

Microbial Carbonate Precipitation by Urease Producing Bacteria in Cementitious Materials

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ABSTRACT

Biocalcification, also known as microbiologically induced calcite precipitation (MICP), is a new phenomenon involving the activity of enzyme urease by living organisms. Urease positive bacteria are used to improve the overall strength, performance and behaviour of mortar specimens. The investigation has measured the variation in compressive strength and self healing of concrete using these bacteria. Nowadays a broad range of products are available on the market for the protection of concrete surfaces. Several of these products are organic coatings consisting of volatile organic compounds. The air polluting effect of these compounds during manufacturing and coating has led to the development of new formulations such as inorganic coating materials. In present study *Bacillus cohnii* and *Bacillus megaterium* were tested for their urease positive activity. Effect of nickel ion concentration on the growth of bacteria and effect on carbonatogenesis activity (calcite precipitation) was investigated varying the nickel ion concentration in culture medium. Different doses of bacterial biomass were added to find the optimum amount of bacteria (particular dose) to be used to obtain maximum compressive strength. Mortar specimens with *B. cohnii* and *B. megaterium* were casted to study their effect on the compressive strength. The result showed a significant increase in the compressive strength and calcite precipitation in bacteria incorporated mortar. The qualitative and quantitative analysis of mortar cubes was accomplished by performing SEM-EDX and XRD. The SEM micrograph showed that bacteria are precipitating calcium carbonate on the cell wall of bacteria. The EDX analyses showed enhanced calcium concentration in bacteria treated mortar specimens. The XRD analysis revealed that the calcite forming bacteria induced the crystallization of rhombohedral calcium carbonate. The increased calcite precipitation in mortar was visualized and quantified.

Keywords—Biocalcification, Microbiologically induced calcite precipitation (MICP), *Bacillus cohnii*, *Bacillus megaterium*, Nickel, Compressive strength.

INTRODUCTION

Bacteria from various natural habitats have frequently been reported to precipitate calcium carbonate both in natural and laboratory conditions. Different types of bacteria, as well

as abiotic factors (salinity and composition of the medium) seem to contribute in a variety of ways to calcium carbonate precipitation in a wide range of environments [1]. Bacteria

based self healing agent is believed to remain hibernated within the concrete for up to 200 years. When cracks appear on a concrete structure and water starts to seep in through, the spores of bacteria starts microbial activities. In the process of precipitating calcite crystals through nitrogen cycle the soluble nutrients are converting to insoluble calcium carbonate. The calcium carbonate solidifies on the cracked surface, thereby sealing it up. It mimics the process by which bone fractures in the human body are naturally healed by osteoblast cells that mineralize to reform the bone. The consumption of oxygen during the metabolic biochemical reactions to form calcium carbonate also helps in arresting corrosion of steel thereby increasing the durability of steel.

Use of bacteria based mineralization concept has lead to the potential invention of a new biomaterial that can fill the cracks and fissures. Bacteria are incredibly diverse and abundant and many bacterial species contribute to the precipitation of mineral carbonates in various natural environments. Urease is nickel dependent enzyme found in plants, bacteria and fungi that hydrolyses urea into carbon dioxide and ammonia thereby enhancing the pH of surrounding. Alkaline pH is the primary condition by which bacteria promote calcite precipitation. As a result of the negative charge or -ve potential of the bacterial cell surface, calcium ions bond to the cell wall. If high concentrations of calcium ions are available close to the bacteria and carbonate ions are present in super-saturation level; precipitation of calcium carbonate crystals will occur on the bacterial cell wall.

Concrete is the most widely used man made construction material. It has specialty of being cast in any desirable shape but plain concrete however possesses very low tensile strength, limited ductility and little resistance to cracking [2]. Repair, rehabilitation and

strengthening are the major part of the construction activity in the recent past. All building materials are porous and the porosity of building material along with ingress of moisture and other harmful chemicals such as acids, chlorides, and sulfates affect the material and seriously reduce their strength and service life. An additive that seals the pores and cracks and thus reduces the permeability of the structure would immensely improve its life [3].

