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Original Research Article

Isolation of Cement Degrading Bacteria and Screening of Their Efficacy for Biocementation

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ABSTRACT

In this present investigation two bacterial strains were isolated from cement dumping site of RAMCO Cement factory, R.R.Nagar, Tamilnadu. These two strains were analyzed for based on their morphological, biochemical characteristics, the strain was identified as Bacillus species and Pseudomonas species. Bacillus species which growth in different cement concentrations as a sole nutrient source the maximum growth rate was observed at 3g supplementation of cement. The cement degrading bacteria Bacillus and Pseudomonas were studied on the effect of various parameters such as pH, temperature, incubation time, inoculum concentration. Gravimetric determination of cement degradation is a weight reduction process of cement cubes. In these Biodegradation, the maximum weight reduction was observed Bacillus in 72 hours (1.22g) and Pseudomonas in 72 hours (1.16g). Bioweathering action of silicates were tested for Ammonium molybdate yellow analysis method. These are measured at 400 nm. In Bacillus species the measurement of the bioweathering action of silicate is 0.68 and Pseudomanas in 0.72. In these production of 2-ketogluconic acid by bacterial isolate to evaluate silicate weathering. The hydrolysis of urea by the widely distributed enzyme urease is special that it is one of the few biologically occurring reactions that can generate carbonate ions without an associated production. When this hydrolysis occurs in a calcium rich environment calcite (Calcium carbonate) precipitates from solution forming a solid crystalline material. The binding strength of the precipitated crystals is highly dependent on the rate of carbonate formation and under suitable conditions.

Keyword: *Bacillus*; *Pseudomonas*; Bioweathering; 2-Ketogluconic acid; Biocementation

INTRODUCTION

Now a days, cement and concrete industries are huge. These industries can make many environmental issues such as Air pollution, Water pollution, Energy consumption and CO_2 emissions [1]. Cement dust released by building demolution and natural disasters can be a

major source of dangerous air pollution. The presence of some substance in concrete including useful and unwanted additives can cause health concerns due to toxicity and radioactivity. Cement is usually a grey powder before being mixed with other materials and water. Cement powder causes allergic reactions at skin contact and is irritating to skin, eyes and lungs.

MICROBIOLOGICAL ACTIVITY OF CEMENT DEGRADATION

The purpose of the work has to expand the current knowledge of microbiological activity on the mineralogical properties on the concrete. It was hypothesized that perhaps certain strains of bacteria might find the unique environment within concrete suitable habitation, and through metabolic activity, damage the concrete either directly or indirectly. The goal of this study to expand the knowledge regarding the evidence of microbiological activity in concrete from two bacteria cultures such as *Bacillus* (BA1) and *Pseudomonas* (PS1). Most research works have indicated that *Bacillus* plays a key role in the cement biodegradation process [2].

BIOWEATHERING ACTION OF SILICATE BY USING BACTERIA

Some bacteria play an important role in mobilization of silica and silicates in nature. Part of this microbial involvement is manifested in the weathering of rock silicates and aluminosilicates. Bioweathering action of silicates seems not to be restricted to corrosive agents that have been excreted by appropriate microorganisms in to the bulkphase but can also involve microbes attached to the surface of silica or silicates [3]. Microbes have a significant influence on the distribution and form of silicon in the biosphere. Those organisms that assimilate silicon clearly act as concentrators of it. Those that degrade silica, silicates act as agents of silicon dispersion.

BIOCEMENTATION

Calcium carbonate precipitaton by bacteria has been done by using ureolytic bacteria are known as Biocement or Calcium Carbonate. Microbiologically induced calcium carbonate precipitation (MICP) is a bio-geochemical process that induces calcium carbonate precipitation within the soil matrix [4]. Biocement is made up of naturally occurring microorganisms. Thus it costs much less to produce, consumes much less energy, and is more environmentally friendly.

MATERIALS AND METHODS

Collection and processing of the sample

The soil sample was collected from the cement dumping area site at R.R.Nagar 10Km from Virudhunagar town, Tamilnadu, India. Soil sample was collected using sterile scalpel, at 4cm depth and transferred to sterile polythene bag. Then the sample was used for further microbiological analysis.

Serial dilution method [5].

The soil sample was serially diluted. 1g of cement soil sample was mixed with 99 ml of sterile distilled water in the conical flask. The diluted samples were used for the isolation of bacteria and these are identified as Morphological and Biochemical characteristics.

