Journal Homepage: http://dergipark.gov.tr/ejens

EJENS

European Journal of Engineering and Natural Sciences

DergiPark AKADEMİK

CODEN: EJENS

Development of Ecological Biodesign Products by Bacterial Biocalcification

Tugba Keskin[1*], Irem Deniz[2] , Alpcan Aric[1], Burak Talha Yilmazsoy[1], Ozge Andic-Cakir[3-4], Ayşegül Erdoğan[4], Didem Altun[5], Ayça Tokuç[5] , Burcu Filiz Demirci[5], Aylin Sendemir-Urkmez[1], Gülden Köktürk[6], Feyzal Özkaban[5]

[1]1Bioengineering Department, Engineering Faculty, Ege University, Izmir, Turkey

2Bioengineering Department, Engineering Faculty, Manisa Celal Bayar University, Turkey

3Civil Engineering Department, Engineering Faculty, Ege University, Izmir, Turkey

4Ege University Application and Research Center for Testing and Analysis, Izmir, Turkey

5Department of Architecture, Faculty of Architecture, Dokuz Eylul University, Izmir, Turkey

6Department of Electrical and Electronics Engineering, Faculty of Engineering, Dokuz Eylul University, Izmir, Turkey,.

**Corresponding Author email:keskin.tugba@gmail.com*

Abstract

Biodesign is an interdisciplinary field in which biological processes are combined with many different fields to produce environmentally friendly and economically feasible products. Within the scope of this study, first $CaCO_3$ production potential of *Sporosarcina pasteurii* has been observed and optimized, and then the capability of hardening of the sand is examined. The optimum $CaCl_2$ concentration for maximized $CaCO_3$ formation was found as 50 mM. The ecological urban element was designed and its mold was produced by 3D printer at lab scale. The *Sporosarcina pasteurii* was mixed with sand and filled into the mold. The sand was mixed with 50 mM $CaCl_2$ solution every day until hardening is observed. At the end of one week, a sitting element from hardened sand was produced. The $CaCO_3$ formation was observed with XPS analysis. Thus, an interdisciplinary approach was used to produce ecological biodesign products.

Key words

Biocalcification, Biodesign, Urban living elements, Sand hardening, *Sporosarcina pasteurii*

1. INTRODUCTION

With the progress of technology, great changes and developments especially in the social sense are emerging. The adverse effects of increasing technology and production capacities on nature and human health are also increasing at the same rate. The elimination of the negative environmental conditions or the reduction of the minimum environmental burden has been the focus of many researchers, along with developing bioengineering approaches. Increasing production capacities, which are the biggest reason of globalization, result in increasing harm to the human health of the emerging products [1].

On the other hand, the products related to the building sector causes the most damage to the environment in human activities. In this point of view, environmentally friendly, sustainable design products that are compatible with nature are now a developing field of study. A number of structures under the heading of sustainable architectural design are criticized by different approaches such as high material or resource consumption during the production process or afterwards, negative associations with the natural environment, and inability to integrate with the socio-cultural structure. Today, all kinds of urban furniture, architectural / structural / industrial materials / products (children playgrounds, seating elements, kiosks, exhibition stands, etc.) can be produced and used by processing natural materials (wood, stone, etc.) [2]–[4].

Biodesign is a study field, in which organisms are used as a design input, to create sustainable, functional, durable and non-health threatening products related to taking inspiration from natural processes [5]. It also investigates natural building designs and structures for human welfare. Cooperative studies between engineers and designers are needed with respect to build progressive, bio-based, natural forms using microorganisms as a factory to produce a structure that is both functional and has aesthetics. One way to produce products can be by utilization of mineralization and biocalcification processes inherent in nature.

Formation of minerals in nature is known as a very slow process that can take as long as a geological time. Many of the mineral types are evoluted by microbial activity which is very dependent on microorganism strain. Ureases are the main enzymes used in mineralization processes. Ureases are homologous and Ni-dependent enzymes which can be found in many living organisms such as plants, bacteria etc. Ureases hydrolyze urea into ammonia and CO_2. *Sporosarcina pasteurii* is a wide spread soil bacterium. It has very large capacity of urease production [6].

One of the main activities of the role of microorganisms in nature is the microbial cementation, in other words biocalcification. Biocalcification can be performed by different strains of bacteria, *Sporosarcina pasteurii*, *Sporosarcina urea*, *Sporolactabacillus* etc[7].

