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Full Length Research Paper

Calcium carbonate precipitation by different bacterial strains

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Bacteria are capable of performing metabolic activities which thereby promote precipitation of calcium carbonate in the form of calcite. In this study, it is shown that microbial mineral precipitation was a result of metabolic activities of some specific microorganisms. Concrete microorganisms were used to improve the overall behavior of concrete. It was predicted that bacterial calcium carbonate ($CaCO_3$) precipitation occurs as a byproduct of common metabolic processes such as urea hydrolysis. In this study, ureolytic bacteria that were capable of precipitating calcium carbonate were isolated and further their urease activity was tested based on the production of urease. Scanning electron microscopy (SED) analysis revealed the direct involvement of these isolates in calcium carbonate precipitation. The production of calcite was further confirmed by x-ray diffraction (XRD) and energy-dispersive x-ray (EDX) analysis.

Key words: Bacteria, urease activity, microbial mineral precipitation, scanning electron microscope-energy-dispersive x-ray, x-ray diffraction.

INTRODUCTION

Bacteria are ubiquitous in every habitat on Earth, growing in soil, acidic hot springs, radioactive waste water and deep in the Earth's crust, as well as in organic matter and the live bodies of plants and animals. Bacteria have a wide range of shapes, ranging from spheres to rods and spirals. The primary role of bacteria in the precipitation process has been ascribed to their ability to create an alkaline environment through various physiological activities (Douglas and Beveridge, 1998). Certain negatively charged nature and specific functional groups of microbial cell walls favours the binding of divalent cations (Ca^{2+} and Mg^{2+}), thereby making microorganisms ideal crystal nucleation site (Rivadeneyra et al., 1998). Specific proteins present in biological extracellular polymeric substances cause the formation of different calcium carbonate polymorphs (Kawaguchi and Decho, 1999). Bacterial deposition of a layer of calcite on the surface of the specimens resulted in a decrease of capillary water uptake and permeability towards gas. The type of bacterial culture and medium composition had a profound impact on calcium carbonate crystal morphology. Microbial mineral precipitation (biodeposition) involves various microorganisms, pathways and environments. Considerable research on carbonate precipitaton by bacteria has been done by using ureolytic bacteria. These bacteria are able to influence the precipitaton of calcium carbonate by the production of urease enzyme. This enzyme catalyzes the hydrolysis of urea to co_2 and ammonia, resulting in an increase of the pH and carbonate concentration in the bacterial environment (stocks et al., 1999). Immobilization technique for remediation of cracks in concrete, where microbial cells are encapsulated in polymers has been adapted to enclose in the gap to enhance the strength for selective concentration (Bang et al., 2001). Microbial calcite precipitation (mcp) occurs as a by-product of common microbial metabolic process, such as urea hydrolysis, photosynthesis, sulfate reduction. These different metabolic processes increase the alkalinity (pH and dissolved inorganic carbon) and thereby favouring the calcium carbonate precipitation (Knorre and Krumbein, 2000). Calcium carbonate precipitation is a general process in the bacterial world under appropriate conditions (Bang et al., 2001). Some

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bacteria and fungi can induce precipitation of calcium carbonate extracellularly through a number of processes that include photosynthesis, ammonification, denitrifycation, sulfate reduction and anaerobic sulphide oxidation (Castainer et al., 2000; Riding, 2000).

Bacillus pasteurii produces intracellular urease constitutes close to 1% of the cell dry weight (Braissant et al., 2002). *B. pasteruii,* a common soil bacterium can induce the precipitation of calcite. As a microbial sealant, $CaCO_3$ exhibited its positive potential in selectively consolidating simulated fractures and surface fissures in granites and in the consolidation of sand. Besides this, a durability study on concrete beams treated with bacteria, exposed to alkaline, sulfate and freeze-thaw environments were also studied. The durability performance increased with increase in the concentration of bacteria. Microbial calcite precipitation was quantified by x-ray diffraction (XRD) analysis and visualized by scanning electron microscopy (SEM) (Ramachandran et al., 2001). Bio-deposition of a calcium carbonate layer on degraded lime stone by five different strains of the *Bacillus sphaericus* group and one strain of *Bacillus lentus* was studied (Dick et al., 2006). It was found that *Bacillus* strains were capable of depositing calcium carbonate, but different in amount. Furthermore, to obtain protective calcite layers, these strains were tested for their ureolytic driven $CaCO_3$ precipitation. The best calcite precipitating strains were characterised by high ureolytic efficiency, homogeneous calcite deposition on limestone cubes and a very negative f-potential. Bio-mediated production of calcite crystals by calcinogenic bacteria has great applicable value for the restoration of deteriorated calcareous monuments, because of its high purity and coherency (Lee et al., 2003). Weathered concrete samples made with Portland cement or with blast furnace slag cement and fouled by lichens were treated with *Thiobacillus* bacteria and an appropriate nutrient (Nitrogen and Carbon) by submersion or sprinkling. SEM and XRD analysis revealed a dense layer of calcite and vaterite crystals (Lee et al., 2003). Biomineralisation of calcium carbonate is one of the strategies to remediate cracks in building materials because cracks not only influence the service durability on concrete structure, but also harmful for the structure safety (Zhong and Yao, 2008). Bacterial deposition of a layer of calcite on the surface of the specimens resulted in a decrease of capillary water uptake and gas permeability (Muynck et al., 2008). Durability of mortar specimens with different porosity was affected by bacterial carbonate precipitation (biodeposition). The surface deposition of calcium carbonate crystals decreased the water absorption with 85% depending on the porosity of the specimens (Muynck et al., 2008).

Microbial calcite precipitation was quantified by XRD analysis and visualized by SEM (Ramachandran et al., 2001). The specimens with bacteria did not develop any micro cracks, as they did not expand much unlike control specimens when subjected to alkali aggregate reactivity, sulfate attack, drying shrinkage and freeze-thaw.

MATERIALS AND METHODS

Isolation

Calcium carbonate precipitating strains were isolated from Rhizopheric soil (tulsi plant) and alkaline soil. The samples were suspended in a sterile saline solution (0.85% NaCl), diluted properly and plated on precipitaion agar containing urea (20 g/l), $NaHCO_3$ (2.12 g/l), NH_4Cl (10 g/l), Nutrient broth (3 g/l), $CaCl_2.2H_2O$ (25 g/l). Incubation was done at 28°C. Colonies were assessed every 5 days with a stereo microscope (Zeiss) and selected as positive based on visual crystal formation within 10 days. Positive isolates were purified through repetitive dilution and plating as described earlier.

Microscopy and crystal nucleation site development

There is considerable geological evidence that micro organisms function as crystal nucleating agents during mineral precipitation. Apart from the metabolic processes, microbes also specifically catalyse the nucleation of calcium carbonate by reducing the required activation energy barrier (for nucleus formation). The essence of this catalysis is the surface of the heteronucleus (in case of the bacteria) should match well with the crystal or can say the interfacial energy between the bacteria and the crystal should be smaller than the interfacial energy between the crystal and the solution (Warren and Haack, 2001). Crystal precipitating colonies were studied after 5 and 10 days cultivation with stereomicroscopy. Digital images were captured with a CCD (charged coupled device) camera. Large crystal aggregates that is precipitated within a single colony of these isolates were subsequently harvested from the agar surface, washed in sterile water and dried (28°C, 3 days). The dried aggregates were ground to be appropriate particle size for XRD analysis, using a McCrone micronising mill. The grounded samples were then mounted in a sample holder and analysed (Xpert software).

Gram staining

Gram staining method was used to determine the morphology of the bacterial strains. Slide with a bacterial smear was placed on a staining rack. The slide was stained with crystal violet for 1 to 2 min and then the slide was flooded with Gram's iodine for 1 to 2 min. Decolourization was done by washing the slide slowly with acetone (2 to 3 s). Slide was then thoroughly rinsed with water to remove the acetone. The slide was flooded with safranin counter stain for 2 min and then washed again with water. The excess water was blotted and then dried in hand over Bunsen flame (Bergey et al., 1994). In Gram-positive bacteria, the dark purple crystal violet stain was retained by the thick layer of peptidoglycan and the Gram-negative bacteria, the thin peptidoglycan layer in the periplasm does not retain the dark stain and the pink safranin counter stain stains the peptidoglycan layer. Eight isolates showed Gram positive character while only two isolates showed Gram negative characters. Furthermore, for the confirmation of the streaking on EMB agar (Eosin methylene blue agar) (Sigma-Aldrich) was also done which showed the same observation as only 2 that was isolated showed growths on EMB, which indicated that stains are Gram negative. EMB medium is partially inhibitory to the growth of Gram positive bacteria and thus, Gram negative growth is most abundant.