Calcium carbonate is one of the most common minerals widespread on earth (4% by weight of the earth's crust). The rate of microbiological calcium carbonate precipitation correlated with cell growth and microbial rate of precipitation was significantly faster than that of chemical precipitation [4]. Calcite precipitation plays an important role in many geological processes, including early diagenesis of marine sediments, hydrochemical evolution of karst streams, formation of travertine and speleothem and the relevant global carbon cycle. Broth of bacterial culture is incorporated in mortar but a more approachable means to incorporate bacteria into mortar is to use dry biomass rather than using bacterial culture (suspension) as using former is much easier and practically accurate. There is a need to identify the bacteria having urease producing activity which in-turn can contribute to self healing properties in concrete. This paper deals with evaluation of bacteria for their urease positive activities, effect of addition of nickel ion on the growth of bacteria in mortar. The evaluation of different bacterial dosage have also been studied in terms of calcite precipitation and compressive strength of concrete.

II. MATERIAL AND METHOD

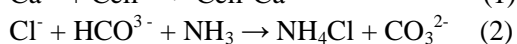
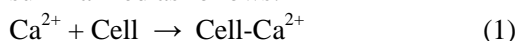
2.1 Bacterial sources

The cultures of *Bacillus cohnii* (MTCC 10221) and *Bacillus megaterium* (MTCC

10086) were obtained from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology, Chandigarh. Both the strains of bacteria which were used for investigation were found gram positive and endospore forming. The urease positive test was carried out using phenol red indicator, which change the color of urea based broth from yellow to pink. The bacterial strains were maintained constantly on nutrient agar slants and nutrient agar plates at pH 8.5 and were preserved in deep freezer (-80°C) All the inoculated plates were incubated at 30°C in Innova 44 shaker incubator. Colonies were observed after 24 and 48 hrs for *B.cohnii* and *B.megaterium*, respectively.

2.2 Media composition for growth of bacteria.

The bacteria were cultured in Himedia urea based broth. Calcium chloride hydrate was used as a source of calcium for precipitation of calcium carbonate. Aseptically 20g/liter of 40% urea and calcium source (calcium chloride) was also added after sterilization. Calcium carbonate precipitation is the end product to be visualized and the possible biochemical reactions (Eq. 1 2 & 3) in urea-calcium chloride medium to precipitate calcium carbonate at the cell surface can be summarized as follows:



2.3 Cement: Ordinary Portland cement of 43 grade was used. The cement has been tested for various properties as per IS: 4031-1988 [5] and found to be confirming to various specifications of IS: 12269-1987 [6].

2.4 Sand: Natural river sand well graded passing through 60 µm sieve was used. Specific gravity was found to be 2.63. Fineness modulus was found to be 2.1, water absorption 0.26% and confirming to zone III.

2.5 Water: All mortar mixing was done using ordinary water.

2.6 Preparation of bacterial biomass in powdered form

The bacterial culture obtained by inoculating bacterial cells in urea based broth. The optical density was taken on Janway7305 uv-vis spectrophotometer at 600 nm. The bacterial culture was then centrifuged on multifuge X3 centrifuge at 8000 rpm, 4°C for 20 minutes and pellets were dried to obtain biomass.

2.7 Viability test of bacterial biomass

The obtained bacterial biomass and bacteria isolated from 240 days old mortar were streaked on nutrient agar plates and incubated for 24 and 48 hrs for both *Bacillus cohnii* and *Bacillus megaterium* respectively. The colonies were visualized by trinocular research microscope.

2.8 Nickel ion concentration in culture medium

Urea based broth containing calcium chloride of 24.6 mM and 0.1M nickel chloride stock solution was prepared. The nickel ion concentration from 0 µM to 1000 µM was added to the broth prior inoculation of *Bacillus cohnii*. Addition of nickel ion to the broth was accompanied by inoculation of bacterial culture and incubation for 24-48 hours at 30°C in Innova 44 shaker incubator. Optical density of control (0 µM) and all the five concentration (5, 10, 100, 500, 1000 µM) of nickel ion containing bacterial culture was taken using uv-vis spectrophotometer at 600 nm in order to determine the particular concentration at which optical density and thus bacterial growth was found maximum.

2.9 Effect of nickel ion concentration on calcite precipitation using *Bacillus cohnii*

Ethylenediamine tetra acetic acid (EDTA) solution of 0.01M was prepared and standardized using 10.00 ml aliquot of standard calcium carbonate solution. 5 ml of aliquot of all the 6 samples of culture medium

(*Bacillus cohnii*) with or without nickel ion were titrated with EDTA solution to ascertain the amount of calcium carbonate precipitated. To the aliquot of taken sample, ammonia-ammonium chloride buffer was added followed by calmagite indicator. The EDTA titration was carried out up to end point i.e. from initial “wine red” color to an end point of “steel blue” and three reproducible titrations were obtained. The estimation of calcium ion using EDTA titration was carried out [7]. The result was also compared with XRF of samples.

