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CALCIUM CARBONATE PRECIPITATION OF ALKALIPHILIC BACTERIA ON ORDINARY PORTLANDITE CEMENT WITH A VISION ON RECYCLED CONCRETE AGGREGATES APPLICATION

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Abstract

Recycled concrete aggregates (RCA) can substitute natural aggregates in concrete with the disadvantage of needing a major quantity of cement and water as consequence of their moderate quality. The objective of this study is to precipitate calcium carbonate crystals over RCA by consuming atmospheric CO₂ with a green and sustainable process achieved by microorganisms (MO), improving the mechanical and durability properties of RCA.

Bio-carbonation is the result of the metabolism of MO, which must possess three main characteristics; the ability to precipitate calcium carbonate, grow under alkaline conditions (between 11 and 13 pH) and to form complex colonies known as biofilm.

In this study, a selection of bacteria isolated from RCA was done with the aim to obtain the species with the capacity to grow in alkaline conditions and to form biofilm. Even if these bacteria are not able to precipitate calcium carbonate crystals, we could fusion their characteristics of growth with other bacteria that perform calcium carbonate synthesis such as *Bacillus Halodurans*.

Microbiological techniques were employed to perform isolation and selection of wild species. An incubation process over a mortar disks was performed to observe the possible behaviour of bacteria to colonize a surface. As result, three wild bacteria species growing in liquid nutrient broth pH 11 were obtained. The isolated bacteria grew under a matrix made of nutritive agar culture over a mortar at pH 13 through biofilm formation. Spherical structures (possibly calcium carbonate crystals) of around 20 – 30 micrometres appeared near to the zones were bacteria developed.

The experimental design of this study opens a new gate of research that seeks an evolution in the use of concrete before and after its life span having as aim a tangible contribution in the reduction of atmospheric CO₂ generating RCA of high quality.

Keywords. Bio carbonation, bacteria, metabolism, biofilm, calcareous rocks and calcium carbonate crystals.

Introduction

High energy consumption, use of large amounts of raw materials and generation of high volumes of construction rubble are current concerns of the construction and demolition industry (C&D) [1]. Recycling is part of the solution. Thus, recycled concrete aggregates (RCA) obtained from crushing and sieving old concrete can replace natural aggregates in new concrete.

Replacement can reach 100% [2,3], but it is generally limited to much lower values, due to the moderate quality and cost of RCA [4,5]. Rougeau et al. [6] shew that durable concrete can be obtained by using a high level of RCA if the quality of the new matrix is improved by adding more cement. In fact, as summarized by Sedran [7], the rough surface of RCA and their low strength inherited from original concrete generally increase the cement demand on new concrete for a given strength compared to concrete with natural aggregates.

Moreover, the high porosity of RCA is a source of variability of the total water content of concrete at industrial scale. Higher RCA content requires more cement and water.

Gonzales and Etxeberria [8] analysed the durability of a High Performance Concrete (HPC) made with 20%, 50% and 100% of RCA. They found that mechanically the HPC made of RCA (HPC – RCA) had almost the same behaviour as a HPC made of original concrete, but emphasizing that porosity and humidity have a negative impact in the durability of HPC – RCA. In addition, they recommend the improvement of HPC – RCA with mineral admixtures to increase its durability.

Zaharieva et al. [9] compared the surface permeability of RCA with normal concrete aggregates (NCA). They conclude that the long-term performance of concrete made with RCA is more permeable than that made of NCA, affecting the durability.

Some studies analyse the performance of silica fume and fly ash in concrete and RCA, saying that these compounds can reduce their permeability and porosity [9,10]. The main disadvantages of using these materials are related to the cost, malleability and quantity to be employed without having negative effects [11,12].

The mortar remaining attached to RCA is the main cause of these drawbacks. Nowadays the reduction of the attached mortar in RCA is an effective technique to enhance their quality [14]. Ultrasonic cleaning method, heating, rubbing, ball milling and the addition of acidic solvents to reduce the attached mortar on RCA are employed. On the other hand these techniques generate energetic costs and in some cases pollutants [15].

An alternative solution is to improve the quality of the matrix itself by natural carbonation. Calcium carbonate precipitation is a natural phenomenon that occurs as consequence of the interaction between atmospheric CO₂ and the portlandite Ca(OH)₂ found on the concrete surface. When there is a relative humidity higher than 30 % these compounds react to form calcium carbonate Ca(CO₃) crystals, sealing pores and decreasing permeability [16,17,18]. The main disadvantage of this process is the time (several months) that it requires to be accomplished.

