BIORESTORATION

Melda Guler, Gulseren Coskun, İzmir Science High School, Turkey, meldaguler04@gmail.com

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ABSTRACT

The cement production causes global warming, environmental pollution and climate changes. Also this production is too costly. Especially the cracks of buildings cause collapsing of them. The demolition pollutes the environment and restructing durations are costly.This project aims to develop bond to reduce environment pollution about cement. The research showed that "the biomineralization" method can be effective. Cement includes %60-70 of calcium carbonate. That is if CaCO3 is produced with microorganisms, it can be eco-friendly bond.

Keywords : *Fine-grained soil, Mechanical properties, Biomineralization, B. megaterium (ATCC 14581), Calcium Carbonate (CaCO3)*

1. Introduction

There is a very rapid population growth in the world. As of 31 December 2021, the population residing in Türkiye increased by 1 065 911 people compared to the previous year and reached 84 680 273 people (TUIK, 2022).

Rapid population growth has led to the need to build housing and business areas. This situation has led to an increase in investments in the construction sector and the use of more cement. Increasing cement production is both costly and brings along various environmental problems (Gürtuğ, Sesalı, Yıldırım, 2016). In addition, due to factors such as earthquake and building age, cracks may occur in the structures. As the buildings become uninhabitable due to cracks, the building must be completely demolished. This causes both environmental pollution and high costs.

1.1. Cement Production

Cement whose raw materials are limestone and clay; sand, gravel, etc. used to bind materials. Calcium hydrates (C_3S, C_2S, C_3A) and calcium ferrite (C_4A) form the composition of cement. When cement interacts with water, hydrated calcium silicate (C_2SHx, C_3S_2Hx), hydrated calcium aluminate (C_3AHx, C_4AHx) and slaked lime $Ca(OH)_2$ are formed, which is the primary cementitious product. Hydrated calcium silicate and hydrated calcium aluminate are formed by the reactions between $Ca(OH)_2$ and minerals in clay ($Al_2Si_2O_5(OH)_4$).

C_3S: $2(3CaO.SiO_2) + n\ H_2O \rightarrow 3CaO.2SiO_2\ (n\text{-}3)\ H_2O + 3Ca(OH)_2$

C_2S: $2(2CaO.SiO_2) + n\ H2O \rightarrow 3CaO.2SiO_2\ (n\text{-}1)\ H_2O + Ca(OH)_2$

1.2. Problems Due to Cement

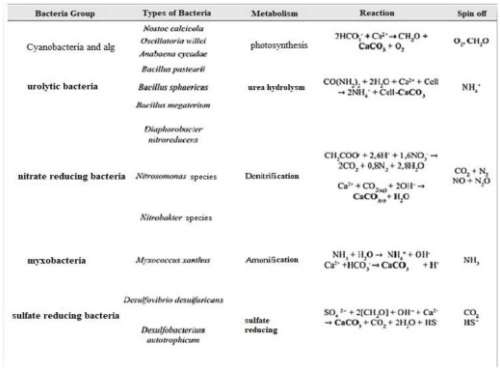
During the production of a bag of cement, approximately 25 kg of coal is burned. As a result of the burning of coal, the increase in CO_2 in the air causes global warming; NOx, SOx gases cause acid rain, which in turn causes climate change and environmental pollution. (Udara et al., 2019). The fact that 5% of the worldwide CO_2 production is caused by cement production shows the worldwide impact of the problem (Kara, 2020). Acid rain affects the chemical and biological structure of the soil. It causes significant damage on historical artifacts and buildings. (https://www.mgm.gov.tr/FILES/genel/brosurler/asit-yagmurlari.pdf Access Date: 18.12.2022)

High temperature, dust, toxic and allergic chemicals and heavy metals (Cd, Cr, Cu, Hg, Pb, Se, Zn etc.) released during cement production also mix with soil, streams and underground waters and adversely affect minerals and living things living there. Cement contains high amounts of materials such as Mn, Mg, TiO2 (Marlowe and Mansfield, 2002; EIA Guide, 2009). Cracks, especially in old buildings, can cause buildings to collapse. Demolition pollutes the environment and rebuilding processes are costly.

