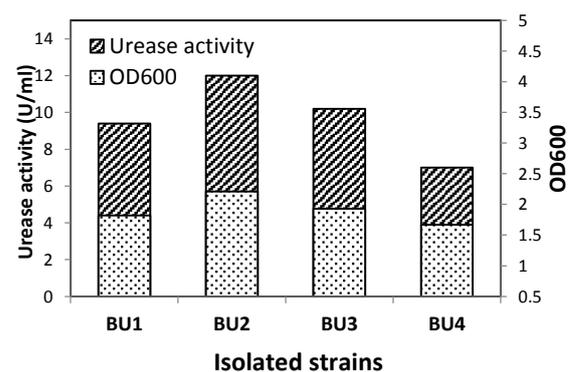


Figure 1: The biomass concentration and urease activity of different isolates cultured in the same conditions

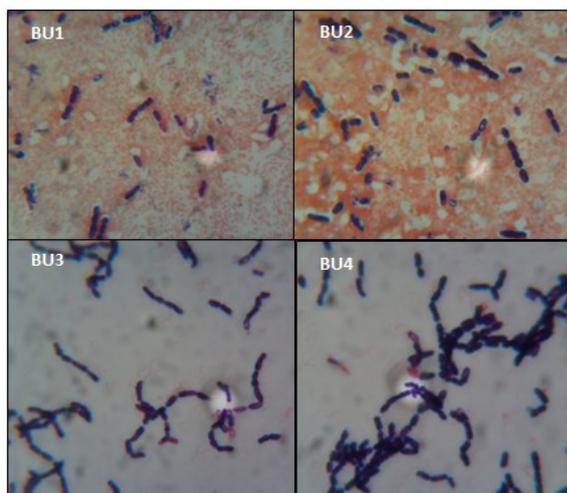


Figure 2: Microscopic images of the isolates BU1, BU2, BU3 and BU4 (40X).

II. Calcite precipitation

Straight after inoculation in NB-U/Ca, a white calcite precipitates seemed in the solution and its density increased during incubation. After 7 days of incubation, calcite precipitants were collected and weighed. All the four isolates precipitated calcite. The maximum amount of calcite (19.13g/l) was produced by BU2, followed by BU3 (18.9g/l). The bacterial isolates BU1 and BU4 precipitated 17.8 and 16.06 g/l of calcium carbonate, respectively, as shown in Figure 3.

III. Compressive strength

The compressive strength of cement mortar samples cured in water and that samples cured with different bacteria in NB-U/Ca growth solution are signified at different cell concentration (OD_{600}) in Figure 4. It can be seen clearly, that the strength values of bacterial cement mortar specimens are greater than those of control specimens. Furthermore, the tendency of compressive strength improving after 28 days may be assigned to the performance of bacteria within the cement mortar specimens.

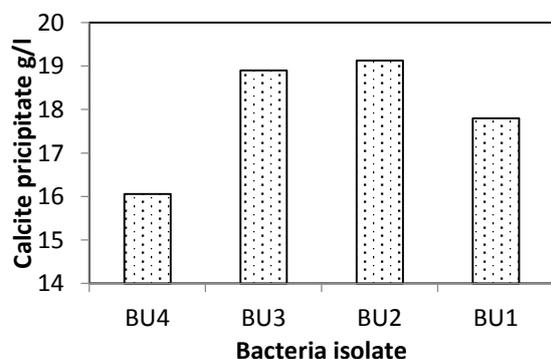


Figure 3: Calcite precipitation by different bacterial isolates (BU1, BU2, BU3 and BU4)

Throughout the growth of the bacterial cell, the calcite production increase on the surface besides through the cement mortar. When the pores in the cement mortar were blocked by this precipitate, the nutrients and oxygen may do not reach to the bacteria. Finally, the bacteria may convert into endospores and may work as organic fibers. This is supplementary with compressive strength improvement of the bacterial cement mortar samples.