Traditionally, gram-positive, aerobic or facultative anaerobic, sporulating, rod-shaped bacteria belong to the *Bacillus* family. This causes many heterogeneities in terms of the characteristics that must be defined within the *Bacillus* family. In 1889, the microorganism identified by Miquel Chester as *Bacillus pasteurii* was defined in 2001 by Yoon et al.[8], and its name was changed to *Sporosarcina pasteurii*. The cell size of *Sporosarcina pasteurii* varies between 1.3-4 ɳm and the spores size is between 0.8-1.3 ɳm. They live at a maximum temperature of 30-45 ° C and a minimum temperature of 5-15 ° C. They can be isolated from soil, water and mud layers and they are non-pathogenic microorganisms with a biosafety level of 1. *Sporosarcina pasteurii* is not conventional because its living conditions can withstand high pH conditions as well as environments containing ammonium salts at high concentrations. In most studies, urea has been used instead of the ammonium salt because these organisms have the ability to break down the urine [9].

The metabolic pathway of *Sporosarcina pasteurii* is as follows.

1 mol urea is converted to 2 mol ammonia and 1 mol CO_2.

$CO(NH_2)_2+H_2O \rightarrow NH_2COOH+NH_3$

$NH_2COOH+H_2O \rightarrow NH_3+CO_2+H_2O$

The pH of the soil medium is increased and the enzymatic reaction occurs by the addition of $CaCl_2$ and $CaCO_3$ precipitates. By the $CaCO_3$ metabolism, the negative effect of atmospheric release and global warming would be prevented [10].

$CO_2+H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^{-}+H^{+} \leftrightarrow CO_3^{+2}+2H^{+}$

$CO_3^{-2}+Ca^{+2} \rightarrow CaCO_3$

The microbial cementing ability demonstrated by this microorganism is widely used as an innovative and promising biotechnological application such as rehabilitation and reinforcement of cement materials [11]. In recent years, the selective cementation activity with the precipitation of $CaCO_3$ by microbiologically induced calcium carbonate biocalcification, was used for filling of cracks with calcite layers[12], blending of self healing mechanism with bacterial immobilization techniques [13], [14]. Another new application area is the use of *Sporosarcina pasteurii* to reduce the hydraulic conductivity in environmental processes. Magnus Larsson proposed this solution as a solution to desertification, including soil remediation, solid phase sequestration of contaminants, and carbon sequestration technologies[15].

In summary biocalcification is a process that is used in healing cracks in reinforced concrete buildings, soil stabilization and environmental processes. Recent studies were also showed that it can be used for hardening sand[16].

This study aimed to develop an architectural design product that is in harmony with the nature and suitable to human health with partnership of bioengineering and architecture disciplines via bacterial calcification to form an architectural structure with the support of 3D printing technology of poly-lactic acid (PLA). For this purpose, *Sporosarcina pasteurii* (*Bacillus pasteurii*) was chosen to use the urea mechanism to precipitate $CaCO_3$ thus hardening sand by utilizing urea. Even though there are limited studies that make use of bacterial calcification to harden sand, this is the first study to show a successful design element built with the help of a 3D printed mold.

2. MATERIALS AND METHODS

Sporosarcina pasteurii was obtained from the DSMZ culture collection and grown in Caso Agar DSM 33 medium. Growth medium content of bacterium; For 1L; 15 g peptone from meat, 5 g peptone form soymeal, 5 g NaCl, 20 g urea. For $CaCO_3$ production in broth medium 10, 30 and 50 mM $CaCl_2$ was added to the medium. For biocalcification DSM 33 medium with urea and 50 mM $CaCl_2$ was used.

2.1. Inoculum Preperation

Sporosarcina pasteurii was obtained from the DSMZ culture collection and grown in Caso Agar DSM 33 medium. Growth medium content of bacterium; For 1L; 15 g peptone from meat, 5 g peptone form soymeal, 5 g NaCl, 20 g urea. For $CaCO_3$ production in broth medium 10, 30 and 50 mM $CaCl_2$ was added to the medium. For biocalcification DSM 33 medium with urea and 50 mM $CaCl_2$ was used.