Growth of bacterial isolates cement as substrate

To determine the optimum medium substrate concentration for *Bacillus* (BA1) and *Pseudomonas* (PS1) growth rates. The isolated *Bacillus* and *Pseudomonas* species were inoculated into silicate bacteria medium with different substrate concentration (cement) ranges such as 1g, 2g, 3g, 4g and 5g respectively. The effects of substrate concentration on *Bacillus* and *Pseudomonas* growth rates were recorded.

Effect of various parameters on the growth of cement degrading bacteria

Effect of pH

To determine the optimum medium pH for maximum *Bacillus* (BA1) and *Pseudomonas* (PS1) growth rate. The isolated *Bacillus* and *Pseudomonas* species were inoculated into the silicate bacteria medium with different pH ranges such as 2, 3, 4, 5, 6. The effect of pH on *Bacillus* and *Pseudomonas* on growth rates were recorded.

Effect of temperature

To determine the optimum medium temperature for *Bacillus* (BA1) and *Pseudomonas* (PS1) growth rate. The isolated *Bacillus* and *Pseudomonas* species were inoculated into silicate bacteria medium with different temperature ranges such as 25°C, 30°C, 35°C, 40°C. The effect of temperature on *Bacillus* (BA1) and *Pseudomonas* (PS1) on growth rates were recorded.

Effect of inoculum concentration

To determine the optimum medium inoculums concentration for *Bacillus* (BA1) and *Pseudomonas* (PS1) growth rate. The isolated *Bacillus* and *Pseudomonas* species were inoculated into silicate bacteria medium with different inoculums concentration ranges such as 0.5%, 1%, 1.5%, 2%, 2.5% and 3%. The effects of inoculums concentration on growth at *Bacillus* and *Pseudomonas* were recorded.

Bioweathering of cement silicates

Determination of silicate in the sample [6]

The ammonium molybdate yellow analysis was performed by mixing 0.2 ml of sample (*Bacillus and Pseudomonas*) to 5 ml of 1N. H_2SO_4, followed by 5ml of 0.3M ammonium molybdate. The sample (*Bacillus and Pseudomonas*) were allowed to react with 5minutes and then it was measured at 400nm with a UV spectrophotometer.

Production of 2-ketogluconic acid by *pseudomonas* (PS1) [7]

Pseudomonas strains all of which were able to produce 2 ketogluconate from glucose. The action of these bacteria was tested in glucose containing basal medium. It was found that dissolution of silicates in these cases resulted from the complexation of the cationic components of the silicates by 2-ketogluconate.These reactions were tested for Glucose containing basal medium.

Characterization of 2-ketogluconic acid by paper chromatography [8] and HPLC Analysis [9]

Samples were taken at regular intervals and paper chromatograms were run with the descending technique to determine the acids present. The solvent system employed was n-Butanol-Formic acid-Water (4:1.5:1). After equilibration, the papers were first irrigated with the solvent for 7 to 8 hr at 25°C, then air-dried, and sprayed with an alkaline solution of 0.04% bromeresol green in 95% ethanol (pH adjusted to 11.5 to 11.8 with NaOH). Acids appeared as yellow spots on a blue background. When dry, the papers were sprayed again with 0.1% orthophenylenediamine in 95% ethanol containing 1% HNO_3, and heated at 100°C for 4 to 5 min. The 2-ketogluconic acid appeared as a yellowish-green spot. The gluconate content represented as the sum of the concentrations of gluconolactone and gluconic acid was determined in the culture supematant obtained after centrifugation (15 min, 8000 rpm).The crude supernatant were analysed for HPLC analysis at Science Instrumentation Centre, ANJAC, Sivakasi.

Urease activity

The isolated *Bacillus* (BA1) and *Pseudomonas* (PS1) were tested for urease activity. This was done by streaking the purified cultures on urease test agar and incubated at 24 hours.

Phenol hyphochlorite assay method [10]

The Urease positive isolate *Bacillus* (BA1) was further tested for the urease activity. This was determined in the media according to the phenol hyphochlorite assay method. Ammonium chloride (50-100µM) was used as standard. The culture filterates (250µl) were added to the mixture containing 1 ml of 0.1M potassium phosphate buffer (pH 8) and 2.5ml of urea (0.1M). The mixture was incubated at 37°C for 5 minutes followed by addition of phenol nitroprusside and alkaline hyphochlorite. 1ml each and incubated at 37°C for 25 minutes. Optical density was measured at 626 nm.

Screening of Biocementation [11]

To 500 ml of water samples, 100ml (3M) concentrations of filtered urea and 100ml inoculums were added and incubated for one week at 37°C. After incubation the deposits of calcium carbonate was filtered using normal filter paper. After filtration the paper containing the deposits is kept inside the hot air oven at 35°C for 6hours.