2.10 Preparation of bacteria incorporated mortar cubes

Mortar cubes were prepared in 50.8 mm³ cube mould, composed of one part of cement, four parts of standard sand by mass and (P/4 + 3) per cent (of combined weight of cement and sand), water (where P is the percentage of water required to produce a paste of standard consistency) and prepared, stored and tested in the manner described in IS 4031: Part 6 (1988) [8]. Four different doses of bacterial biomass ranging from 0.25 to 1.0 gm were incorporated into mortar specimens. Control mortar specimens were casted without the addition of bacterial biomass. Casting was undertaken in two sets for two different strains, each set comprise of control specimens, biomass incorporated specimens and biomass and calcium lactate incorporated specimens for four different doses (0.25, 0.50, 0.75 and 1.0 gm) of biomass (incorporated in every specimen except control). In each set and for each dose (taken), total 36 cubes were casted for each dose, the first 12 were for control, 12 were used for biomass and another 12 were used for biomass and calcium lactate.

2.11 Standardized method for crack generation

Cracks of 0.3 mm width and 10-15 mm depth were generated on mortar cubes of dimension 50.8 mm³ through insertion of aluminum plate

(0.3 mm). The aluminum plate was inserted into the wet mortar mixture to a depth of 25 mm and removed [9] (Figure 1(i)).

Demolding was done after 24hrs. After demolding all specimens were cured in water at room temperature (27± 2°C) (Figure-1(ii)). The compressive strength of six cubes for each bacterial strain and calcium source was conducted using universal testing machine after 28 days of curing. The compressive strength test was conducted similarly for control.



Figure-1(i). Cubes with aluminum plate



Figure-1(ii). Cubes with cracks

2.12 SEM - EDAX Analysis

SEM-EDAX analysis was carried out on the broken samples of the mortar cubes after 28 days of curing. Samples were gold coated with a JFC-1200 fine coater prior to examination. SEM micrographs were obtained using a ZEISS Ultra- Plus FEG-SEM. The morphology of bacteria and remediated crack was analyzed with SEM at various magnifications [1x, 2x, 5x, 10x]. Calcite layer formed by the bacterial isolates were completely dried at room temperature and then examined by SEM.

2.13 XRD Analysis

The XRD analysis was employed to determine the crystalline form of the crystals and to determine chemical composition of the precipitate that occurred due to bacterial mineralization. XRD-spectra were obtained using Rigaku, DMax 2200 X-ray diffractometer with a Cu anode (40 kV and 30 mA) and scanning from 10 to 80° 2 θ. Calcite layer was crushed and grinded using motor pestle before mounting on to 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 Centre for Diffraction data.

III. RESULTS

3.1 Viability test and urease activity

Fresh bacterial biomass and bacteria isolated from mortar were streaked on nutrient agar plate showed colonies of *Bacillus cohnii* and *Bacillus megaterium* (Figure 2) indicating the viability of the bacteria in the dried form. Both bacteria were found urease positive as color of urea based broth change from yellow to pink.