2.2. Bacterial Staining and Growth Curve

The Schaeffer-Fulton method was used for bacterial staining. The growth broth and the broth with $CaCl_2$ was prepared on microscope and air dried and heat fixed. The malachite green was added for endospore staining. Then the slide was rinsed with water to remove malachite green. Then the broth on slide was stained by safranin this secondary stain was again washed with water and air dried. The bacteria were observed under 1000X (oil immersion) with total magnification. The vegetative cells were observed by pink/red color.

Using the broth medium the growth performance of *Sporosarcina pasteurii* was measured at 600 and 660 nm of optical density during 24 h.

2.3. $CaCl_2$ Concentration Optimization in Broth Media

$CaCO_3$ concentration was reacted with an optimum concentration of $CaCl_2$. 10, 30 and 50 mM $CaCl_2$ was added to broth medium to start $CaCO_3$ formation. $CaCO_3$ was separated from broth medium by filter paper (Advantec 5, USA).

2.4. Bacterial Solidification

In bacterial solidification experiments, preliminary experiments were performed on petri dishes. First bacteria were grown on petri dishes agar medium. One petri without inoculums was used as control. The two petri dishes (with and without inoculums) were added with equal amounts of sea sand so that the height would be 2-3 cm. 10 mL of 50 mM $CaCl_2$-containing growth medium was added to the sand. Inoculum was added to one of the petri dishes. The petri dishes were left open in the incubator at 30°C. The feed medium was depleted for one week as the petri dishes appeared to solidify.

2.5. Design of urban, architectural and industrial elements

If the bacterial calcification process is directed, it is thought that it is possible to produce architectural, urban and industrial design products. Today, many industrial products contain harmful and toxic substances for health. Particularly the construction sector is one of the most harmful activities for environment within human activities. The designs, produced by this method, will be organic and ecological because they are shaped like processes in nature. It will not contain harmful and toxic substances found in the urban environment, architectural structures or in many other things we use in everyday life.

Basically, here is to direct the bacteria through engineering methods, without leaving it to natural growth process, for generating creative design products. By using this method, a vase or a plate, or a seating element or a playground for the city can be produced. These biodesign products will be a healthy and ecological alternative with a completely natural, non-toxic character. The goal is to make this even more advanced, so that it can be used on larger scale, in architectural structures or production of buildings. Very different from the construction methods of today, buildings can be produced from soil or from sand, by bacteria - in the direction of architects and engineers' designs. Although there are a variety of researchers study on this method, there is no detailed research on the directing of bacteria, nor is there an architectural structure produced by this method.

2.6. $CaCO_3$ Analysis

The presence of $CaCO_3$ was revealed by XPS analysis. The measurements were performed with a Thermo Scientific Model K-Alpha XPS instrument using monochromatic Al Kα radiation (1486.7 eV). Survey spectra and high-resolution spectra were acquired using analyzer pass energies of 50 eV. The X-ray spot size was 300 µm for single point analysis. Data were analyzed using Avantage XPS software package. Peak fitting was performed using Gaussian/Lorentzian peak shapes and a Shirley/Smart type background.

3. RESULTS AND DISCUSSION

3.1. Bacterial Staining Results and Growth Curve of Bacteria

Determination of life cycle of microorganisms is very important for optimal process performance. The growth curve of *Sporosarcina pasteurii* was sketched by measuring the optical density of 600 and 660 nm by taking samples every 2 h during 24 h and the graph was sketched time vs OD (Optical Density). The results showed that the most active times of *Sporosarcina pasteurii* is between 6-8 h (Figure 1). According to this result the inoculation of all processes was done at 6 h.