RESULTS

Isolation of cement degrading bacteria from cement dumping site at R.R.Nagar

Cement dumping soil samples were taken from RAMCO Cement factory, R.R.Nagar, Tamilnadu. for this study. These soil samples were serially diluted and of two bacterial strains were isolated. They were designated as namely BA1, PS1.

Identification of strains based on Morphological and Biochemical characteristics

The bacterial isolates BA1 and PS1 were identified based on morphological and biochemical analysis. This bacterium BA1 was Gram positive, rod shaped, endospore forming and motile bacteria, and the bacterium PS1 was Gram Negative, rod shaped bacteria. (Table.1). According to Bergey's manual of determinative bacteriology, the selected bacterium was identified as *Bacillus species* and *Pseudomonas species*.

Growth of bacterial isolates cement as substrate

To determine the optimum medium cement substrate concentration for *Bacillus* (BA1) and *Pseudomonas* (PS1) growth rate. In *Bacillus* the maximum growth rate was observed Substrate concentration 3g attained at the OD of 0.84. In *Pseudomonas* the maximum growth rate was observed in Substrate concentration 3g attained at the OD of 0.89 (Fig.1).

Effect of various parameters used for cement degrading bacteria

Effect of pH

To determine the optimum medium pH for maximum *Bacillus* (BA1) and *pseudomonas* (PS1) growth rates were recorded. In *Bacillus (BA1)* the maximum growth rate was observed in pH 4 attained at the OD of 0.76. In *Pseudomonas* (PS1) the maximum growth rate was observed in pH 4 attained at the OD of 0.81. (Fig.3)

Effect of temperature

To determine the optimum medium temperature for maximum *Bacillus* (BA1) and *pseudomonas* (PS1) growth rates were recorded. In *Bacillus (BA1)* the maximum growth rate was observed in temperature 40°C attained at the OD of 0.75. In *Pseudomonas* (PS1) the maximum growth rate was observed in temperature 40°C attained at the OD of 0.78. (Fig.4)

Effect of inoculums concentration

To determine the optimum medium inoculums concentration for maximum *Bacillus* (BA1) and *pseudomonas* (PS1) growth rates were recorded. In *Bacillus* the maximum growth rate was observed in inoculums concentration at 2% (0.66). In *Pseudomonas* the maximum growth rate was observed in inoculum concentration at 2% attained at the OD of 0.62.(Fig.2)

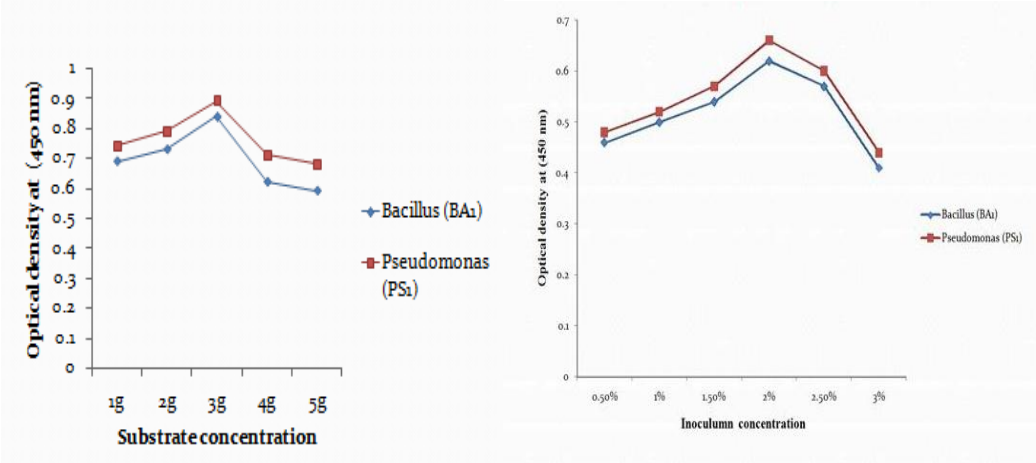

Fig.1&2: Effect of substrate and inoculum concentration on cement degrading bacteria