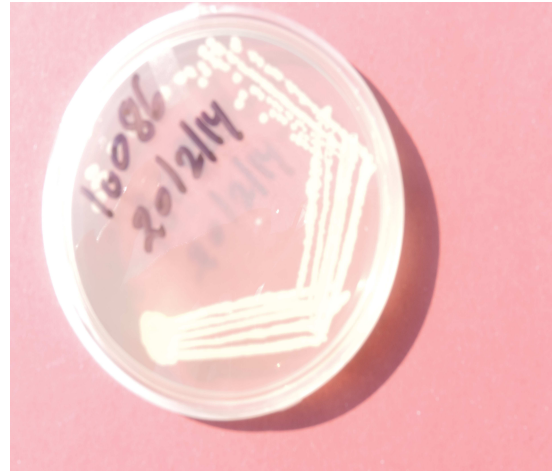
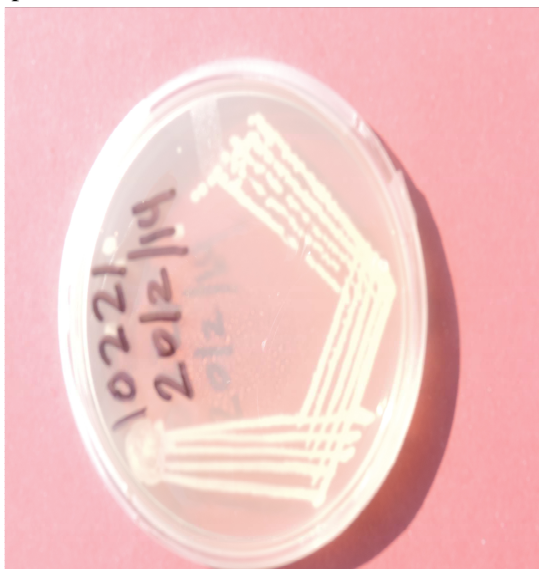
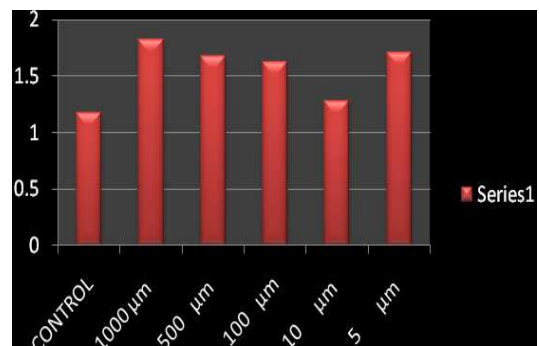


Figure 2 *Bacillus cohnii* (i) and *Bacillus megaterium* (ii)

3.2 Effect of nickel ion concentration in culture medium

Optical density readings of all the concentrations (of nickel ion) showed increased cell number with respect to control (Figure 3) indicating that nickel ion can act as growth enhancer. As increased bacterial growth means increased pellet and thus increased dry biomass in grams so by incorporating nickel ion, it is possible to extract more amount of dry biomass from culture medium in one batch as compared to the control. Further, incorporation of nickel ion served the purpose of increasing the urease activity as well as increased calcium carbonate precipitation as urease is nickel ion dependent enzyme.



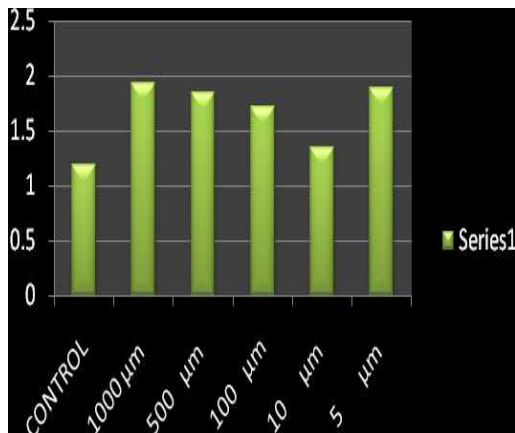


Figure 3. Optical density & nickel ion concentration (y&x) *B. cohnii* and *B. megaterium* after 24 and 48 hrs, respectively.

3.3 Effect of nickel ion concentration on calcite precipitation using *Bacillus cohnii*

The calcite precipitation was found to be higher in nickel ion containing culture medium in comparison to control sample. More specifically, to serve the purpose of increased calcite precipitation, nickel ion concentration of 5µm was found appropriate. The XRF of samples also confirmed the same finding.

3.4 Compressive strength

The compressive strength of investigations is showed in graphs. All the values are the average of the three trails in each case in the testing program of this study.

The compressive strength results of mortar cube test specimens with *Bacillus cohnii* and *Bacillus megaterium* in four that the compressive strength increases in bacterial mortar specimen as different doses are shown in Figure 5. The results of 28 days samples shows compared to control. Further, the mortar specimens with dry biomass and calcium lactate showed heightened increment in compressive strength in comparison to control and dry biomass containing



Figure 4. Universal Testing Machine

specimen. It was concluded that the addition of bacteria with dose of 0.5 gm increases the compressive strength to a value considerably greater than the other three bacterial biomass dose (0.25, 0.5, 0.75 and 1.0 gm).

The improvement in compressive strength by *Bacillus* sp. is probably due to precipitation of calcium carbonate on the bacterial cell surfaces and within the pores of cement–sand matrix, which plug the pores within the mortar. It is in agreement with the findings of other researchers [10,11,12,13]. The overall trend of an increase in compressive strength up to 28 days might be attributed to the behavior of bacterial cells within the cement mortar matrix.