1.3. Biomineralization and Microbial $CaCO_3$ Formation Mechanisms

The product that is frequently used in cement-based products is calcium carbonate. $CaCO_3$ is a fairly solid compound. 65-70% of the cement component is CaCO3, while the remaining part is composed of clay components (Yıldırım, 2019). Biomineralization is defined as the mineralization created by living things (Knoll, 2003). Various biopolymers are formed as a result of biomineralization. Calcium carbonate precipitation takes place by various microorganisms in soil, water and marine environments (Cura et al., 2001). This event occurs in various ways depending on the type of microorganism: cyanobacterial photosynthesis, sulphate reduction, denitrification, ammonification, urea hydrolysis (Gürtuğ et al.,2016) (Table 1).

Table.1: Microbial $CaCO_3$ formation mechanisms due to bacterial groups (Gürtuğ et al., 2016)



Bacteria Group	Types of Bacteria	Metabolism	Reaction	Spin off
Cyanobacteria and alg	*Nostoc calcicola* *Oscillatoria willei* *Anabaena cycadae*	photosynthesis	$2HCO_3^- + Ca^{2+} \rightarrow CH_2O + CaCO_3 + O_2$	O_2, CH_2O
urolytic bacteria	*Bacillus pasteurii* *Bacillus sphaericus* *Bacillus megaterium*	urea hydrolysm	$CO(NH_2)_2 + 2H_2O + Ca^{2+} + Cell \rightarrow 2NH_4^+ + Cell\text{-}CaCO_3$	NH_4^+
nitrate reducing bacteria	*Diaphorobacter nitroreducens* *Nitrosomonas* species *Nitrobakter* species	Denitrification	$CH_3COO^- + 2,6H^+ + 1,6NO_3^- \rightarrow 2CO_2 + 0,8N_2 + 2,8H_2O$ $Ca^{2+} + CO_{3(aq)}^{2-} + 2OH^- \rightarrow CaCO_{3(s)} + H_2O$	$CO_2 + N_2$ $NO + N_2O$
myxobacteria	*Myxococcus xanthus*	Amonifikation	$NH_2 + H_2O \rightarrow NH_4^+ + OH^-$ $Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$	NH_3
sulfate reducing bacteria	*Desulfovibrio desulfuricans* *Desulfobacterium autotrophicum*	sulfate reducing	$SO_4^{2-} + 2[CH_2O] + OH^- + Ca^{2+} \rightarrow CaCO_3 + CO_2 + 2H_2O + HS^-$	CO_2 HS^-

1.3. Urolytic Bacteria and Bacillus megaterium (ATCC 14581)

Microorganisms with urease enzyme attract calcium ions in the environment electrostatically, thanks to their negatively charged membranes, and cause the formation of calcium carbonate ($CaCO_3$) precipitation (Gürtuğ et al., 2016). The resulting $CaCO_3$ precipitation can provide self-healing by filling cracks in cement-based materials (De Muynck et al., 2010).

The mechanism of urea hydrolysis and $CaCO_3$ formation is given in Table (2), and the calcium carbonation process in the bacterial wall is given in Figure (1).

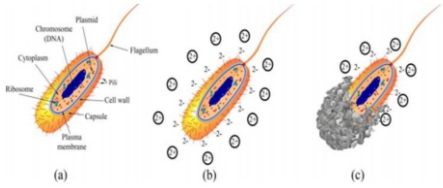


Fig1: Calcium carbonation process in the bacterial wall. (a) bacterial structure, (b) negatively charged bacteria attract positive structures, (c) biomineralization (Seifan et al. 2016).

Table 2: Urea hydrolysis and $CaCO_3$ formation (Gürtuğ et al., 2016)

$$CO(NH_2)_2 + H_2O \longrightarrow NH_2COOH + NH_3$$
$$NH_2COOH + H_2O \longleftrightarrow NH_3 + H_2CO_3$$
$$H_2CO_3 \longleftrightarrow HCO_3^- + H^+$$
$$2NH_3 + 2H_2O \longleftrightarrow 2NH_4^+ + 2OH^-$$
$$Ca^{2+} + Cell \longrightarrow Cell - Ca^{2+}$$
$$Cell - Ca^{2+} + CO_3^{2-} \longrightarrow Cell - CaCO_3$$

Tezer and Başaran Bundur (2021) conducted experiments using Sporosarcina pasteurii bacteria in their study. They used diatom and pumice in their work. However, silt and clay were used in this project. In addition, in this study, the effect of CaCO3 formed by B. Megaterium (ATCC 14581) bacteria on the binding of the building material was investigated. The effect of binding was seen with SEM images.