Another solution studied in the present paper, consists in accelerating carbonation thanks to the use of microorganisms. Three hypotheses explain the formation of Ca(CO₃) crystals by

biomass. The first says that the process is a by-product of autotrophic or heterotrophic metabolisms. The second says that the nucleation sites present in the membranes of the microorganisms (MO) attract divalent ions such as Ca^{2+} leading to crystal formation and finally, the third establishes that the process is carried out by extra polymeric substances secreted by MO [19].

Bacteria are the most studied microorganisms in the subject; they are classified depending on their source of carbon. Autotroph species consume environmental or dissolved CO_2 while heterogeneous species consume carbon of organic compounds [20].

The most attractive species are those with the capacity of metabolising CO_2 (g): (i) Aerobic bacteria which source of carbon is organic matter; (ii) Methanogenic bacteria; (iii) Oxygenic photosynthetic bacteria which energy is obtained through photosynthesis; (iv) Anoxygenogenic photosynthetic bacteria which energy is obtained through photosynthesis without the production of oxygen.

Environmental conditions as a relative humidity superior to 30 %, a pH bigger than 11 to favour the abundance of ions such as calcium and a temperature between 25 – 37°C are needed to facilitate bacteria development and as consequence lead to the formation of $\text{Ca}(\text{CO}_3)$ crystals [21].

Studies on this matter as those performed by Medevielle [21], show that some MO such as *Bacillus halodurans* is able to precipitate $\text{Ca}(\text{CO}_3)$ by two metabolic pathways. The carbonic anhydrase pathway, which consumes CO_2 (g) and the Urease pathway, that metabolise Urea [19]. All the tests performed during her experiments over RCA shew the same obstacle during each treatment, the non-homogeneity during crystal precipitation, linked to bacteria inoculation and dissemination.

Due to the capacity of *B. halodurans* to precipitate $\text{Ca}(\text{CO}_3)$ a possible association with other bacteria to colonise a surface is analysed. Biofilm is a matrix of extra polymeric substances and proteins [22], it plays an important role in bacteria surface colonisation (Figure 1 A).

A computer database comparison was done between the genes responsible of biofilm formation in *Bacillus subtilis* and those present in *B. halodurans* (similar species) [23,24,25,26]. As result *B. halodurans* lacked of the principal genes responsible to form biofilm. The result of this analysis shows the possible incompetence of *B. halodurans* to colonise a large area on a surface and shows its vulnerability against other microorganisms or abiotic factors such as alkalinity [22].

Bio mineralization through biofilm is less studied, but it can contribute with mineral uptake, stock and even it can influence in the crystal shape [27]. Related to the environment in which they exist, calcareous rock bacteria was studied by Kaue et al. to observe their capacity of $\text{Ca}(\text{CO}_3)$ synthesis through biofilm, founding calcite and vaterite precipitation [19]. Then if a consortium of bacteria is used to explode the best quality of each one in colonization and bio mineralization, a better result in homogeneity during crystal formation can be achieved. Calcite, aragonite and vaterite are common polymorphs of calcium carbonate crystals (Figure 1 B).