Urease activity

All the isolates were tested for urease activity. This was done by streaking the purified cultures on urease test agar (Himedia) and inoculating test broth with viable liquid cultures as well as filtrates of the liquid culture.

Phenol hypochlorite assay method

The urease positive isolates were further tested for the urease activity. This was determined in the media according to the Phenol hypochlorite assay method. Ammonium chloride (50 to 100 µM) was used as standard. The culture filtrates (250 µl) were added to the mixture containing 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1 M). The mixture was incubated at 37°C for 5 min followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 min. Optical density was measured at 626 nm and one unit of urease is defined as the amount of enzyme hydrolyzing 1 µmol urea/min (Natarajan, 1995).

SEM and XRD analysis

The morphology and chemical constituents of the bacteria were analyzed with SEM and XRD. Samples were completely dried at room temperature, and then examined at accelerating voltages ranging from 30 to 35 kV by an SEM (Zeiss EVO50). Samples were gold coated with a sputter coating Emitech K575 prior to examination. XRD spectra were obtained using an X'Pert PRO diffractometer with a Cu anode (40 kV and 30 mA) and scanning from 3 to 60°. Each bacterial sample was crushed and ground before mounting onto a glass fiber filter using a tubular aerosol suspension chamber (TASC). The components of the sample were identified by comparing them with standards established by the International Center for Diffraction Data. All experiments were performed in triplicate.

EDX analysis of ureolytic isolates

Mineral constituents of the isolates were further characterized by EDX analysis. Presence of high amounts of calcium in the bacterial isolates confirmed the presence of calcite in the form of calcium carbonate. The isolates were grown at a higher rate in the presence of oxygen and consequently induced active precipitation of calcium carbonate around the surface area.

RESULTS

Isolation of calcium carbonate producing bacteria

Presence of urea in the media (composition of media: urea (20 g/l), $NaHCO_3$ (2.12 g/l), NH_4Cl (10 g/l), Nutrient broth (3 g/l), $CaCl_2.2H_2O$ (25 g/l) hydrolyzes the media (due to the occurrence of the urease enzyme) which in turn increases the pH, as it (bacteria) utilizes urea as a nitrogen source and also as a source of energy. The addition of urea and calcium chloride in the medium supports the microbial growth. The bacterial cell surface with a variety of ions could not specifically induce mineral depositions by providing nucleation site (Ferris et al., 1986, 1987). In this study, out of 10 strains which were isolated in the laboratory strains 3, 4 and 5 were found to be best on the basis of calcite formation. All the strains have utilized the urease obtained from Jack bean. Hydrolysis of one mole of urea results in the release of two molecules of ammonia and one mole of carbon dioxide.

Growth profile of ureolytic bacteria

The growth profile studied up to 120 h. It was observed from graph that in strains 1 and 2 the optical density has increased up till 48 h which is 0.65 and 0.601 respectively which keep on decreasing up till 120 h linearly, whereas in strains 3, 4 and 5, the optical density kept on increasing till 120 h. The maximum growth observed 1.084 in strain 4 followed by strains 5 and 3 which is 1.013 and 0.867 respectively (Figure 1). The pH of the medium was significantly increased with the increase in growth of these isolates. The ability to grow at high pH by strains 3, 4 and 5 suggests that it can be used in building materials such as cement to enhance the calcite precipitation where the pH of the proximal environment is highly alkaline (pH 11 to 12) (Achal et al., 2009). In most cases, the active carbonatogenesis seems to start first and to be followed by passive one, which induces the growth and shape modifications of initially produced particles that how the bacteria were encased within the calcium carbonates systematically (Figure 2) (Castainer et al., 2000).

Crystal nucleation site development

Based on morphological differences, ten isolates were selected for further study. It was observed that large crystal aggregates were precipitated within single colonies on the precipitation agar. The isolates were termed calcium precipitating bacteria (strains 1 to 10). Morphologically, distinct groups of crystal aggregates were distinguished. A total of 10 isolates were selected based on visual differences in the precipitate morphology. Strains 1 to 5 all produced large, white crystals within 20 to 48 h with aggregates taking up as much as 98% of the total colony surfaces (Figure 3a to e). Strains 6 to 7 precipitated at similar rates but produced whitish and transparent crystal aggregates. Strains 8 to 10 precipitated at noticeably slower rates (about 3 to 5 days for crystallization). Crystal precipitating colonies were studied after 5 and 10 days cultivation with stereomicroscopy. Digital images were captured with a CCD camera. Large crystal aggregates that precipitated within a single colony of these isolates showed crystal nucleation site development (Figure 3a to j).

Urease activity

A change in coloration following incubation (5 days at

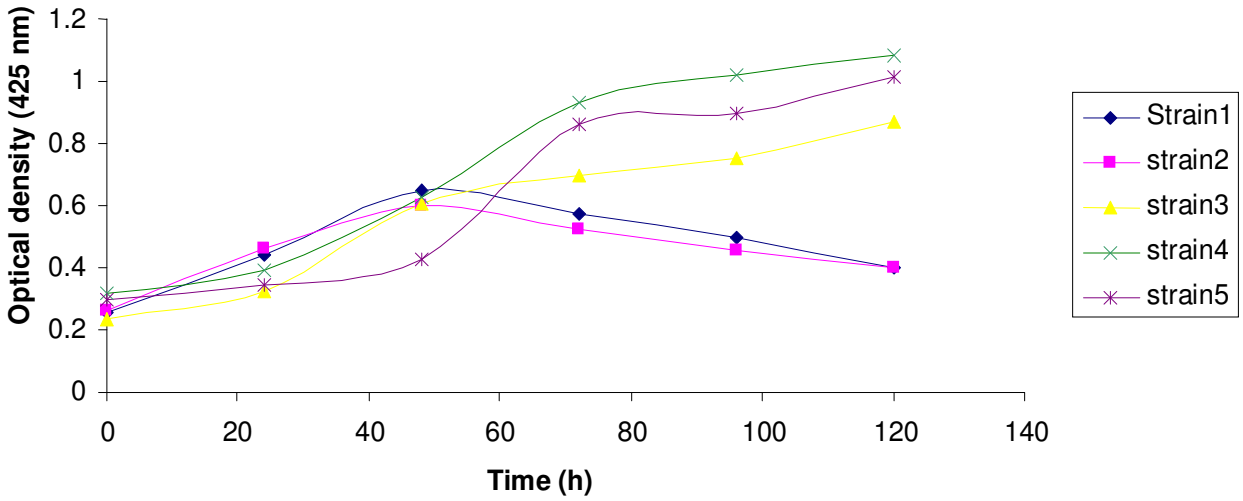

Figure 1. Growth profile of different Bacterial strains.

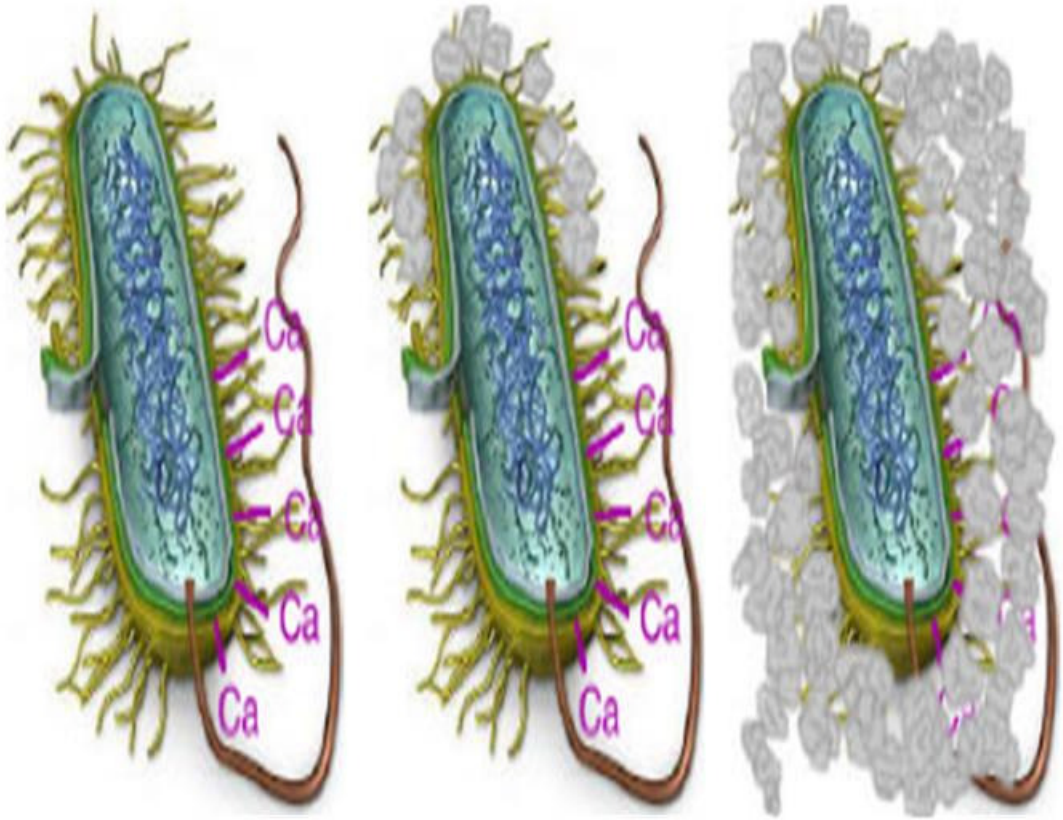

Figure 2. Calcium carbonate formation on cell wall of bacteria (Castainer et al., 2000).