2. Material and Method

2.1. Materials and Devices Used

The materials used in the experiments were of analytical purity and were obtained from Sigma Aldrich, Fluka companies. The devices used are of high sensitivity (Table 3).

2.2. Preparation of Medium and Growth of Bacteria

B. megaterium (ATCC 14581) was used in this study. B. megaterium (ATCC 14581) obtained from Ege University Science Faculty Biochemistry Department Biotechnology Laboratory culture collection was first line inoculated on Nutrient agar and then inoculated into Nutrient broth, which is a liquid medium.

Nutrient agar was prepared in distilled water as 20.0 g/L, then sterilized in autoclave at 121 °C 1.5 ATM for 15 minutes, cooled to 45-50°C and poured into sterile petri dishes 12.5 mL each. The nutrient agar divided into Petri dishes was expected to freeze. On the other hand; 3 g Nutrient Broth, 5 g urea, 0.7 g $CaCl_2$ (calcium chloride) were added to 250 mL pure water and dissolved and a liquid medium was formed.

Bacteria inoculation was carried out after the medium was brought to room temperature. In order to prevent microbial contamination during sowing, the sowing surfaces were sterilized with 70% ethanol. The loop, which was turned into incandescent in a spirit stove, was used for the inoculation of B. megaterium (ATCC 14581) cells in nutriene tagar after cooling (Fig. 2).

Table 3: Materials, Devices and Media Components Used

MATERIAL	COMPANY
Nutrient Broth	Fluka
Urea	Sigma Aldrich
Calcium chloride	Bio Basic Inc.
Yeast Extract	Oxoid
Peptone	Fluka
Beef Extract	Fluka
Bacterium (B. megaterium (ATCC 14581)	Ege University Faculty of Science Department of Biochemistry Biotechnology Laboratory Culture Collection
Low plasticity silt (Maximum diameter) 63x10-6 m)	Aydınlar madencilik
Clay (kaolin)	UTELKA Ltd. Şti.
DEVICE	**COMPANY**
Precision Scale	Daihan Biomedical
Autoclave Device	Hmc
Shaking Incubator	New Brunswick Scientific
Spectrometer	Perkin Elmer
pH Meter	Hanna Instruments
Free Pressure Device	Multiplex 50
Weighing	Adventurer Ohaus
Magnetic Stirrer	VELP Scientifica
Coating Device	Leica EM ACE600
SEM (Scanning Electron Microscope)	Thermo Scientific Apreo S

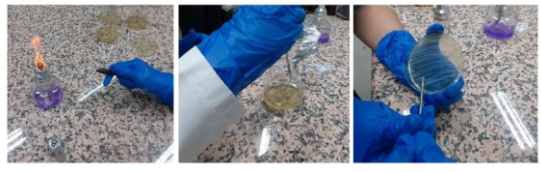


Fig2: Calcium carbonation process in the bacterial wall.

B. megaterium (ATCC 14581) was inoculated for 120 hours at 37 °C with a stirring speed of 120 rpm. Bacterial growth was monitored spectrophotometrically every 24 hours, and an absorbency value of 1.5 was observed at OD600, which determined the transition to log phase. The sample, whose transition to the log phase was detected and $CaCO_3$ formation did not occur yet, was kept in the incubator for 120 hours and studied with two different cultures with $CaCO_3$ formation.

Bacteria were divided into 2 groups in order to examine the effectiveness of the growth stages of the bacteria on the building materials. Bacteria that have just entered the log phase have just started the $CaCO_3$ generation process. B. megaterium (ATCC 14581) bacteria entered the log phase after approximately 24 hours and therefore the first group of bacteria was incubated in the incubator for 24 hours. The other group is the bacteria in the group where the log phase has ended, that is, the $CaCO_3$ formation has been completed. Since the bacteria formed $CaCO_3$ after an average of 4 days, the bacteria in the second group were also incubated for 4 days. In order to examine the effect of the substances formed as a result of the metabolic activities of B. megaterium (ATCC 14581) on the pH value of the medium, which is 7.4, measurement was made with a pH

meter. No significant difference was observed in the pH measurement.