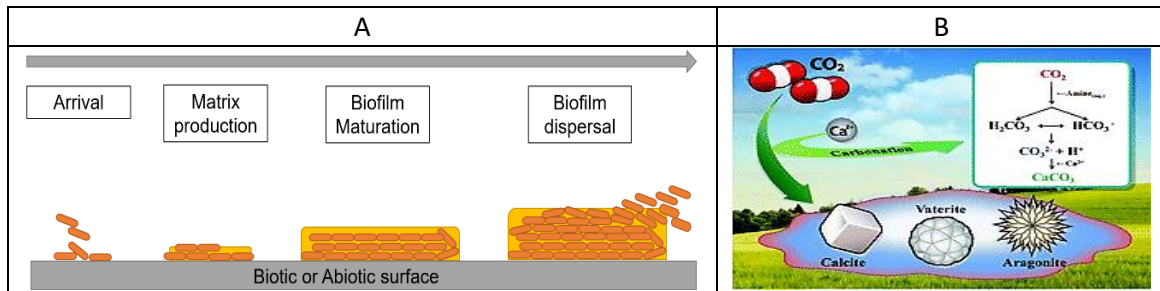


Figure 1. A - Stages of biofilm formation. First bacteria or fungi cells arrive into a surface (rod shaped structures) and start cell differentiation for matrix production, and then cells begin to form chains and the volume of the biofilm increases. In the biofilm maturation, cells are well organized and are surrounded by an extra polymeric substance and in the biofilm dispersal, cells secrete substances that allow them go out of the biofilm and reach other areas [22][28]. **B -** Extracted from “CO₂ mineralization into different polymorphs of CaCO₃ using an aqueous-CO₂ system” It show the conversion of Carbon dioxide into the main calcium carbonate crystals [29]

This study, analyse the capacity of wild bacteria isolated from recycled concrete aggregates to form biofilm and to grow under alkaline conditions. Bacteria were isolated and selected based on their capacity to grow at pH 11 in nutrient broth media. As a final point, their capacity to develop over mortar disks (cement paste + sand) through a matrix made of nutrient agar broth was tested, incubating the disks for 60 days at 25°C with a 99.5 % of humidity.

Methodology and Materials

Isolation of Microorganisms

RCA (pH 10) were collected from two different stocks located in Nantes, France and transported in sealed plastic bags. Samples were identified with their stock ID code and renamed for an easier utilisation, the RCA took from the stock MH-13-092 were labelled as GRR (Figure 2. b)) and from the stock MH-15-145 were GB (Figure 2. c)). One aggregate out of five was used to the manipulation from each stock sample.

Two Petri plates with nutrient broth 1.5 % agar pH 7 (sterilised at 121 °C – 1.5 AT – 15 min) were used to perform bacteria isolation by contact. The area of the Petri plate was divided into five zones with the aim of putting a different part of the RCA in contact with the solid media (Figure 2 A).

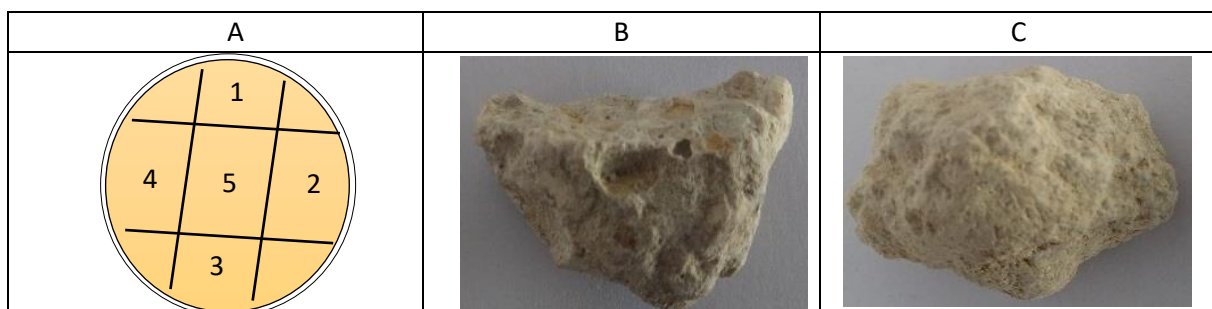


Figure 2. A - Division of the Petri plates in five zones to perform bacteria isolation by contact, **B -** Granulat GRR and **C –** Granulat GB

All the process was done in an airflow laminar cabinet (Angelantoni Industry Steril VBH36c2) to keep sterile conditions. At the end of the manipulation, both Petri plates were incubated at 37°C for 96 h.

Selection of microorganisms based on the resistance to alkaline pH and biofilm formation

From the Petri plates used to isolate MO by contact with RCA, all the colonies present after 96 h of incubation were marked and numbered on the bottom of the Petri plate. By working in the airflow laminar cabinet all colonies were inoculated in test tubes with 6 ml of liquid nutrient broth pH 7 and incubated at 37°C, 300 rpm for 96 h.

After 96 h of incubation 1 ml of the culture media was inoculated in test tubes with 6 ml of liquid nutrient broth at pH 9 (verified with a Melter-Toledo potentiometer 51302928) and incubated for 168 h, 300 rpm at 37°C. The process continued for the species that survived the increment of pH and for those that formed biofilm. The MO that grew at pH 9 were inoculated in test tubes with 6 ml of liquid nutrient broth at pH 11 and the process was repeated with liquid nutrient broth pH 13.