28°C) on urease agar and urea broth was recorded as a urease positive reaction. In all cases, urease activity was cell associated. When organisms utilize urea, ammonia is formed and this in turn makes media alkaline which then produces red pink colour (Figure 4a to j). The strain specific ureolytic activity and found some strains have high urea affinities whereas some have lower affinities (Natarajan, 1995). By using Phenol hypochlorite assay method, it was estimated that strains 3, 4 and 5 showed maximum urease activity. The highest productivities in all media were obtained in 120 h. After 120 h, urease production was decreased in the biomineralisation media

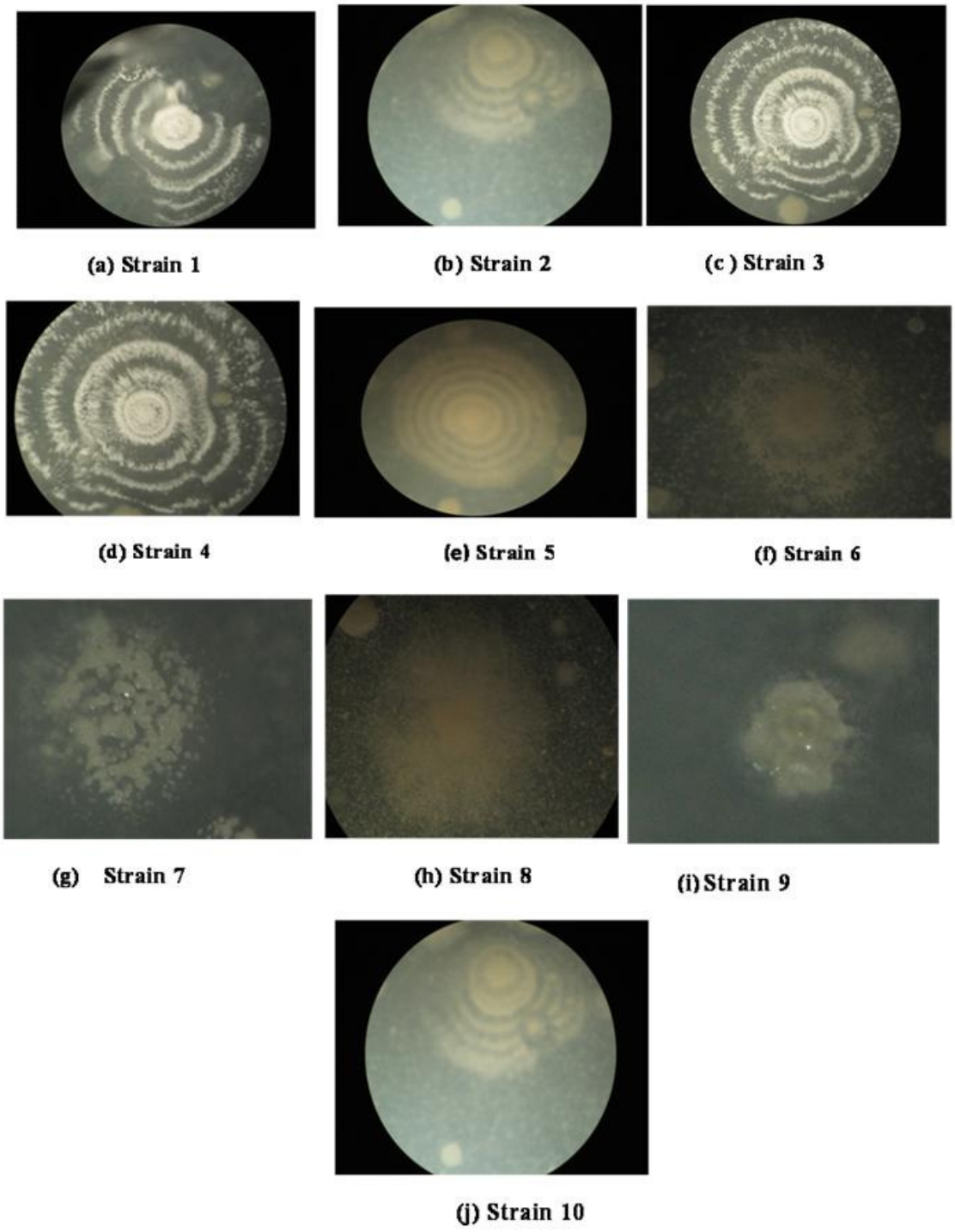

Figure 3. Strains (a to j): Stereomicroscopic images of calcium carbonate crystals precipitated within bacterial colonies on semi-solid growth media and classed according to morphological differences.

(Figure 5).

SEM and XRD analysis of bacterial isolates

To determine the presence of microbial calcite precipitation, the pure bacterial cultures were examined under SEM. Strains 3, 4 and 5 was found to produce the maximum urease activity. So, these strains were further chosen for SEM and XRD. The SEM analysis revealed distinct calcite crystals embedded with bacteria. This indicated that the bacteria served as the nucleation sites