2.3 Use of Bacteria in Soil Stabilization-Free Pressure Tests

It was thought that the bacteria could easily create $CaCO_3$, and at the same time, it would be appropriate to use materials with large macro-cavities. For this purpose, the material classified as SP (poorly graded medium sand-D50=0.4 mm) according to the Unified Soil Classification System was chosen because it could not hold itself and its unconfined compressive strength was zero. For the preparation of samples with a relative density of 50%, 400 g of sand (0.5-2.0 mm in size) and 50 ml of B. megaterium (ATCC 14581) culture (Log phase start and CaCO3 formation complieted) were used and the first samples were prepared. The prepared molds were cured 7 day. At the end of the period, they were dispersed during the demoulding process with the Sample Extraction System. Because $CaCO_3$ could not sufficiently bind the sand used and frictions affected the sample during demoulding. For this reason, it was decided to change the material used.

In the second experiment, samples were prepared by adding 50 ml of B. megaterium (ATCC 14581) culture to a mixture of 70% silt and 30% clay. The clay-sand mixture prepared with log phase bacterial cultures could not be removed from the mold and dispersed. The sample, which was prepared and cured with the cultures whose $CaCO_3$ formation was completed, could be removed from the mold and the unconfined compressive strength was measured. However, the compressive strength was as low as 65 kPa. It was decided to perform an optimization process for the log phase and for the $CaCO_3$ formation phases.

2.4 Optimization Process for Bacteria to Generate $CaCO_3$

The effect of B. megaterium (ATCC 14581) on CaCO3 formation was investigated by changing the content of the medium with optimum reference values for the growth of bacteria. $CaCl_2$ and urea were added to support the formation of $CaCO_3$.

The amount of only one substance at a time was changed in the media to observe the optimization. No changes were made in the procedures applied in bacterial sowing (Table 4).

Table 4: Substance Components and Amounts to be Used in Optimization (For 1 L medium)

	Substance	Reference Quantities(g)	Quantities Used in Optimization (g)			
Nutrient Broth Content	Peptone	5	1	2.5	10	15
	Yeast Extract	2	0.25	1	4	6
	Beef Extract	1	0.1	0.5	2.5	5
	NaCl	5	1	2.5	7.5	10
Additions	CaCl2	3	0.1	1	6	9
	Urea	20	5	10	40	60

For the bacterial cultures obtained, samples were formed with 0.63 mm, 350 g sand and 50 mL medium. It was compressed into 196 cm^3 molds at Standard Proctor Energy (592.7 kJ/m3). In this context, it was compressed in 3 layers with 25 blows to each layer with the hammer released from a height of 30.5 cm. The mold was separated from the lower part and the upper and lower surfaces were smoothed with the help of a knife. The mold was removed with the help of a sample extractor. The prepared molds were kept at room temperature for 14 days (Fig. 3).

Fig.3: Preparation of Molds and Demoulding Process

The unconfined compressive strengths of the samples were determined according to the ASTM D2166/D2166M standard. The experiments were carried out at a loading speed of 1.27 mm/min. The dimensions of the unconfined pressure test instrument used are 500 x 500 x 1470 mm. The load cell of the free pressure device has a capacity of 5 kN. In the simplest terms, the unconfined compressive strength is the ratio of the applied load at the time of failure to the cross-sectional area at the time of failure. For example, assuming that the volume does not change (Eq. 1).

$$q_u = \frac{P}{A_0}(1-\varepsilon) \quad (1)$$

In this formula, q_u, A_0 and Ɛ show the unconfined compressive strength, cross-sectional area and unit deformation before the test, respectively. The secant modulus (E50), which reflects its resistance to elastic and plastic deformation, is an important parameter that contains information about deformation behaviour (Wang et al., 2013). The stress-strain ratio, which corresponds to 50% of the unconfined compressive strength in the stress-strain graph obtained from the unconfined compression test, is defined as the secant modulus (Lorenzo and Bergado, 2006).

Two controlled trials were conducted in this project. In the first experiment, the optimum amount of medium required for the survival and reproduction of the bacteria was taken as reference. However, the desired result could not be obtained in the prepared samples. For this reason, optimization has been made. The amount of substances contained in the medium has been changed. Thus, CaCO3 production of B.megaterium (ATCC 14581) bacteria and its effect on the building material were observed. In the second experiment, the amount of substance was kept constant and the effect of the bacterial phase on the strength was investigated. The sample was created with the media in which the bacterium had just entered the log phase and the media where $CaCO_3$ was formed. The strengths and stiffness (secant modulus) were examined with a 14-day curing period. The measurement results were graphed and the samples with the highest strengths were determined (Table 5). Bacterial mineralization products in these samples were examined using scanning electron microscopy (SEM). Mineral formations were determined by SEM images and it was investigated whether bacterial cells combined with carbonate crystals.