Microscopic observation of microorganisms

MO were observed in a Scope A1 microscope SNT 12 V 100 W after 96 h of incubation. 70 µl of media with MO were put on the crystal slide and fixed by flame with a burner Bunsen, in the next second's crystal violet was added abundantly on the slide with the MO. After 50 seconds the slide was washed 3 times with distilled water. All the process was done in the laminar airflow cabinet.

Mortar pictures were taken after 60 days with the camera of the microscope Axiocam 506 color at 50X and 100 X.

Biofilm formation of MO over mortar

Two mortar disks (pH 13) of 5.8 cm of diameter x 2.2 cm of length were used to observe the capacity of RCA MO to grow over it (both were washed with distilled water before the process). MO were incubated in liquid nutrient broth at pH 11, 300 rpm for 96 h. Inoculation was done by layers. First, 1ml of liquid nutrient broth pH 7 was spread over the disks, after 15 minutes when the media was almost absorbed by the mortar 1 ml of each MO was added to the first disk. To finish, after 15 minutes 1ml of nutrient broth 1.5 % agar was added to cover the surface. When the media with agar was almost solid, 1 ml of the media with agar was supplementary added.

Incubation

Both disks were placed in a desiccator of 15 L with an airflow entrance activated each 2 days for 4 hours (Figure 3). Humidity was provided by 1.5 L of water providing 99.5 % of humidity. The incubation chamber was cleaned with ethanol 70 % and to avoid any contamination by the airflow entrance, a filter of 0.2 microns was used. The period of incubation was for 60 days.

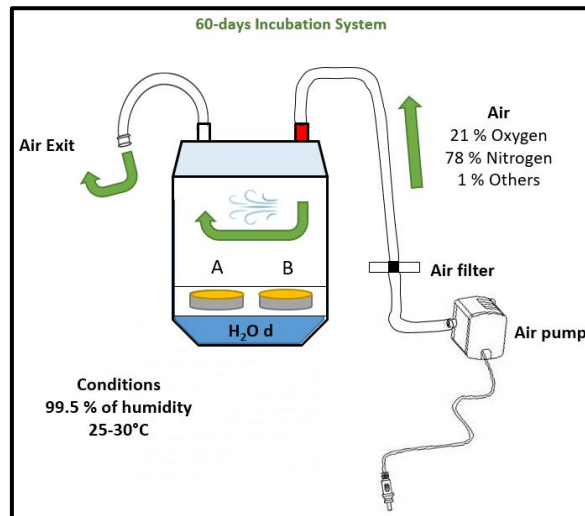


Figure 3. Incubation system supplied by air each 2 days for 4 hours. A is the disk treated with bacteria and B the control disk, both present in the Figure 2. Distilled sterile water was put at the bottom of the incubator to generate humidity in the system.

Results

Isolation of Microorganisms

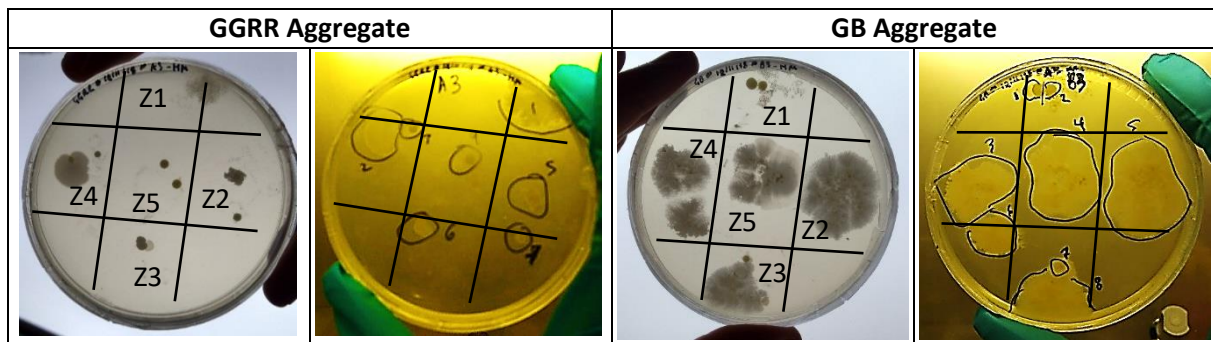


Figure 4. Microorganisms isolated from RCA. Bacteria and fungi colonies correspondent to the GRRR and GB granulates. Bacteria morphology GRRR (zone 2,3,4 and 5) GB (zone 1, 3) and fungi morphology GRRR (zone 1, 2 and 4) GB (zone 2,3,4 and 5) in the Petri plates.