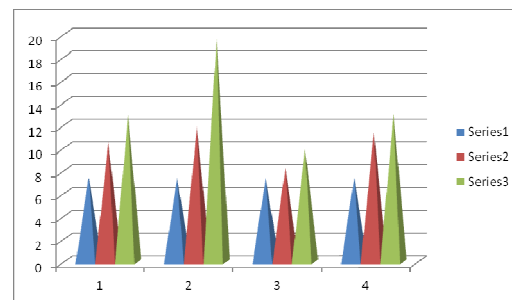
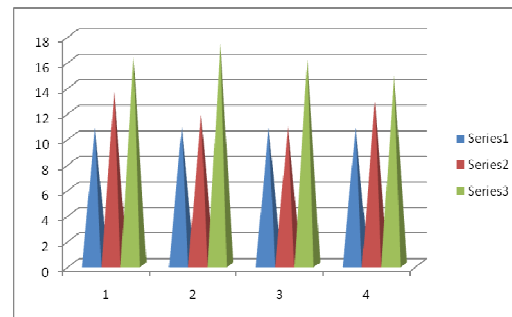
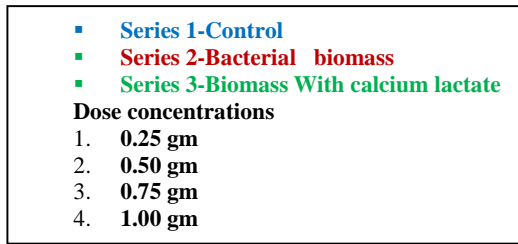


Figure 5. Comparative Compressive Strength



(Mpa) of Mortar at different doses

3.5 SEM- EDAX analysis

The enhancement in compressive strength of bacteria incorporated mortar specimens could be attributed due to the microbial calcite precipitation. The mortar samples were taken off and examined under SEM with various magnifications. Figure 6(i) is scanning electron micrograph of bacteria-free (control) mortar matrix indicating calcium% 12.03. mortar samples were taken off and examined under SEM with various magnifications. Figure 6(i) is scanning electron micrograph of bacteria-free (control) mortar matrix indicating calcium% 12.03.

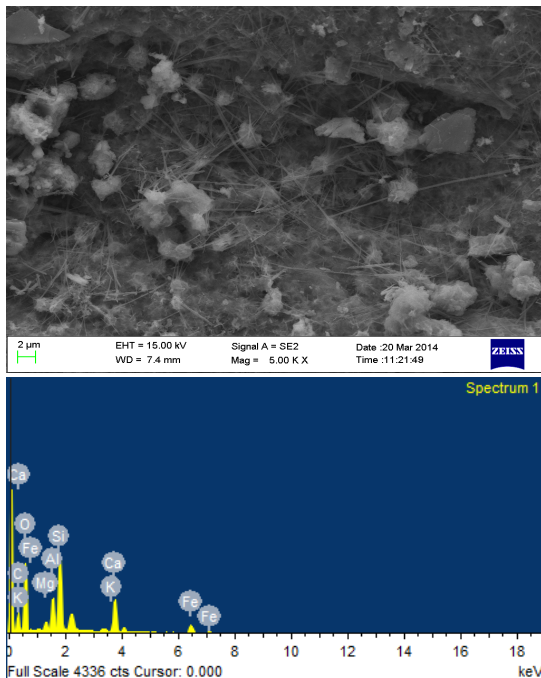


Figure 6(i): SEM and Energy-dispersive X-ray spectrum of control (untreated) specimen.

Fig.6 (ii) shows micrographs of the specimen prepared with *B.cohnii*. The sample showed calcite crystals grown on the cell wall of rod shaped bacteria. They had distinct and sharp edges, indicating a full growth of the crystals. The result is in agreement with other researcher [2] which showed that *Sporosarcina pasteurii* helped in calcite precipitation in sand column.

The presence of calcium was evident and the precipitation was inferred to as calcite (CaCO_3) crystal. Calcium in this treated specimen was found to be 26.26%.