Table 5: Variables

TABLE OF VARIABLES		
Independent variable	**Dependent variable**	**Control Group**
The amount of any substance in the medium	Strength of the obtained sample	Sample waiting temperature, ambient Other component amounts of the medium
Bacterial phase added to the sample (log phase and CaCO3 formation states)	Strength of the obtained sample	Sample waiting temperature, ambient Other component amounts of the medium

3. Results and Discussion

The unconfined compressive strength and secant modulus results are given in the graphs below, which are drawn according to the crimping results. In each graph, samples with the medium removed from the incubator (a) at the beginning of the log phase of the bacterium and (b) removed from the incubator when CaCO3 formation of the bacterium is completed are included. Graphs are named according to the amount of items changed for optimization (Fig. 4- 8).

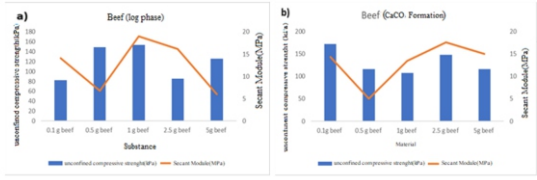


Fig.4: Graphs of unconfined compressive strengths and secant modulus according to different beef extract amounts. a) Log phase b) $CaCO_3$ formation

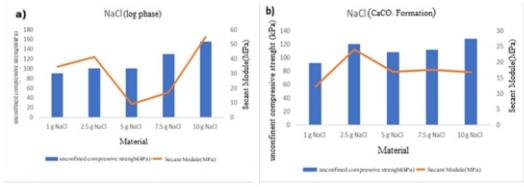


Fig. 5: Graphs of unconfined compressive strengths and secant modulus according to different NaCl amounts. a) Log phase b) $CaCO_3$ formation

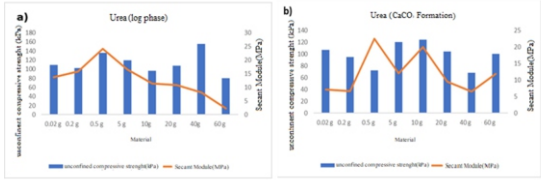


Fig. 6: Graphs of unconfined compressive strengths and secant modulus according to different Urea amounts. a) Log phase b) CaCO3 formation

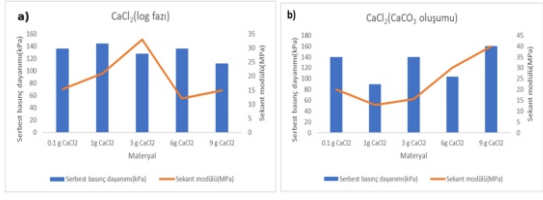


Fig. 7: Graphs of unconfined compressive strengths and secant modulus according to different CaC_{12} amounts. a) Log phase b) $CaCO_3$ formation

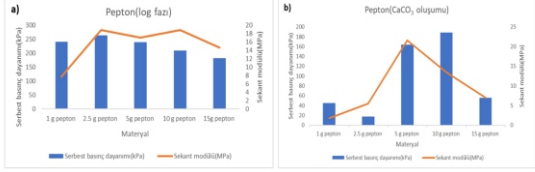


Fig. 8: Graphs of unconfined compressive strengths and secant modulus according to different Yeast extract amounts. a) Log phase b) $CaCO_3$ formation

All graphics were examined and samples with high unconfined compressive strength were determined. 2.5 g peptone (log phase); 10 g peptone ($CaCO_3$ formation); 4 g yeast extract (log phase); 0.25 g yeast extract (log phase); 6 g yeast extract $CaCO_3$formation); 0.1 g beef extract ($CaCO_3$) formation); Free compressive strengths were higher in 40 g urea (log phase) samples.

B. megaterium (ATCC 14581) is a carbonate-precipitating bacteria. It produces urease, which catalyzes the hydrolysis of urea resulting in the formation of carbonate and ammonium ions. In the presence of Ca2+, CaCO3 is formed due to the precipitation reaction of calcium and carbonate ions. Bacterial urease activity directly determines the productivity of biogenic CaCO3, which is the main crack healing material in the bacteria-based healing system. The amount and rate of decomposable urea were affected by the urea and Ca source (Wang et al., 2017). In this system, urea is the source of carbonate. If more urea is supplied, more $CaCO_3$ can be formed provided that sufficient calcium ions are present, although it has been shown that bacterial growth and urolytic activity are inhibited when the urea content is excessive.