Colonies grew on the designated zones of contact. Fifteen microorganisms grew after 96 h of incubation in both Petri plates. Seven colonies were present in the GRRR plate where five seemed to be bacteria and two fungi. For the GB granulate plate eight colonies grew, four with a fungi appearance and four with a bacteria morphology (Figure 4).

Selection of microorganisms based on the resistance to alkaline pH and biofilm formation

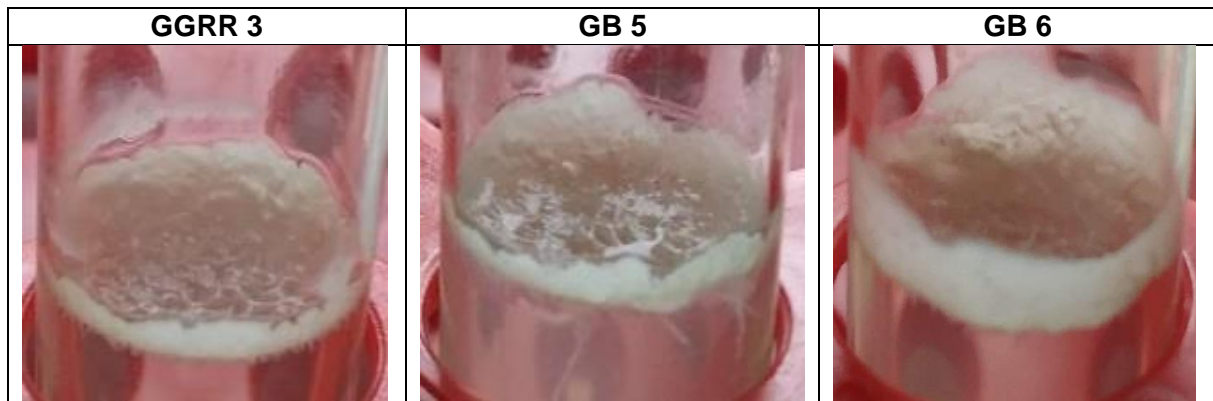


Figure 5. Microorganisms in nutrient liquid media pH 11. GRR 3 is the colony number three isolated from the GRR granulate, GB 5 AND GB 6 are MO isolated from the GB granulate.

Just three out of fifteen MO species were resistant to grow in nutrient liquid broth pH 11. These MO formed a robust biofilm at the top of the culture media (Figure 5). The formed structures had a strong consistency; the borders that had contact with the internal walls of the tube were strongly attached. GRR 3 biofilm seemed to be more compact and dried at the surface than the biofilm of the GB species. These MO were not able to grow at pH 13, a second culture media at pH 12 was done, but the result was the same. Suggesting that these bacteria have as limit of development a pH of 11.

Microscopic observation of microorganisms

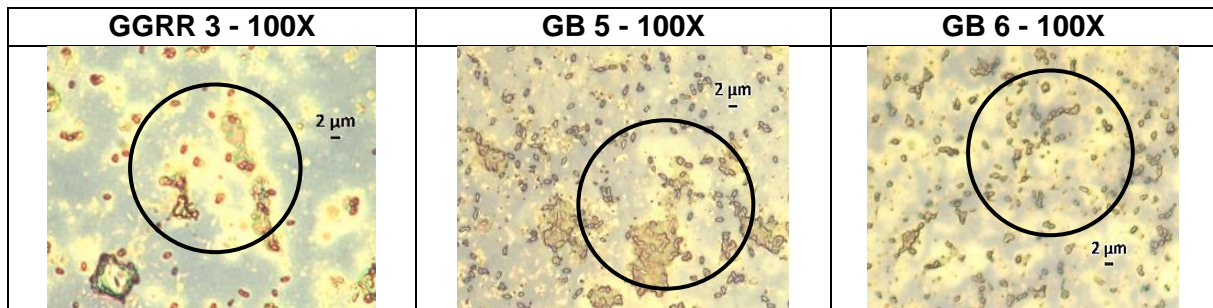


Figure 6. Microscopic observation 100 X of the three species with the capacity of development in nutrient broth pH 11. Crystal violet was used as dye to perform bacteria observation.