This commensurable improve in compressive strength of cement mortar samples cured with different *Bacillus* strains isolated in this work was in agreement with several previous results reported in [15, 16]. Consequently, it was established that the improvement in the compressive strength is mostly because of consolidation in the cement mortar samples with a resulting filling of the pores within its matrix with microbially induced calcite precipitation.

Conversely, high compressive strength was obtained for cement mortar with high bacterial cell concentration up to $OD=1$. The compressive strength of cement mortar specimens cured with bacterial cell concentration of $OD=1$, which gives higher improvement percent in compressive strength than control samples on 28 days-curing and larger than that of samples cured with bacteria concentration of $OD=1.5$. This may be attributed to the high production rate of calcite when using cell concentration of $OD=1.5$. With increasing of the unstructured fractions of calcite, deposits through the pores of cement mortar, leading to a reduction the level of the compressive strength enhancement.

From the experimental results of compressive strength shown in Figure 4, it can be observed that the higher compressive strength value (34.75 MPa) was found for samples cured in the presence of *B. subtilis* (BU2). This is 45.3% higher than the control samples. Bacterial mortar samples cured in the presence of *B. atrophaeus*, *B. aryabhatai* and *B. amyloliquefaciens* have generated compressive strengths of 30.87, 26.97 and 27.54 MPa correspondingly which extent to 29.1, 12.8 and 15.2%, compared to control samples. In corporation of bacteria has improved the compressive strength of concrete. Mortar samples cured using media alone without inoculating bacterial strains did not display any important improvement in compressive strength compared with control samples.

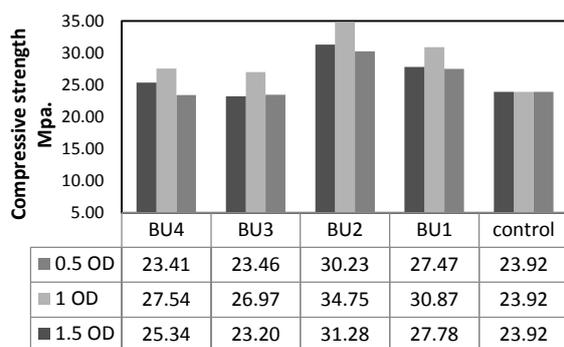


Figure 4: Variation of compressive strength using different cell concentration (OD600) at 28 days for a bacterial isolate

IV. Water absorption of bacterial cement mortar

Water absorption of mortar samples mixed with water and that cured with bacteria concentrations of OD=1 was investigated for all bacterial isolate samples (BU1, BU2, BU3, and BU4) cured under growth media for 28 days. The cumulative-water absorption against time square root for all bacterial samples was calculated and offered in Figure 5. Experimental results revealed that the water absorption of mortar samples in the presence of bacteria and control declines with time; this is because of the gathering of hydrated products, which block the open pores in the samples. Similarly, the values of water absorption of mortar samples cured in the presence of bacteria are lesser than those of control samples. This is assigned to that bacteria biomass and calcite precipitated on the surface and inside the pores of the mortar matrix. The total porosity of the mortar is related to water absorption linearly. It can be noted that sorptivity is minimum (0.155) for BU2 (Table 2) comparison with control and other isolates. So as to estimate the relationship between the sorptivity and the compressive strength, it can be noted that when the sorptivity decreases, the compressive strength increases in a linear manner, as shown in Table 2. The smaller sorptivity amount suggests that cement mortar is thicker. This is because the closing of the pores by calcite, which in turn increases strength. Comparable remarks were similarly stated somewhere else [2,17].

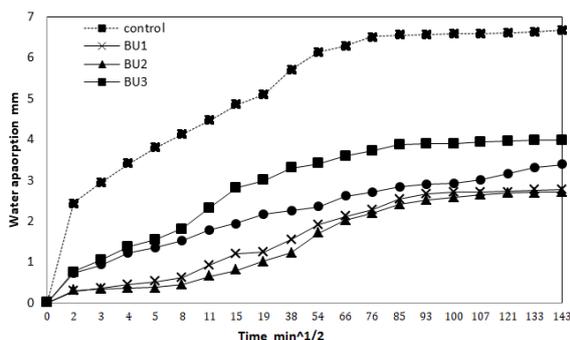


Figure 5: Cumulative water absorption of cement mortar

Table 2: Compressive strength and sorptivity coefficient.