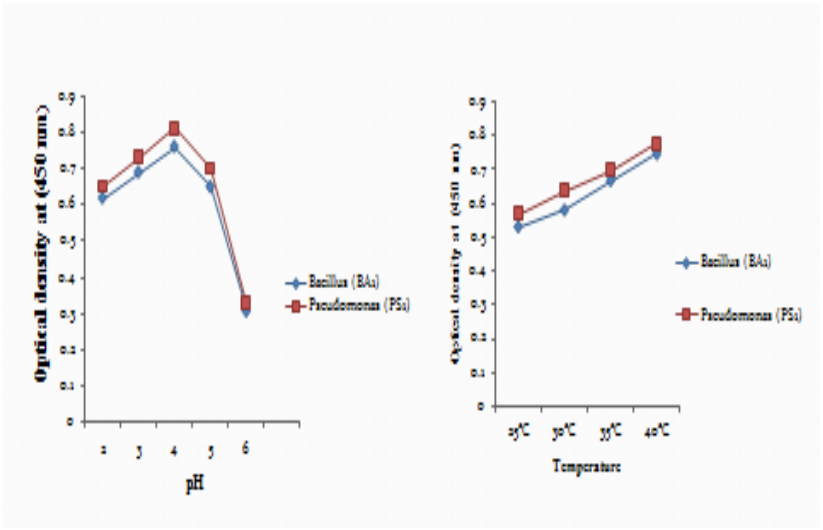

Fig.3&4: Effect of pH and temperature on cement degrading bacteria

Gravimetric determination of cement.

The cement cubes were first immersed in distilled water for 24 hours so that the drying procedure would start with water-saturated samples. The samples were dried in an oven at 80°C for 3 days (the time required for maximal evaporation), and then weighed. At various times during incubation with the bacterial cultures, cement cubes were removed from the cultures for determination of weight changes. (Fig.5, 6, 7)

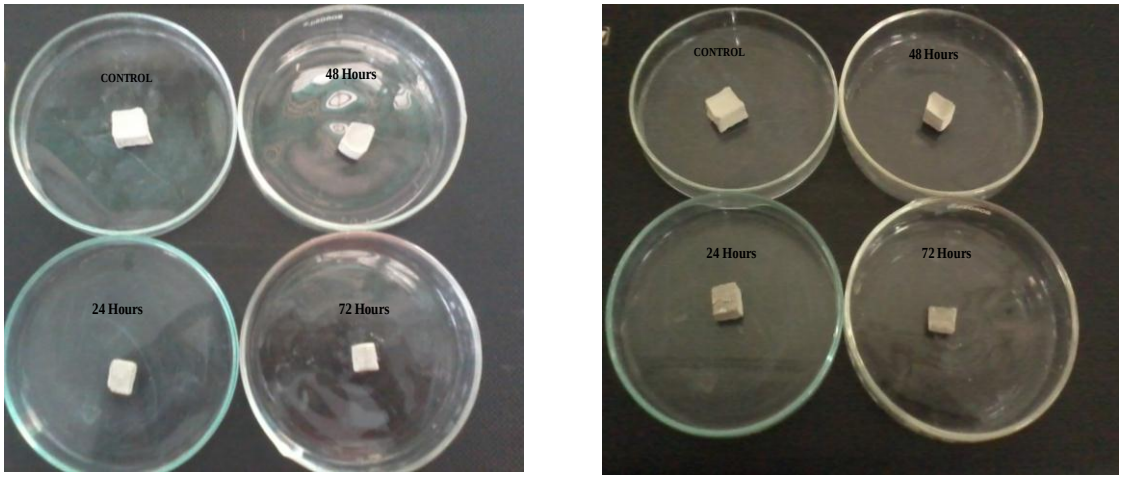

Fig.5&6: Weight reduction of cement cubes using Plate by *Bacillus* and *Pseudomonas* sp

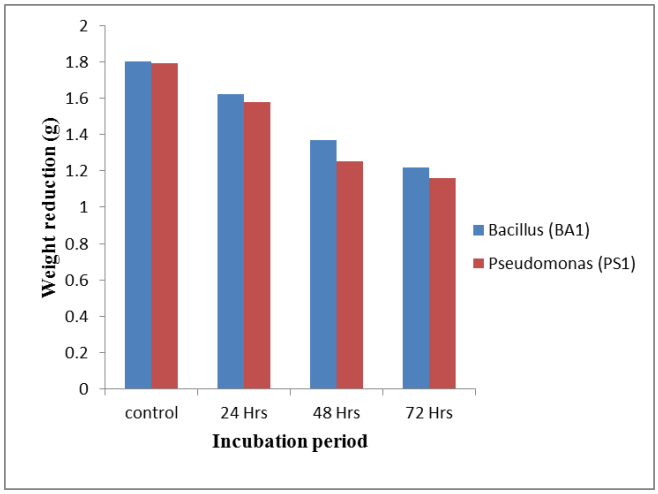

Fig.7: Weight reduction of cement cubes by graph representation

Determination of Silicate In The Sample

The ammonium molybdate yellow analysis was performed by mixing 0.2 ml of sample (*Bacillus, Pseudomonas*) to 5 ml of 1N. H_2SO_4, followed by 5ml of 0.3M ammonium molybdate. The sample was allowed to react with 5minutes and then it was measured at 400nm with a UV spectrophotometer.(Table.1)