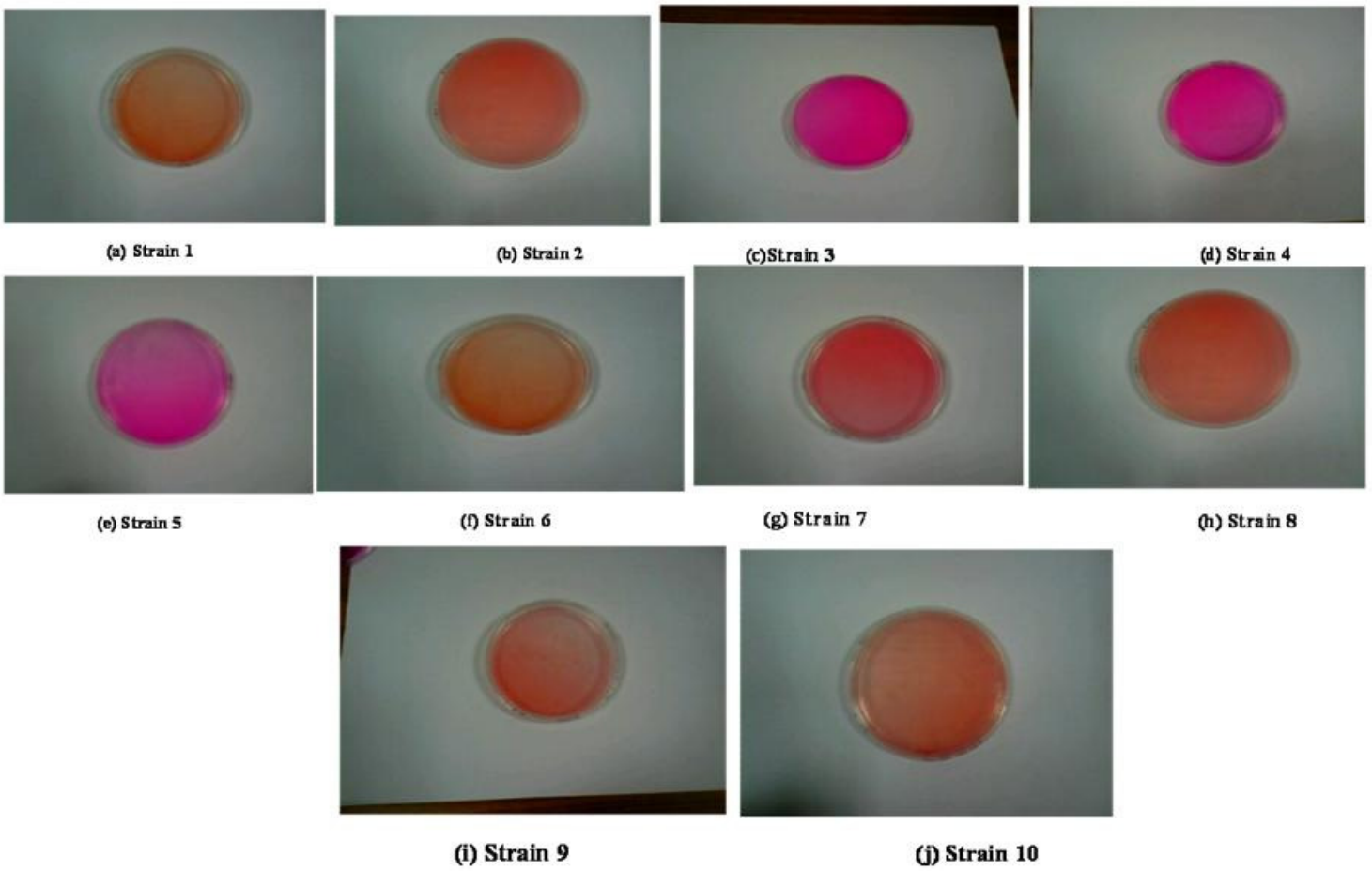

Figure 4. Strains (a to j): A change in coloration by bacterial isolates on urease agar.

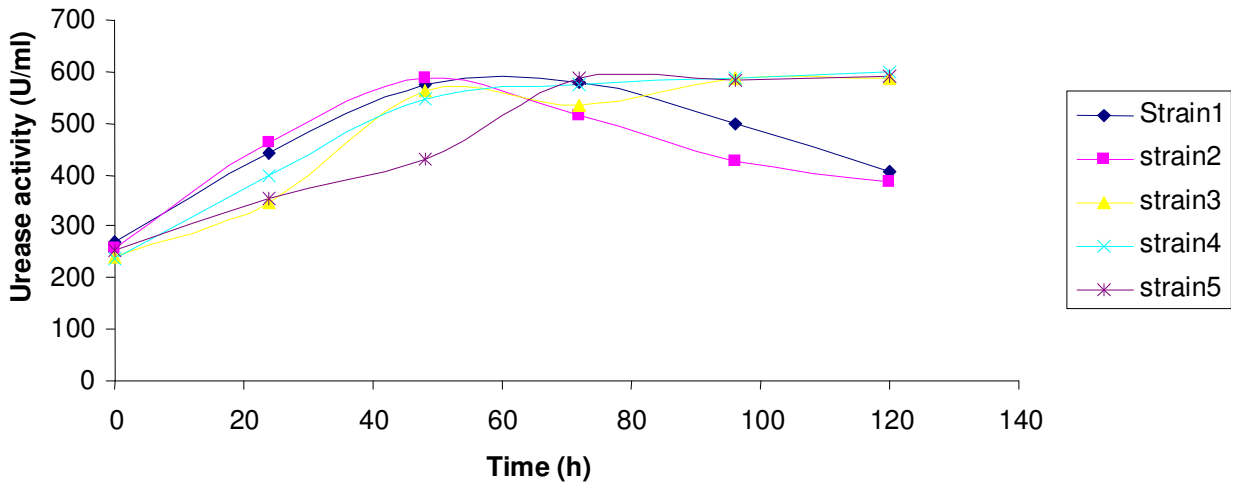

Figure 5. Urease activity of different bacterial strains.

for the mineralization process. High calcium amounts in all the bacterial samples confirmed that calcite was present in the form of calcium carbonate. The presence of crystalline calcium carbonate associated with bacteria indicated that bacteria served as nucleation sites during mineralization process (Achal et al., 2009). For further

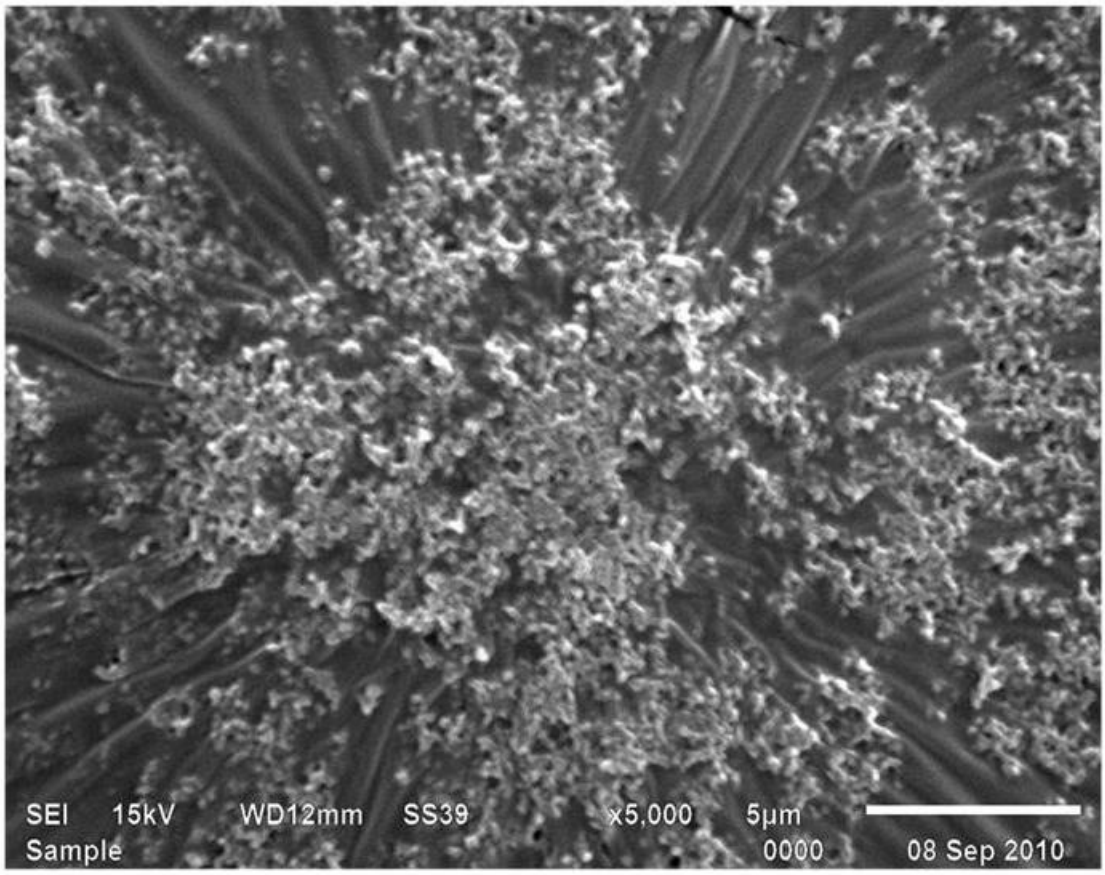

Figure 6. SEM images of strain 1.

confirmation of the carbonate deposits as calcite crystals, XRD analysis was performed. Rhombohedral crystals characteristic for calcite was present in Figures 6 to 10. The influence of the calcium source was limited to the morphology of the crystals. The presence of chloride ions indicated rhombohedral crystals while the presence of acetate ions resulted in spherical ions (Muynck et al., 2008). The increase in the urease activity was observed in strains 3, 4 and 5 which determined a significant growth of urease production. These results suggest that strains 3 and 5 can be used commercially for the crack remediation process in buildings. Bio-mineralization process by *B. pasteurii* by SEM and XRD (Figures 11 to 13) and indicated that calcite is the dominated mineral phase form when bacteria are present (Sarda et al., 2009; Lian et al., 2006).

EDX analysis of bacterial isolates

The isolates which were found to produce the maximum urease activity were strains 3, 4 and 5. Hence, these strains were further analysed by EDX spectra (Figures 14, 15 and 16 respectively). The presence of crystalline calcium carbonate associated with bacteria indicated that bacteria served as nucleation sites during the mineralization process. The maximum amount of calcium was found to be (in weight %) 52.54%, strain 3, 46.42% in strain 4 and 42.25% in strain 5 which was clearly indicated by EDX spectra. The majority of the carbonate deposits were present as calcite crystals as confirmed by XRD analysis. Achal et al. (2010) also studied the microbial concrete, which showed it as a novel strategy to restore or remediate concrete structures by using *Bacillus* species. From these results, it can be concluded that these strains are more efficient with respect to calcite precipitation.