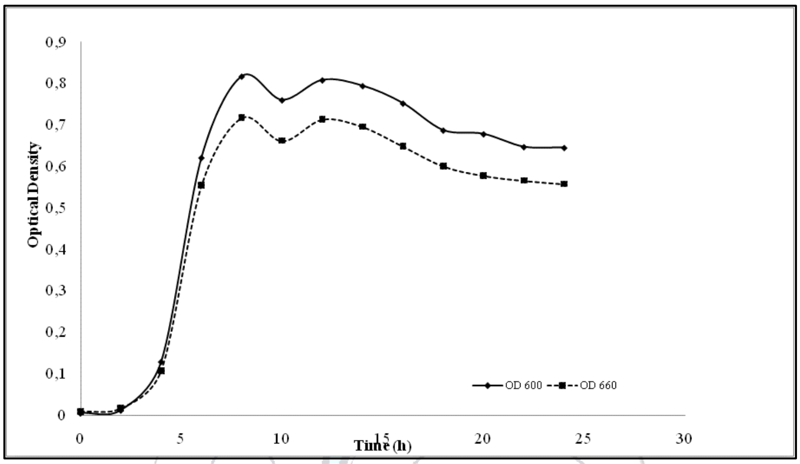

Figure 1. Growth curve of Sporosarcina pasteurii

The bacterial staining was performed according to Schaffer Fulton method. In this method red-purple colors showed the vegetative *Sporosarcina pasteurii*, which means these are active microorganisms (Figure 2 a). In this method the stains were not washed with alcohol or any other chemicals so we can observe the cluggings of $CaCO_3$ formation in the broth medium containing 50 mM $CaCl_2$ (Figure 2 b). The $CaCO_3$ formation was not enough with the broth medium containing 10 mM $CaCl_2$(Figure 2c) and 30 mM $CaCl_2$ (Figure 2 d) .

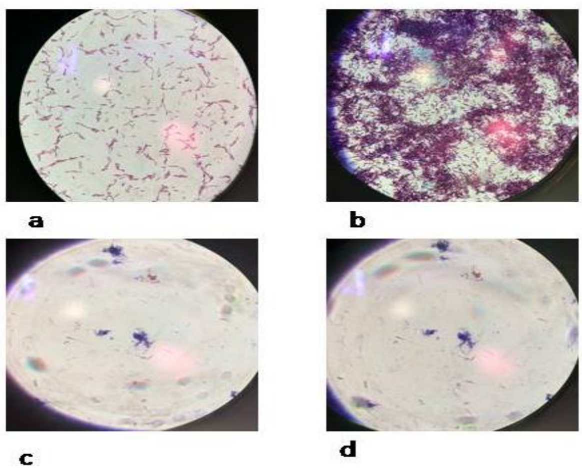

Figure 2. Staining microphotographs of the bacteria(X100; 1024dpi, olympus E 400)

3.2.$CaCl_2$ Concentration Optimization in Broth Media

$CaCO_3$ precipitates were produced by hydrolysis of urea which results in production of ammonia and carbonate. Carbonate binds calcium ions in medium and after this reaction $CaCO_3$ crystals can be formed. $CaCO_3$ (calcium carbonate) concentration is related with an optimum concentration of $CaCl_2$;10 mM, 30 mM and 50 mM $CaCl_2$ was added to broth medium to start $CaCO_3$ formation. Starting from the second hour (Figure 3a) of the process a white powder started to appear which is a mixture of $CaCl_2$ and $CaCO_3$ crystals. The concentration of the $CaCO_3$ increased by incubation at 30 ^oC during 7 days and the photos taken on day 1, day 2 and day 7, respectively. (Figure 3b, c and d)). $CaCO_3$ was separated from broth medium by filter paper and weighted. The weight of the $CaCO_3$ produced from 10 mM, 30 mM and 50 mM are 1.02 g, 1.34 g and 1.62 g respectively. In another study conducted by Dhami et al.[17]. The amount of precipitated $CaCO_3$ from different strains of B. megabacterium MTCC 1684 was between 0.84-0.076. This difference caused from the difference of bacteria and also the medium used.

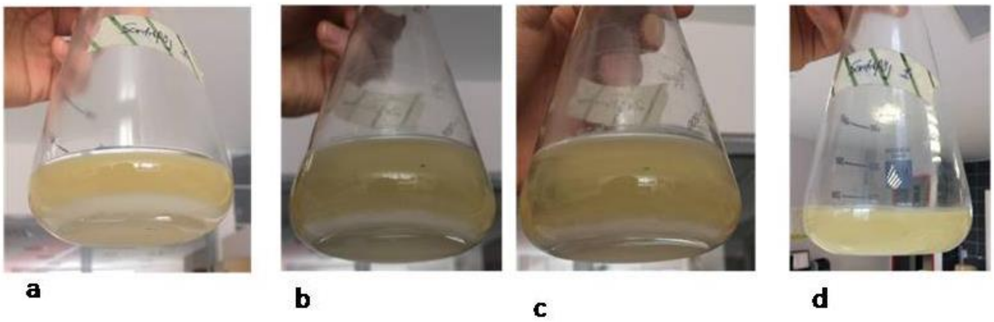

Figure 3. $CaCO_3$ formation during incubation (after; a)1 hour; b) 2 hours; c) 1 day; d) 1 week)

3.3. Bacterial Solidification

Microbially induced $CaCO_3$ is a very important product which can be used to fix cement cracks and fissures in structural formation. The most important application area for microbiologically induced $CaCO_3$ is remediation of damaged concrete. Microbiologically induced $CaCO_3$ formation reaction generally ends with high pH because of series biochemical reactions. *Sporosarcina pasteurii* is plays an important role in this process by producing urease to hydrolyze urea to ammonia and O_2. The produced ammonia will increase the pH of surroundings and calcite precipitation will start[18].