Microscopic observation revealed that the three MO, which developed in nutrient broth pH 11, were bacteria (Figure 6). Due to their morphology, the three species might be part of the coccobacilli group. In the GRR 3 - 100X and in the GB 5 - 100X photos we can observe some biofilm with bacteria agglomeration that could be produced because of the alkalinity. In the GB 6 - 100 X photo, we can just see bacteria agglomeration. In the three cases bacteria seems to measure less than 2 µm. Shining regions on the picture are product of the light reflexion over crystal violet (dye for bacterial observation).

Biofilm formation over mortar and crystal formation

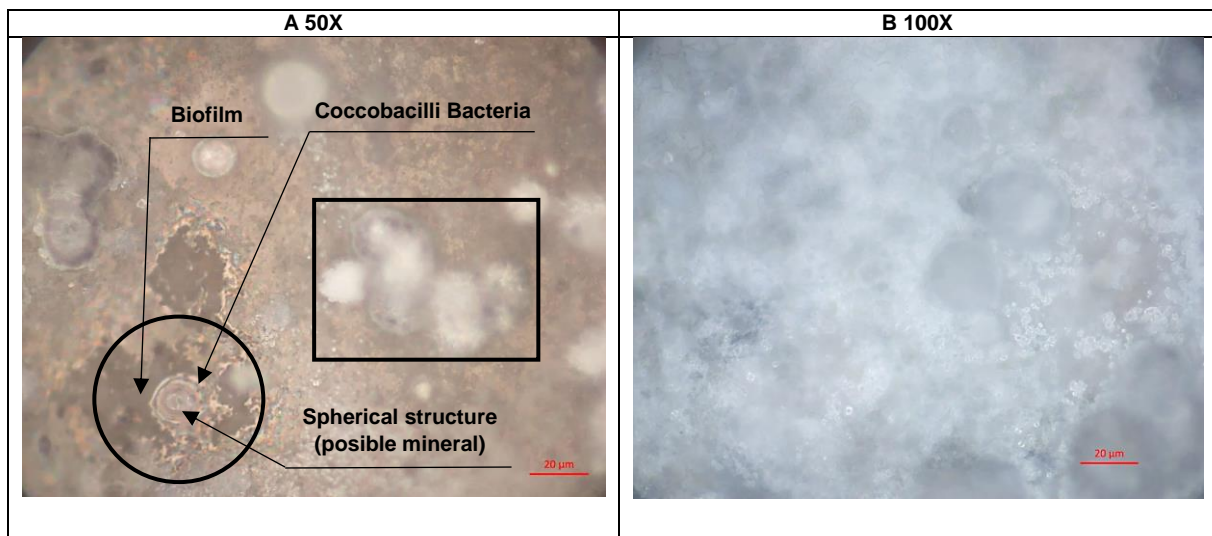


Figure 7. Microscopic observation of bacteria biofilm and crystal formation 50 X A and 100X B in different zones of the mortar. Observation is limited because of the matrix of agar.

Bacteria were still present on the disks after 60 days, many pieces of biofilm were found at the surface of the mortar. In the circle zone of the Figure 7 A three pieces of biofilm surrounding a possible crystal of calcium carbonate are observed. In the rectangular zone an agglomeration of spheres with the same appearance as that surrounded by biofilm are present.

In the Figure 7 B spherical and small structures that apparently must be mineral precipitation are observed. The biofilm lacked of homogeneity, but apparently these spheres and small structures are covering a large area of the granulate. In the control disk any bacteria and spherical structures were present after the 60 days of incubation.

Discussion

Fifteen MO were isolated from the surface of RCA. At the beginning, due to the colonies morphology bacteria and fungi presence was suggested. Commonly fungi have a tendency of dispersion while bacteria grow in pellet [30]. After a microscopic analysis the three species that survived at pH 11 were bacteria.

The three isolated bacteria have a rod-shaped structure in one extreme and a spherical shape in another; because of this, these bacteria might be part of the coccobacilli group, they look almost round [39,40]. Bacteria can change its shape as consequence of adaptation to external conditions. Young, K. [41] states that shape is a vector of evolution remarking that facts as nutrient acquisition, cell division and active motility induce shape changing [42].