DISCUSSION

Numerous diverse microbial species participate in the precipitation of mineral carbonates in various natural environments including soils, geological formations, fresh water biofilms oceans and saline lakes (Peckman et al., 1999; Rivadeneyra et al., 2000). The mechanism of urease in which an organism creates a local micro-environment with conditions that allows optimal extra-cellular chemical precipitation of mineral phases (Hamilton et al., 2003). Bacterial community has very limited

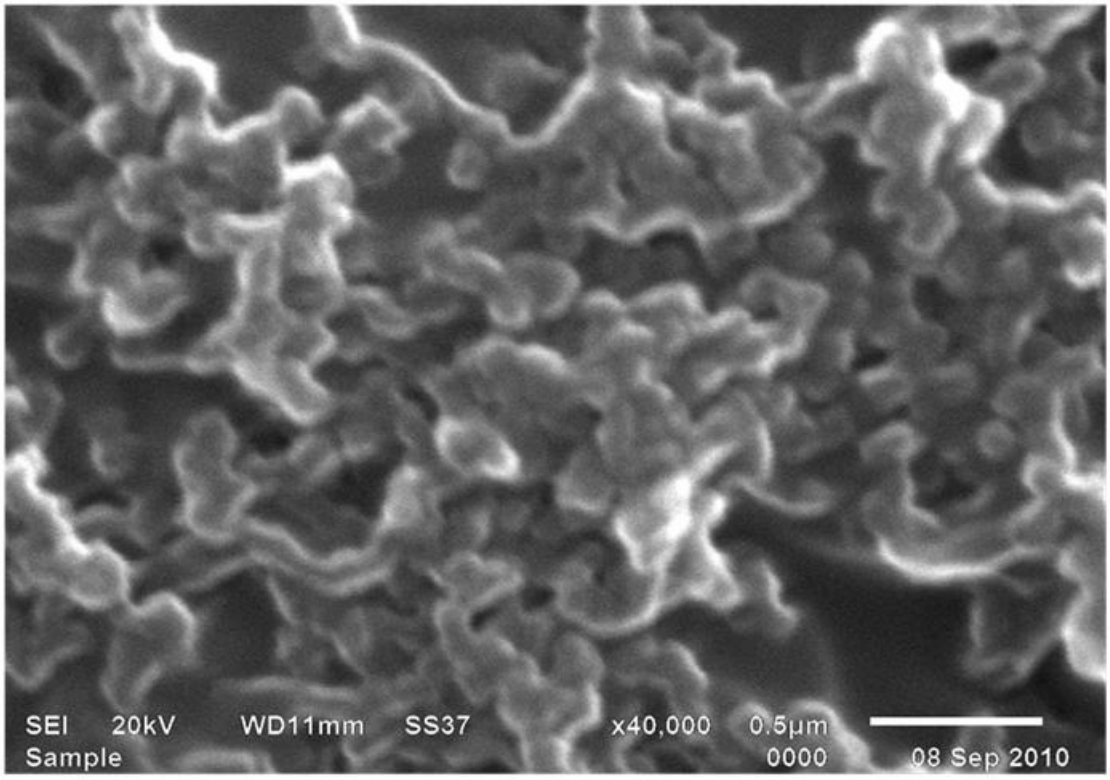

Figure 7. SEM images of strain 2.

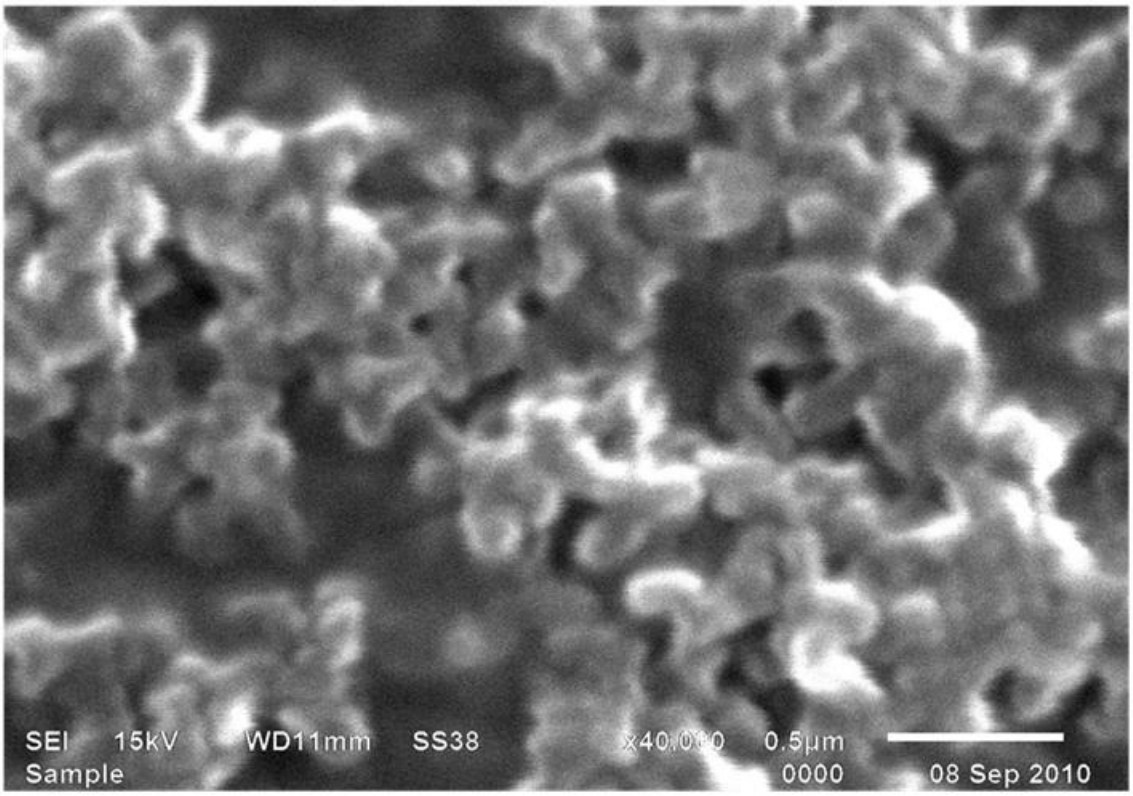

Figure 8. SEM images of strain 3.

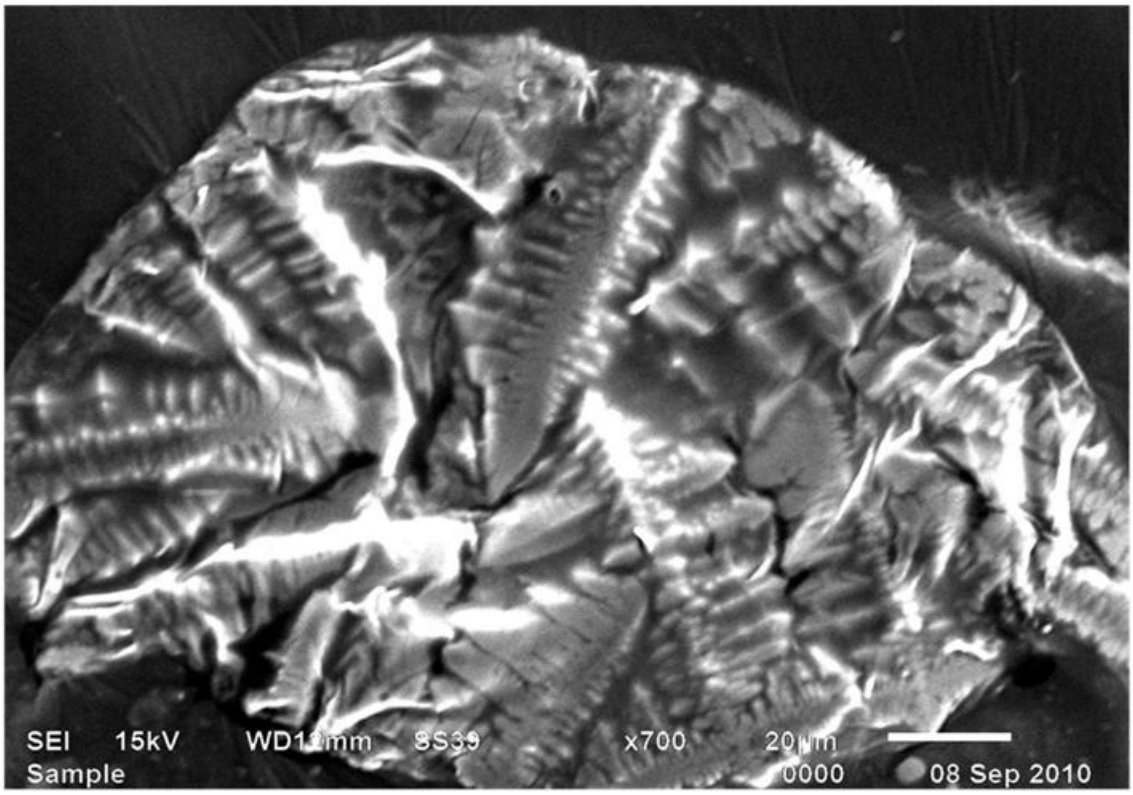

Figure 9. SEM images of strain 4.