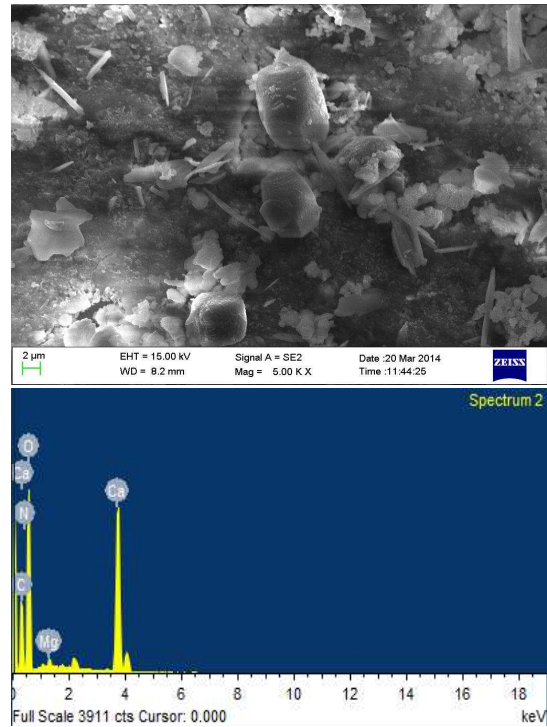
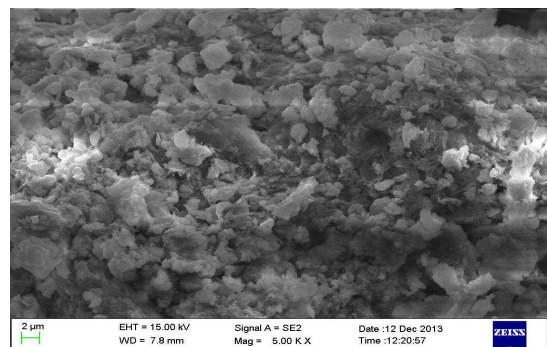


Figure 6(ii)SEM and Energy-dispersive X-ray spectrum of *B.cohnii* treated specimen



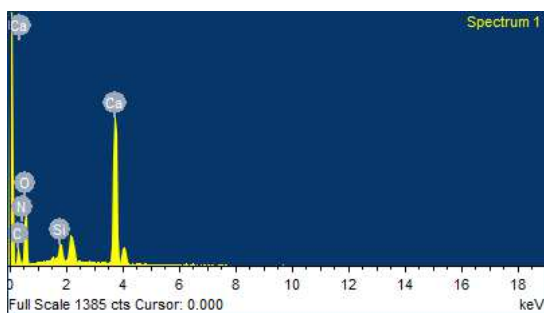


Figure 6(iii) SEM and Energy-dispersive X-ray spectrum of *B.cohnii* and calcium lactate treated specimen

Fig.6 (iii). showed the micrograph of bacterial biomass and calcium lactate. At 5x magnification, the fine structure of the crystallites is easily discerned and evident. Calcium % in this treated specimen was 42.19. The abundant presence of Ca was evident and the precipitation was inferred to as calcite (CaCO_3) crystal.

3.6 X-Ray Diffraction (XRD) Analysis

The sample was analyzed for its chemical characteristic by x-ray diffraction and result (Figure 7) indicated positively as calcium carbonate. The calcite forming bacteria are induced the crystallization of rhombohedral calcite.

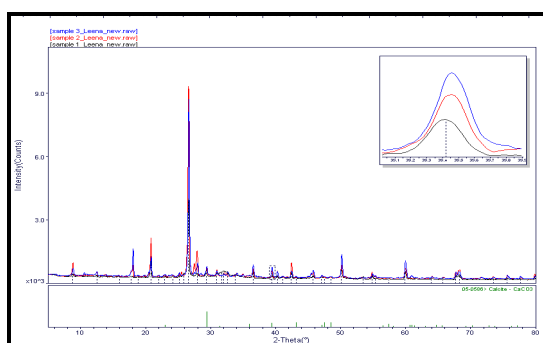


Figure 7. X-ray diffractogram of untreated, bacteria and bacteria with calcium lactate treated specimens.

CONCLUSION

The *B.cohnii* and *Bacillus megaterium* were found urease positive and able to precipitate

calcite within mortar specimen. Nickel as a promoter of urease activity has a profound effect on bacterial growth when incorporated in urea based broth prior to inoculation and the maximum absorbance was found at 1000 μm followed by 5 μm ascertaining that these are the most suitable concentrations for growth of *B.cohnii* and *B.megaterium*. Thus, it can be concluded that nickel ion can be used to enhance bacterial growth and being a co-factor of urease can be used to increase precipitation of calcium carbonate. In addition to this, a dose of 0.5 gm of the dry bacterial biomass can be used as a standard dose to fulfill the aim of crack filling as response (compressive strength) of 0.5gm of dose was found to be best than all other doses used.

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