In our experimental conditions, stress is given by the availability of nutrients, humidity and alkalinity, facts that can consequently affect the morphology of our bacteria. Furthermore, in the microscopic observation we can infer that the GB 5 and GB 6 species are the same due to their structure and size in comparison with the GGRR 3 that looks bigger.

The GGRR 3 bacteria grew in a pelletized colony (Figure 4 GGRR zone 4) while the GB 5 and GB 6 bacteria grew in dispersion (Figure 4 GB zone 2 and 4). This behaviour might be linked to their rod shaped-structure. Krawczyk et al. [31] analysed bacteria by modeling a non linear development and concluded that the rod-shaped species tend to spread much faster than other populations. In addition associated factors of development such as the availability of nutrients, space, presence of other bacteria and motility are considered as factors of development.

Furthermore, chemotaxis might be also responsible for this behaviour. Some bacteria expands in concentric ring patterns, consuming the nutrients in the initial inoculated zone and migrating towards by the action of chemo attractants, which can be produced by the same or other bacteria species. This migration is characteristic of motile bacteria, but non-motile bacteria can also swim chemotactically in semi-solid media [32,33,34].

In this case bacteria dispersate over the Petri plate with nutritive broth 1.5% agar. In fact, we might isolated one non-motile bacteria and two motile bacteria, which presumably moves outwards the inoculation zone by the presence of other bacteria from the same GBR or nutrients distribution.

When all bacteria were cultivated in liquid nutrient broth pH 7 some of them formed biofilm at the top of the media which is characteristic of obligate aerobic bacteria that search the area of major oxygen concentration, in this case located at the top of the test tube [35].

Moreover, when bacteria were cultivated in a media that was each time more alkaline, just some of them used to grow and form biofilm. These bacteria communities are made of exopolysaccharides and proteins, it protects bacteria from the environment helping them to have a better adaptation to a new media [22,36,37,38].

After 60 days of incubation bacteria formed biofilm under the nutrient broth agar matrix that covered almost all the surface of the mortar disk. The observations revealed that only the regions that were covered with the matrix presented microbial growth. These MO grew in the internal part of the matrix, which means that bacteria could be better protected from desiccation and temperature change than on other regions that were not covered with the matrix of agar [43].

Logically bacteria look always for the carbon rich source to develop that is the nutrient agar matrix. Meaning that bacteria might just grow on the agar matrix making any interaction with the mortar, but bacteria could also develop on the mortar looking for minerals [19].

The structures observed by focal microscopy under the matrix must be analysed by Raman microscopy to know their composition because if it is well known the structures of the Ca (CO₃) crystals, their shape depend on the type of microorganism and environmental conditions [44].

The structures found over the mortar have approximately a size between 20 and 30 micrometres. Three hypotheses might explain their formation. First, these bacteria might possess the carbonic anhydrase enzyme which hydrate atmospheric CO₂ converting it in carbonic acid H₂CO₃ and it reacts with the calcium present on the mortar [45]. Second, the proteins present in the biofilm, precipitated Ca (CO₃) crystals affecting their morphology and

their direction of deposition [45,46]. Third, the nucleation of carbonates takes place in the membrane of bacteria as consequence of ion exchange [19].

Conclusion and Perspectives

Three coccobacilli-like bacteria were isolated from RCA. They are able to grow at pH 11 and they possess the ability to form biofilm. The isolated bacteria grew under a matrix made of agar that is in contact with a mortar at pH 13. The development of bacteria is not homogeneous but pieces of biofilm were found all over the surface.

Spherical structures surrounded by bacteria and biofilm were found under the matrix. As any additional source of calcium such as calcium acetate was added to the mortars, precipitation could be the result of the consumption of atmospheric CO₂ and the calcium present over the mortar but further experiments to conclude this hypothesis must be done.

If these structures are any form of calcium carbonate, the porosity of the mortar at the surface might be reduced, which means that it could also reduce the porosity on RCA. Reproduction of results must be done in order to test strength and absorption properties.

Bio mineralization through biofilm structures shall be studied as well as the possible symbiosis with bacteria that perform Ca (CO₃) precipitation. Bio molecular analyses shall be done to know the protein content and exopolysaccharides that forms the biofilm.

Other types of matrix such as a hydrogel nutrient broth or a semi-solid agar broth must be tested to find the best way of bacteria inoculation. Bio molecular analyses to know the specie of the isolated bacteria could be done; this can give more information about their preferable source of carbon and behaviour.

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