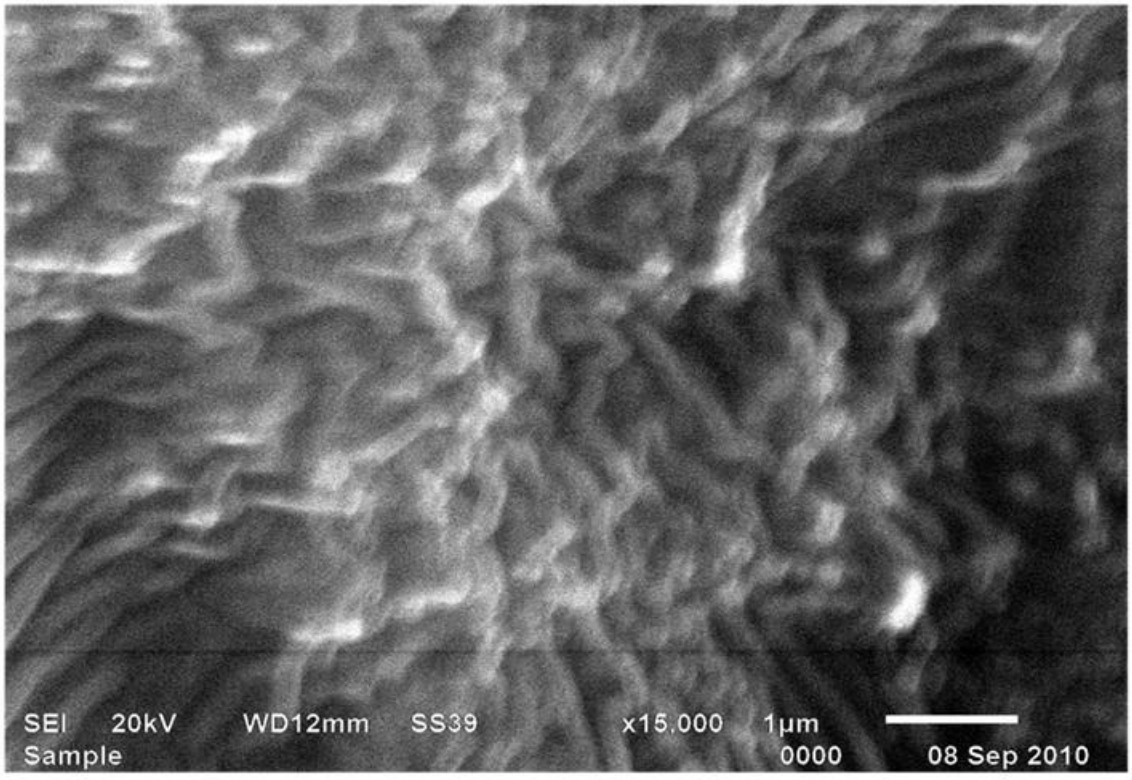

Figure 10. SEM images of strain 5.

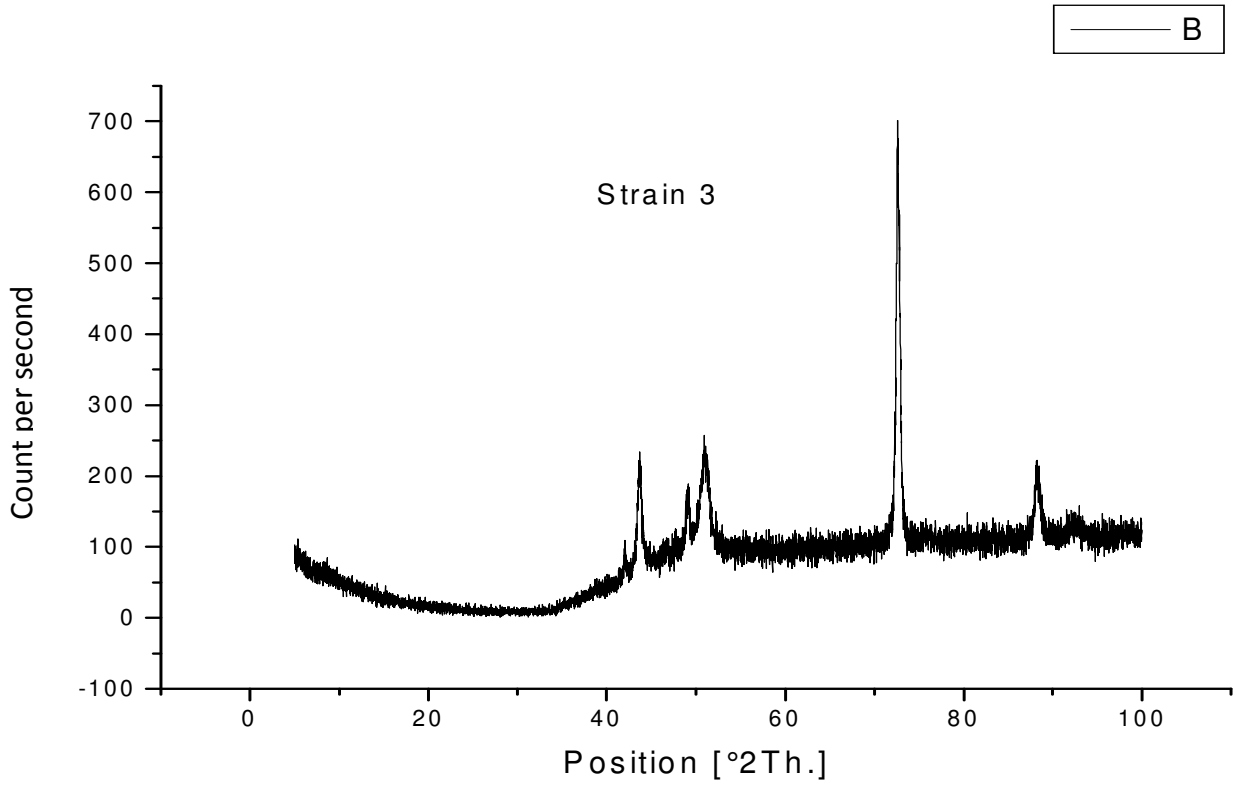

Figure 11. XRD analysis for strain 3.