The strength value of the fine grain silt-clay mixture at the optimum water content of 51 kPa has increased to a maximum of 5-6 times after the optimization studies with the effect of calcite rain. It has been concluded that a strength performance will be seen. The increased rigidity of the material, which evolved from a ductile structure to a brittle structure, again stood out as an increase in performance at low amplitude deformation levels. Strength values, for example, were obtained as a result of cement stabilization and in previous studies (Kalıpcılar et al., 2016) show that the strength of fine-grained soils reaches strength values slightly above the levels obtained with biocalcite in this study, after durability effects such as sulfate effect, even when the cement amount is increased to a very high value such as 15% (Fig. 9). In this context, promising results were observed in the improvement of the mechanical properties of the soils after the optimization of the study.

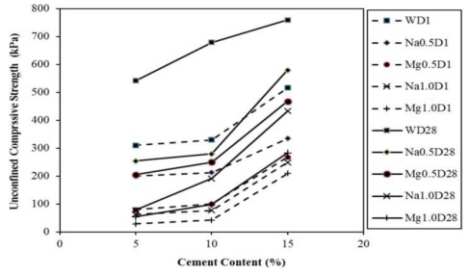


Fig. 9: Strength values obtained after curing of cement stabilized floors up to 28 days (time is indicated by the letter D) after the effect of water (W), Na- and Mg-sulphate (Kalıpcılar et al., 2016; Sezer et al., 2017)

4. SEM Results

Scanning electron microscopy (SEM) analysis performed on powder samples taken from the fracture surfaces of the samples subjected to the unconfined pressure test reveal the reasons for the increase in strength and stiffness. In Figures (10) and (11), bacterial formations, bonding with calcite, especially the bonding of silt particles and matrix structure are clearly seen, thus improving mechanical properties. The better results of the culture samples added in the log phase compared to the CaCO3 formation phase samples show that B. megaterium (ATCC 14581) can produce $CaCO_3$ even underground formation

(Dhami et al., 2013).These images are the proof that there is $CaCO_3$ in the structures formed by silt and clay. While the sample, which we consider as blind, where only the medium is placed, cannot even be removed from the mold, the strength of the mold as a result of $CaCO_3$ production being produced in the ground material shows that $CaCO_3$ is inside the structure.

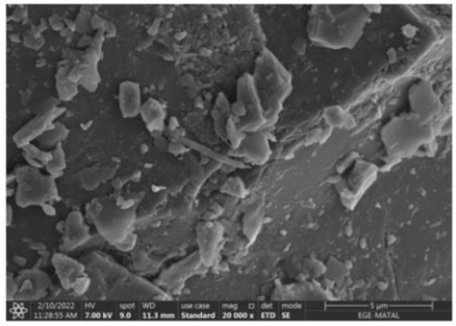


Fig. 10: SEM image of samples prepared using 4g yeast extract (log phase)

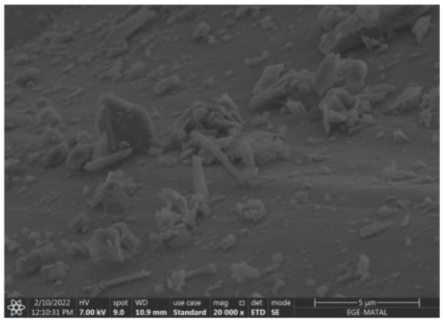


Fig. 11: SEM image of samples prepared using 0.1g beaf extract ($CaCO_3$ formation)

The setup of the project is to move away from the use of cement and to use environmentally friendly biomolecules. It can also be used in the biological repair of potentially damaged columns, floors or structures in construction, which is one of the most important parts of this project. Thus, the structures can be restored and reused at a lower cost without demolition.

5. Conclusion

It has been concluded that biocalcite can be used to add strength to the soil instead of cement. Optimization studies will be carried out in order to further increase the strength. By using this method, environmentally friendly biomaterials will be produced and it will be possible to get rid of the damages of cement. Due to the high cost of cement production, the production of buildings is also costly. This problem will also be solved. In addition, serious contributions will be made to the economy by saving the buildings that have to be demolished due to cracks.

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