In this study, first of all, attempts were made to be sure about the solidification process is carried out by *Sporosarcina pasteurii* in petri dishes. *Sporosarcina pasteurii* is grown on agar plates and the 2-3 cm height sand as put on it. Then the medium with $CaCl_2$ was spread over sand daily until solidification completed. Another petri without inoculums was used as control. The same procedure was applied on it. It was seen that the inoculum-free sand has elasticity (Figure 4a) just because of the agar and can be easily and rapidly disintegrated while the inoculum-containing sand is hardened (Fig. 4b).

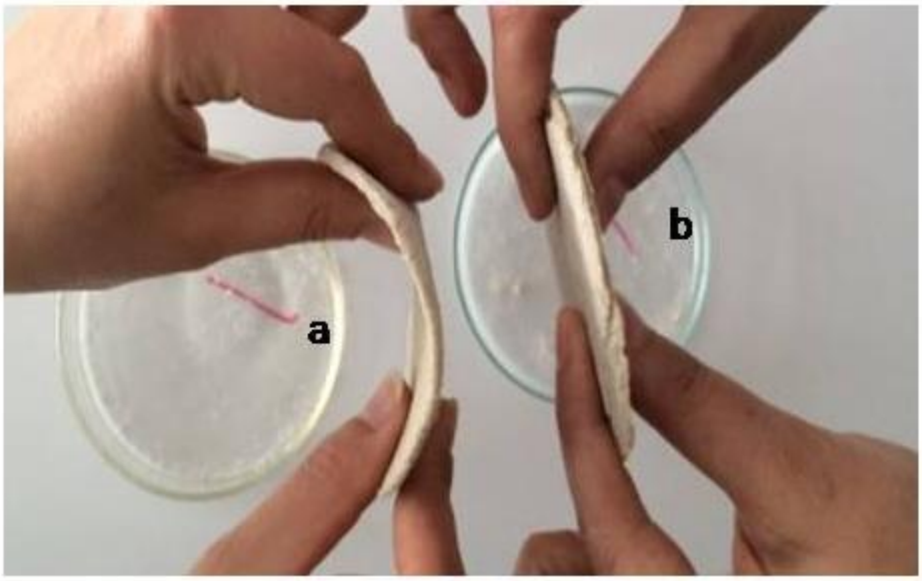

Figure 4. Solidification trials on petri dishes (a. without inoculum; b. with inoculum)

3.4. Production of Environmentally Friendly Design Elements by Bacterial Solidification

After the successful solidification process in petri dishes, a unique design element was designed and taken out from the 3-D printer (Figure 5). The design element that was selected for production with biodesign in this paper is a multipurpose urban furniture. It is part of a product range for parks and can be used to sit or lie on under various weather conditions including next to the sea. The product in this paper was scaled down for laboratory conditions.

Figure 5. 3D printed design

First of all this structure was filled with sand. Inoculum was then added to the sand and evenly distributed throughout. The DSM 33 growth medium containing 50 mM $CaCl_2$ was evenly distributed on the sand too and incubated for 1 week in a 30°C incubator. The design unit obtained at the end of one week is shown in Fig 6.

The ability of CO2 capture by Sporosarcina pasteurii is was analyzed by Okwadha and Li [19] and they found that CO2 usage by ureolytic activity can be used for microbial $CaCO_3$ formation. Another application area where

Figure 6. Environmentally friendly solidified design element

Sporosarcina pasteurii is used is civil engineering. A study conducted by Achal et al.[20] resulted in the increase of durability of concrete and Jonkers et al.[16]showed the self healing capability of concrete by *Sporosarcina pasteurii*.

Stocks and Fisher [7] used narrow plastic columns to detect the solidification ratio and ability of sand hardening by *Sporosarcina pasteurii* was analyzed by SEM and XRD. The analysis showed the formation of calcite crystals which decreased the permeability of solid by 98%.