Bacterial Strain	Compressive strength after 28 day (MPa)	Sorptivity (mm.min ^{-0.5})
control	23.92	0.281
BU1	30.87	0.167
BU2	34.75	0.155
BU3	26.97	0.202
BU4	27.54	0.176

V. Crack remediation

Compressive strength of control and remediated crack mortar samples was investigated, by curing with bacteria (conditions include OD₆₀₀=1 and Urea/Ca=1), and control mortar sample is shown in Figure 6. It was stated that untreated crack mortar has compressive strength lesser than that of control samples.

It can be observed that the higher increase in compressive strength (36.73MPa) was found in mortar samples cured with BU3. This is 59.6% more than untreated specimens. Bacterial mortar specimens cured with BU1, BU4 and BU2 have generated compressive strengths of 36.68, 33.14 and 30.38 MPa respectively, which amount to 59.3,44 and 32%, compared to untreated cement mortar specimens. This is mostly because of chemical bonding among calcite produced by bacteria and sand particles which filled the crack and therefore consolidated it. Though, the calcite precipitation occurred mostly close to the surface of the crack where dense growth of calcite crystals embedded with cells was observed.

Afterward 6 weeks, calcite precipitates were noted within all the cracks of bacterial cement mortar samples. At the finish of the investigation, whole cracks healing was reached in bacterial mortar samples cured with BU3, BU1, BU4 and BU2 (Figure 7 a, b, c and d). In contrast, the crack in the mortar samples cured with *B. subtilis* (BU2) was moderately closed by bacteria, as noted in Figure (4d).

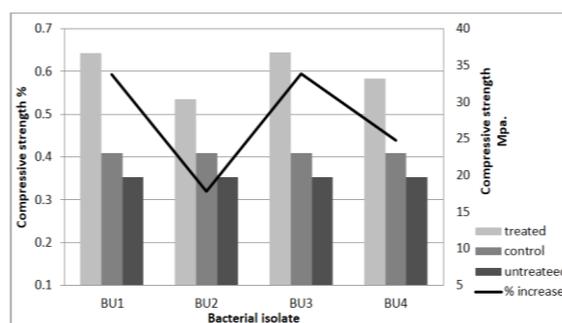


Figure 6: Compressive strength of control, untreated and treated crack treatment of cement mortar.

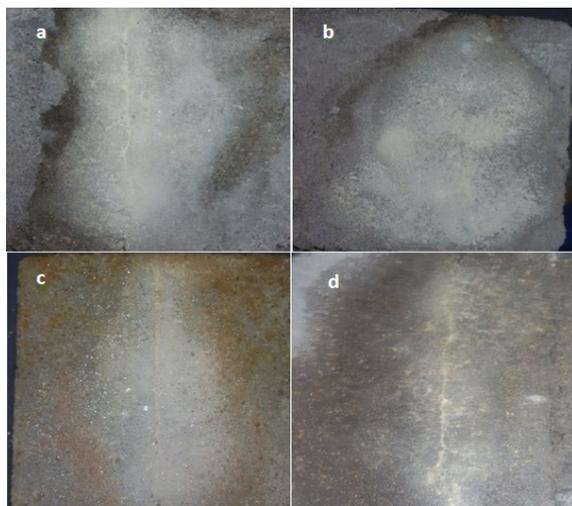


Figure 7: Photos of crack healing, (a) cement mortar sample with *B. aryabhatai* (b) in cement mortar specimen with *B. atrophaeus*, (c) in cement mortar specimen with *B. amyloliquefaciens* and (d) in cement mortar specimen with *B. subtilis*, after the end of healing.