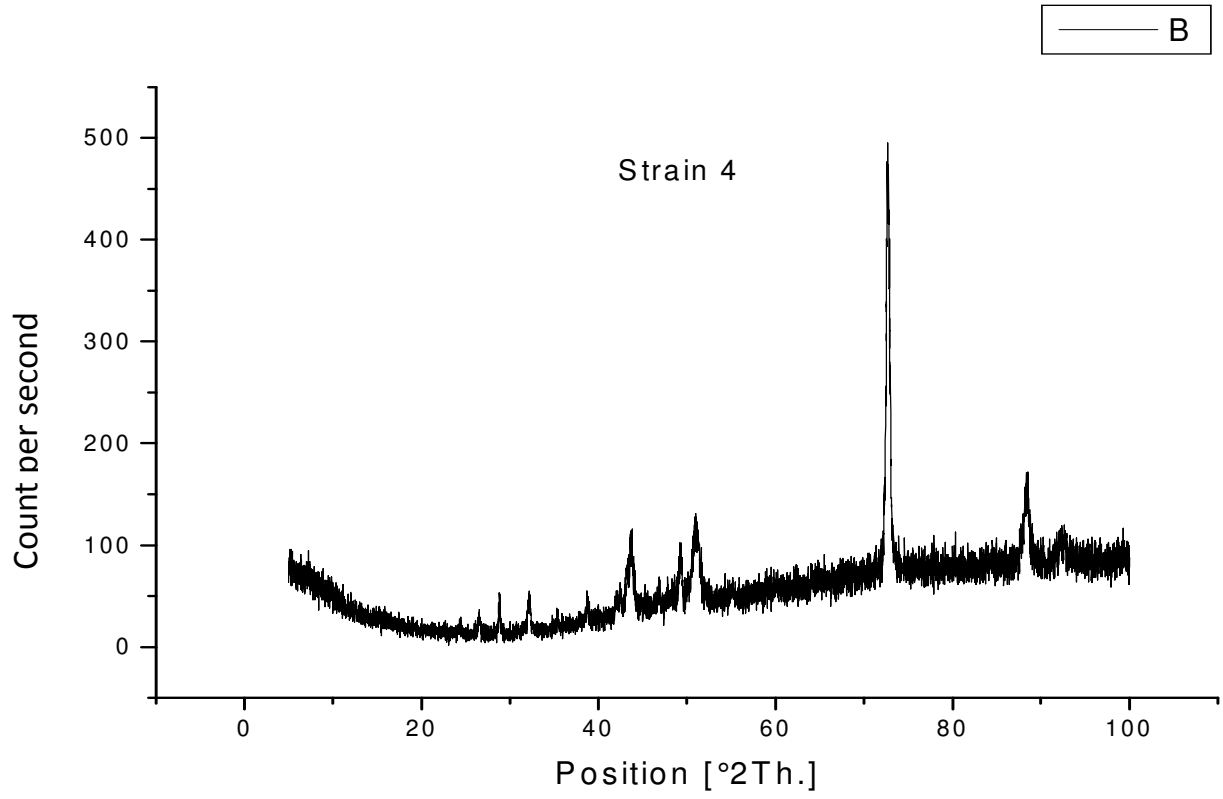

Figure 12. XRD analysis for strain 4.

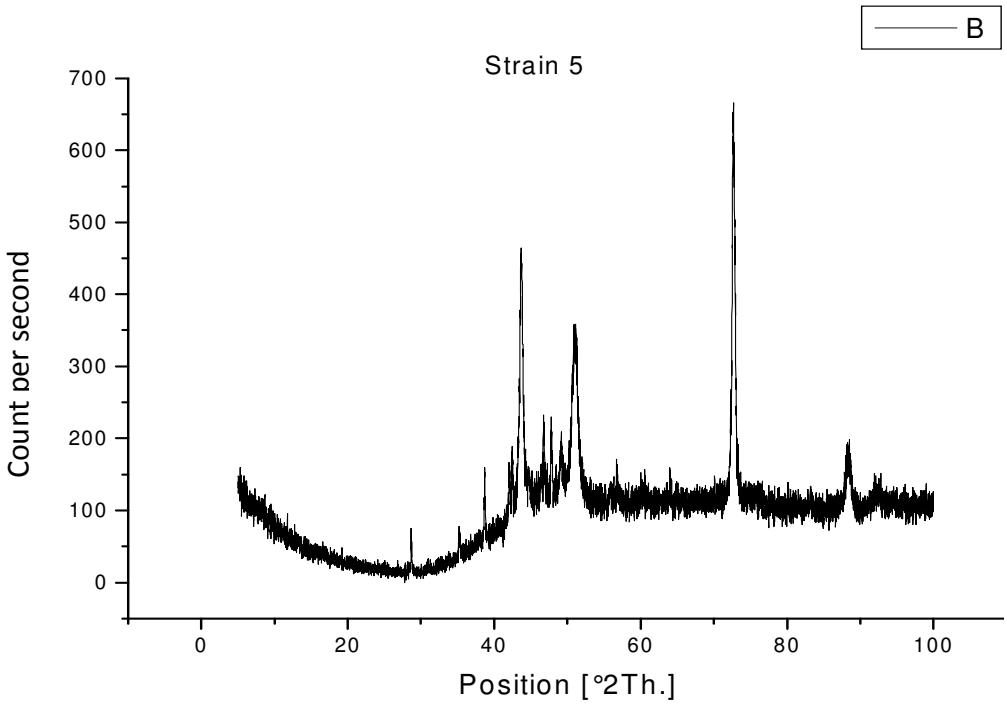

Figure 13. XRD analysis for strain 5.

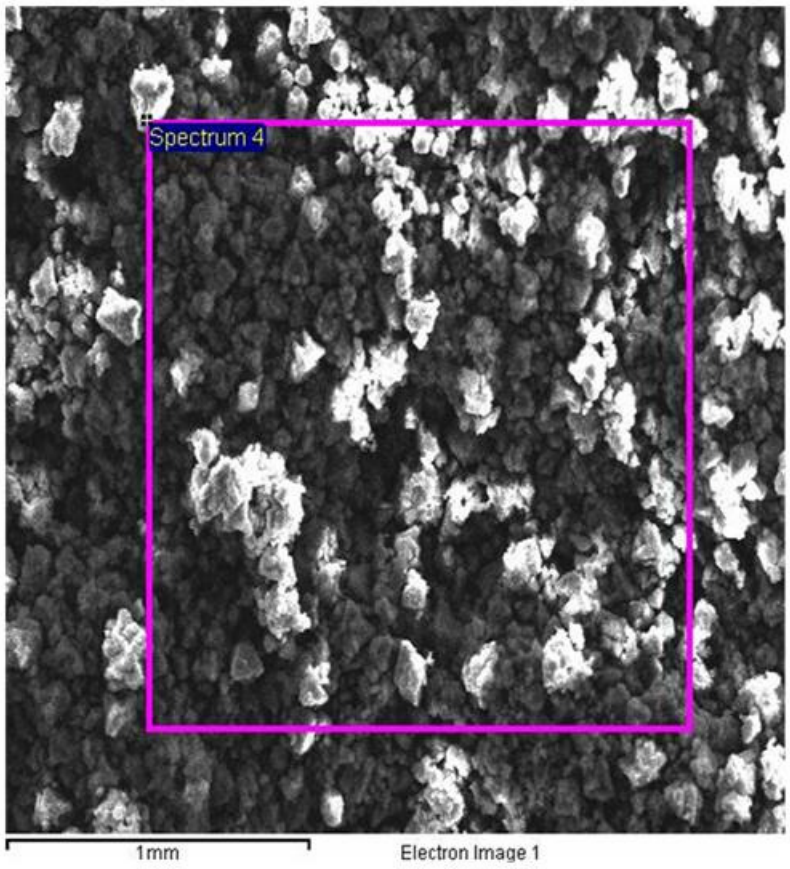

Figure 14. EDX spectra of strain 3.

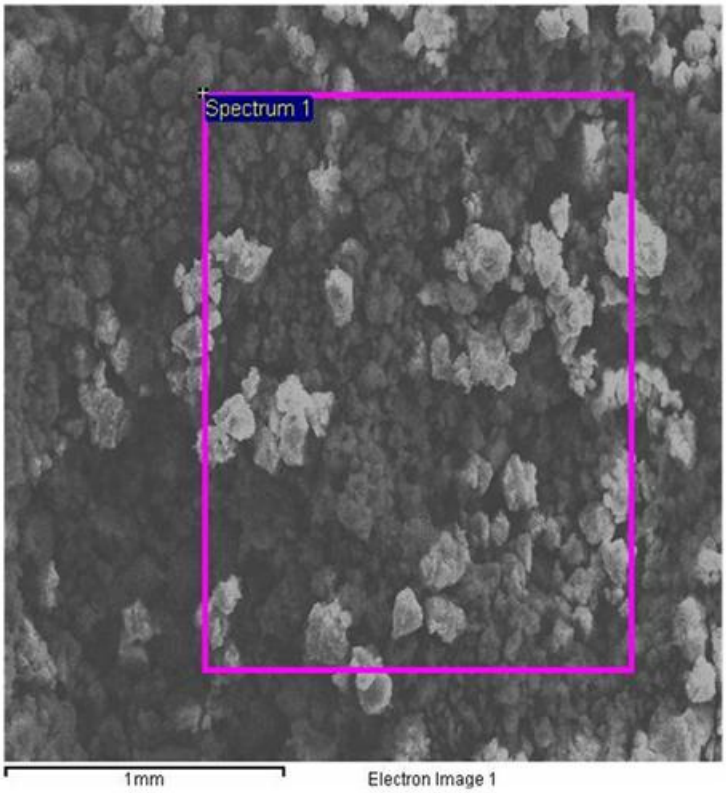

Figure 15. EDX spectra of strain 4.