The sand hardening properties of the bacteria in the work done by Sarmast et al.[21] have been tested in plastic columns. It has been observed to harden for 7 days in a similar quadrant in our study. At the end of this process, the stiffened sections were taken and $CaCO_3$ crystals formation was compared and equal distribution throughout the column was observed. In our study, the possibility of taking the advantage of the sand hardening properties of bacteria to create a sophisticated architectural design elements which can be readily used in daily life.

3.5. Solidification and $CaCO_3$ Analysis Results

Ca 2p peaks have clearly spaced spin-orbit components ($\Delta_{carbonate}$ =3.5 eV). In the present study, Ca 2p region for this material (Figure 7) exhibits a well-resolved doublet with a Ca $2p_{3/2}$ component at 347.1 eV and a Ca $2p_{1/2}$ component at 350.7 eV. The Ca $2p_{1/2}$ and Ca $2p_{3/2}$ bands are separated by ~3.6 eV indicating that the structure is $CaCO_3$.

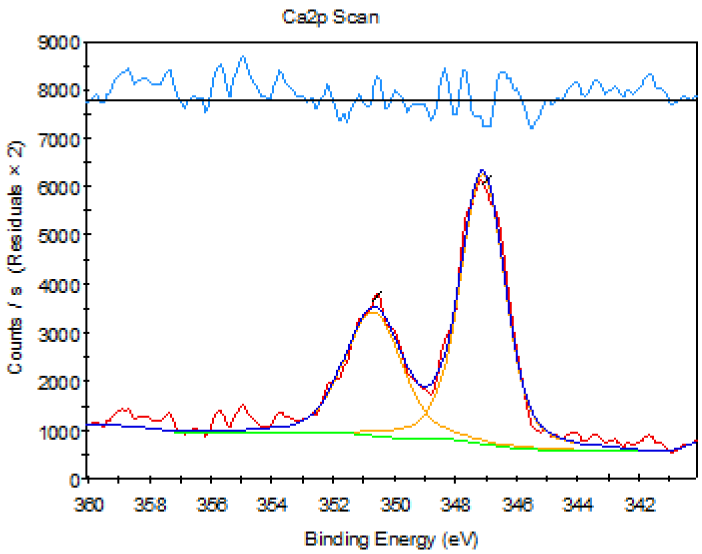

Figure 7. XPS spectrum of Ca in solidified sand

Sarmast et al. [21] used 2 bacterial species (*Sporosarcina pasteurii* is and *Sporosarcina urea*) to test the reaction times of solidifying at sandy soil columns. The results showed that $CaCO_3$ precipitation so does solidification is more favorable with *Sporosarcina pasteurii* and 12% $CaCO_3$ precipitation was observed. In our study according to XPS results 10% $CaCO_3$ precipitation was observed with 50 mM $CaCl_2$ addition.

CONCLUSION

Sporosarcina pasteurii is an environmentally friendly bacterium that can be used in hardening sand. Bacterial solidification was achieved after addition of $CaCl_2$ to the medium. In future studies, real scale designs will be manufactured via harmless environmental processes and elements made by living organisms can be produced at larger scales. The originality of this study is that the resulting products are healthier and ecological alternatives to urban constructions with detrimental health and environmental effects. Besides the novel composition and production of the design products, they aim to be totally natural, non-toxic and durable. From a social point of view, this totally natural alternative production method in the environmentally notorious construction industry gives hope to the future generations for a new way of living.

ACKNOWLEDGMENT

The authors wish to thank Prof. Dr. Nuri Azbar for the technical support by Environmental Biotechnology and Bioenergy Laboratory and Can Aysan for 3D printing support.