VI. Scanning electron microscopy (SEM)

SEM graph of cement mortar samples cured with a bacterial concentration of OD=0.5, 1 and 1.5 are revealed in Figure 8. From SEM annotations, calcite produced by bacteria could be obviously illustrious inside the pores of mortar. Moreover, the precipitated calcite content increases with bacteria concentration (OD). Clearly, SEM graph showed various morphologies of calcite crystals in cement mortar samples prepared at different bacterial concentrations of strain BU2; spherical calcite produced by OD=0.5 which have a smaller size than OD=1 which retain rode shape and spherical crystals. Furthermore, the amorphous calcite part and slight quantity of spherical calcite are detected in cement mortar cured with OD=1.5.

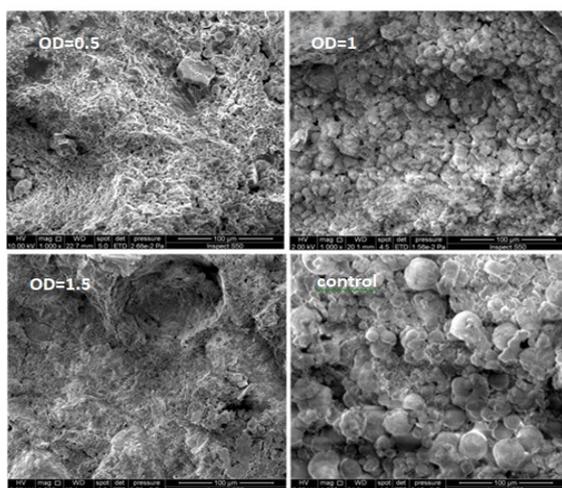


Figure 8: SEM photos of cement mortar cured with different bacterial concentration (OD=0.5, OD=1 and OD=1.5) of bacteria BU2 after 28-days representing the existence of calcite crystals.

Energy dispersive X-ray (EDX) analysis for bacterial strain BU2 at OD=1 showed that new phase produced by isolates, in cement mortar, which was calcium carbonate phase, as shown in Figure 9.

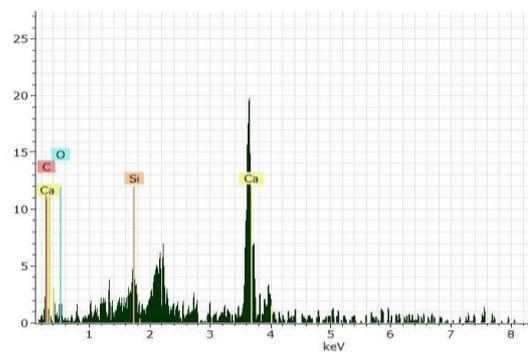


Figure 9: EDX test of precipitated calcite in cement mortar by bacteria.

4. Conclusions

Based on the results of this work, it can be concluded that all the four locally isolated bacterial strains showed great urease-activity. These isolates created endospores and produced calcite. The isolated bacteria were recognized as *B. subtilis*, *B. atrophaeus*, *B. aryabhatai* and *B. amyloliquefaciens*. The urease-activity and calcite formation by *B. subtilis* is close to *B. aryabhatai* and more than other two *Bacillus* species *B. aryabhatai* and *B. amyloliquefaciens*. The compressive strength and sorptivity of the bacterial strains were enhanced for cement mortar because of the deposition of calcite by the bacteria. The compressive strength improved at a bacterial concentration of OD=1 while it decreases at OD=1.5. Although the higher compressive strength values were gained for cement mortar cured with *B. subtilis*, the improvement in compressive strength of cement mortar cured with *B. atrophaeus* is similarly good. A full crack healing was detected in cement mortar samples cured with all bacterial strains. The existence of distinguished calcite crystals was noted in the SEM investigation of cement mortar samples. It can be established that *B. subtilis* (BU2) is appropriate for usage in cement mortar as they have caused in improved strength, while *B. atrophaeus* and *B. aryabhatai* are excellent for the healing of cracks in mortar samples.

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