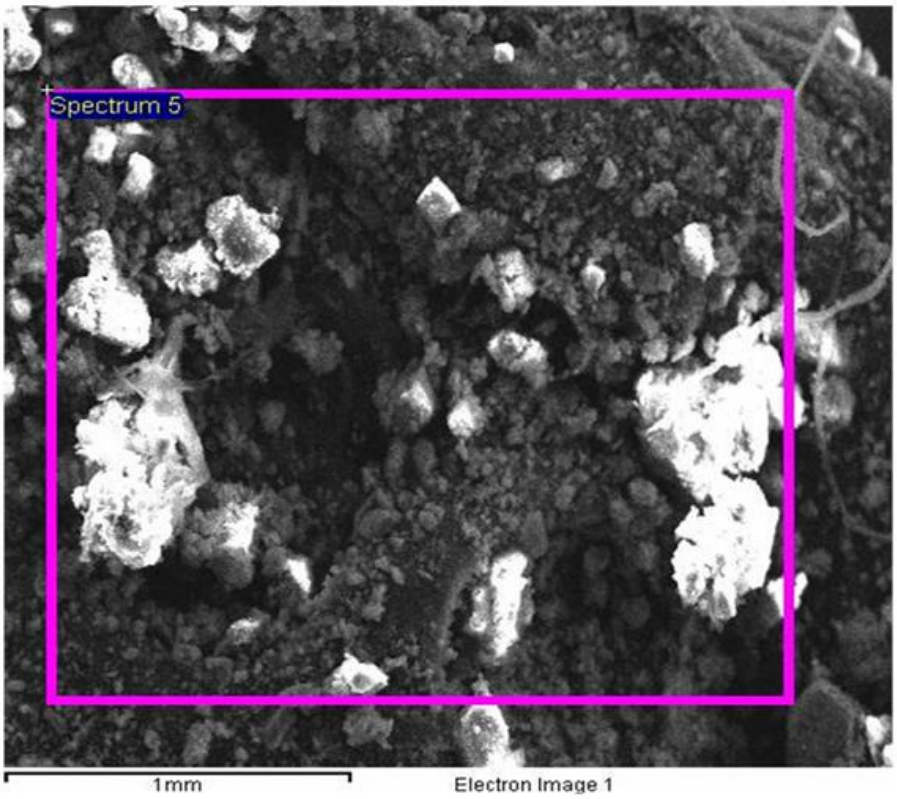

Figure 16. EDX spectra of strain 5.

diversity that can withstand extreme alkaline condition. Urease producing microorganisms were selected on the basis of their survival in alkaline environments. When microorganisms utilize urea, ammonia is formed during incubation that makes the reaction of media (composition explained in isolation earlier explained) alkaline, producing a red pink color due to the presence of phenol red; a pH indicator. Urea test agar (Himedia) in general was used for the selection of bacterial strains. In this mechanism, 1 mol of urea is hydrolyzed intracellularly to 1 mol of ammonia and carbamate:

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

These products of (1) and (2) subsequently equilibrate in water to form bicarbonate and 2 mol of ammonia and hydroxide ion: $H_2CO_3 \rightarrow HCO^{3-} + H^+$ and then: $2NH_3 + 2H_2O \rightarrow 2NH^{4+} + 2OH^-$ (pH increase). This reaction give rise to a pH increase which creates bicarbonate equilibrium: $HCO_{3-} + H^+ + 2OH^- \rightarrow CO3^{2-} + 2H_2O$ (Stocks et al., 1999). This equation shows the increase in pH initially in the local environment around the bacterial cell and propagates in the bulk solution of bacterial cell suspension. Thus, the carbonate concentration will increase. $CO_3^{2-} + Ca^{2+} \rightarrow CaCO_3$ ($K_{Sp} = 3.8 \times 10^9$); K_{Sp} the solubility product. So, the driving force for precipitation of calcium carbonate is the super saturation level S which is defined by the ration of the ionic product. The formation of calcium carbonate is when the solubility product exceeds the solubility, or can say it based on the available amount of calcium or carbonate ions:

$$S = (Ca^{2+}) \times (CO_3^{2-})/K_{So}$$

The process of precipitation is a complex mechanism. This mechanism is a function of the cell concentration, ionic strength and the pH of the medium. The media for the growth of the micro organisms are supplemented with a calcium source such as calcium chloride which is precipitated as calcium carbonate. The high pH of the localized areas without any initial increase in pH in the entire medium, commences the growth of calcium carbonate crystals around the cell (Stocks et al., 1999). Possible biochemical reactions in urea-$CaCl_2$ medium to precipitate "calcium carbonate" at the cell surfaces as follows:

$$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+} + Cl^- + HCO^{3-} + NH_3 \leftrightarrow NH_4Cl + CO_3^{2-}$$

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

In Figure 5, it was observed in strains 1 and 2 that the maximum urease activity was found to be 578 and 512 U/ml respectively in 72 h which kept on decreasing up till 120 h in biomineralization media. The growth profile of bacterial strains which were isolated on the basis of calcium carbonate production was studied and it was observed that bacterial growth was divided into 4 stages: The first log phase where growth and reproduction will occur. During the second log phase, reproduction occurs at an exponential rate, leading to a mass production of carbonate and hydrogen carbonate. It is the stage where actual calcium particles are formed. In the third phase stationary phase, reproduction goes on but not with the same speed as in the log phase. Finally, in the death phases, where the bacterial cell started dying and calcium production also started decreasing (Figure 1). Same trend was found in growth curve of these two strains 1 and 2 which indicates as the multiplication of bacteria decreases after 72 h, even the urease activity also decreased. Different pure culture of *B. pasteurii*, *B. lentus*, Bervibacterium ammonigens and observed maximum urease production in *B. pasteurii* followed by *B. lentus* and Bervibacterium ammonigens (Sarda et al., 2009).

The maximum urease activity was found to be 589, 598 and 593U/ml in 3, 4 and 5 respectively in 120 h after which there was marginal increase due to which the experiment was terminated after 120 h (Figure 5). The percent increase was found in 54.68, 58.5 and 58.9% in strain 3 in 72 to 120 h. In strain 4, 59, 59.7 and 60.5% and in strain 5 it was 56.72, 56.4 and 57.2% which allows the termination of the experiment. The maximum number of calcium carbonate peaks was observed in strains 3, 4 and 5. So; from the aforementioned results it was concluded that strains 3, 4 and 5 were more efficient than other isolated strains that were strains 1 and 2 with respect to calcium carbonate precipitation. Besides calcium carbonate, small amounts of vaterite were also present on closer observation; spherical deposits (vaterite and fluorite) were visible. The bacterial strains were further analysed by EDX spectra and it was concluded that presence of crystalline calcium carbonate associated with bacteria indicated that bacteria served as nucleation sites during the mineralization process. The potential of bacteria to act as self healing agent in concrete has proven to be a promising future. As presently, about 8% of atmospheric carbon dioxide emission is due to cement production, mechanisms that would contribute to longer service life of concrete structures would make the material not only more durable but also self repair, that is the autonomous healing of cracks in concrete. This field appears to be more beneficial as bacterial concrete appears to produce more substantially more crack plugging minerals than control specimens (without bacteria). A promising sustainable repair methodology technique based on the application of mineral producing bacterial strains for ecological engineering purposes is becoming increasingly popular. This is reflected by recent studies where bacteria were applied for the removal of chemicals from waste water streams (Gross et al., 2007) for bio-

remediation of contaminated soils (Chaturvedi et al., 2006) and removal of green house gases from landfills (Jugnia et al., 2008). The applicability of specifically mineral producing bacteria for sand consolidation and limestone monument repair (Gollapudi et al., 1995) and filling of pores and cracks in concrete have been recently investigated (Muynck et al., 2008). In all these studies so far, bacteria or derived ureolytic enzymes were externally applied on cracked concrete structures or test specimens, that is as surface treatment or repair system. An integrated healing agent would save manual inspection and repair and moreover increase structure durability. Addition of such an agent to the concrete mixture would thus save both money and the environment as less maintenance and use of environmental friendly repair material is needed. Microbial carbonate precipitation (biodeposition) decreases the permeation properties of concrete. Hence, a deposition of a layer of calcium carbonate on the surface of concrete resulted in a decrease of water absorption and porosity.

The ability of these bacterial strains to tolerate highly alkaline environments may have important implications for remediation of cracks and fissures in various concrete structures.

Conclusions

In this study, we studied the bacterial isolates on the basis of their urease activity, calcite precipitation and survival at higher pH. The bacterial isolates which showed increased urease activity, calcite precipitation and survival at higher pH, could be used in the remediation of cracks in building materials.

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