REFERENCES

[1] L. S. Wong, "Microbial cementation of ureolytic bacteria from the genus Bacillus: a review of the bacterial application on cement-based materials for cleaner production," *J. Clean. Prod.*, vol. 93, pp. 5–17, 2015.
[2] I. Fjørtoft and J. Sageie, "The natural environment as a playground for children," *Landsc. Urban Plan.*, vol. 48, pp. 83–97, 2000.
[3] I. Fjørtoft and J. Sageie, "The natural environment as a playground for children. Landscape description and analyses of a natural playscape," *Landsc. Urban Plan.*, vol. 48, no. 1–2, pp. 83–97, 2000.
[4] E. M. Campa, "Pensamientos compartidos. Aldo van eyck, el grupo cobra y el arte," *Rev. Proy. Progreso, Arquit.*, no. 11, pp. 64–75, 2014.
[5] M. Taya, "Bio-inspired design of intelligent materials," *Smart Struct. Mater.*, vol. 5051, pp. 54–65, 2003.
[6] D. Olivera-Severo, G. E. Wassermann, and C. R. Carlini, "*Bacillus pasteurii* urease shares with plant ureases the ability to induce aggregation of blood platelets," *Arch. Biochem. Biophys.*, vol. 452, no. 2, pp. 149–155, 2006.
[7] S. Stocks-Fischer, J. K. Galinat, and S. S. Bang, "Microbiological precipitation of $CaCO_3$," *Soil Biol. Biochem.*, vol. 31, no. 11, pp. 1563–1571, 1999.
[8] J. H. Yoon, K. C. Lee, N. Weiss, Y. H. Kho, K. H. Kang, and Y. H. Park, "*Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (larkin and stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosa*," *Int. J. Syst. Evol. Microbiol.*, vol. 51, no. 3, pp. 1079–1086, 2001.
[9] W. R. Wıley and J. L. Stokes, "Requirement of an alkaline pH and ammonia for substrate oxidation by *Bacillus pasteurii*.," *J. Bacteriol.*, vol. 84, pp. 730–734, 1962.
[10] R. Siddique and N. K. Chahal, "Effect of ureolytic bacteria on concrete properties," *Construction and Building Materials*, vol. 25, no. 10. pp. 3791–3801, 2011.
[11] S. J. Park, Y. M. Park, W. Y. Chun, W. J. Kim, and S. Y. Ghim, "Calcite-forming bacteria for compressive strength improvement in mortar," *J. Microbiol. Biotechnol.*, vol. 20, no. 4, pp. 782–788, 2010.
[12] Q. Chunxiang, W. Jianyun, W. Ruixing, and C. Liang, "Corrosion protection of cement-based building materials by surface deposition of $CaCO_3$ by *Bacillus pasteurii*," *Mater. Sci. Eng. C*, vol. 29, no. 4, pp. 1273–1280, 2009.
[13] J. Wang, K. Van Tittelboom, N. De Belie, and W. Verstraete, "Use of silica gel or polyurethane immobilized bacteria for self-healing concrete," *Constr. Build. Mater.*, vol. 26, no. 1, pp. 532–540, 2012.
[14] H. M. Jonkers and E. Schlangen, "Crack Repair By Concrete-Immobilized Bacteria," *Civ. Eng.*, no. April, pp. 1–7, 2007.
[15] B. Mahanty, S. Kim, and C. G. Kim, "Biokinetic modeling of ureolysis in Sporosarcina pasteurii and its integration into a numerical chemodynamic biocalcification model," *Chem. Geol.*, vol. 383, pp. 13–25,

2014.

[16] H. M. Jonkers and M. C. M. van Loosdrecht, "BioGeoCivil Engineering," *Ecological Engineering*, vol. 36, no. 2, pp. 97–98, 2010.

[17] N. K. Dhami, M. S. Reddy, and A. Mukherjee, "*Bacillus megaterium* mediated mineralization of calcium carbonate as biogenic surface treatment of green building materials," *World J. Microbiol. Biotechnol.*, vol. 29, no. 12, pp. 2397–2406, 2013.

[18] S. S. Bang, J. K. Galinat, and V. Ramakrishnan, "Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*," *Enzyme Microb. Technol.*, vol. 28, no. 4–5, pp. 404–409, 2001.

[19] G. D. O. Okwadha and J. Li, "Optimum conditions for microbial carbonate precipitation," *Chemosphere*, vol. 81, no. 9, pp. 1143–1148, 2010.

[20] V. Achal, A. Mukherjee, and M. S. Reddy, "Microbial concrete: Way to enhance the durability of building structures," *J. Mater. Civ. Eng.*, vol. 23, no. 6, pp. 730–734, 2011.

[21] M. Sarmast, M. H. Farpoor, M. Sarcheshmehpoor, and M. K. Eghbal, "Micromorphological and biocalcification effects of *Sporosarcina pasteurii* and *Sporosarcina urea*e in sandy soil columns," *J. Agric. Sci. Technol.*, vol. 16, no. 3, pp. 681–693, 2014.