Table 1: Determination of silicate in the sample

S.No.	Name of the organism	Optical density (400nm)
1	*Bacillus.Sp*	0.68
2	*Pseudomonas.Sp*	0.71

Production of 2-Ketogluconic Acid By Pseudomonas (Ps1)

Pseudomonas strains all of which were able to produce 2 ketogluconate from glucose. The action of these bacteria was tested in glucose containing basal medium. It was found that dissolution of silicates in these cases resulted from the complexation of the cationic components of the silicates by 2-ketogluconate. The growth activity appears on glucose basal medium.These are analyzed by Paper chromatography and HPLC analysis (Fig.8,9,10).

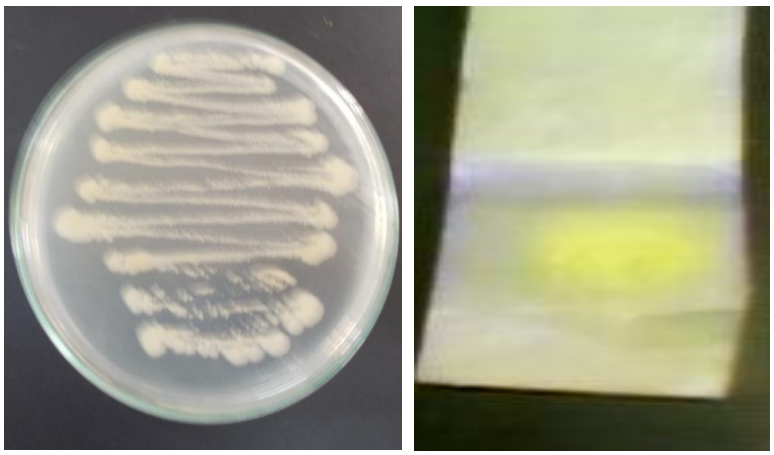

Fig.8 & 9: 2-Ketogluconic acid production by Glucose basal medium and paper chromatography

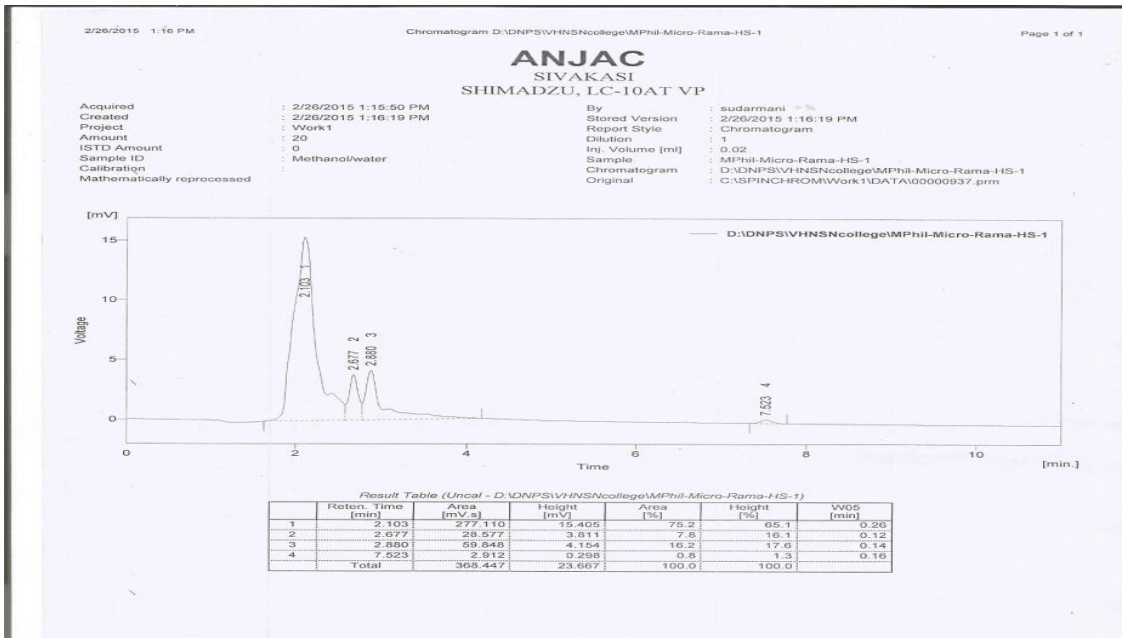

Fig.10: Confirmation of 2-Ketogluconic acid by HPLC analysis

Biocement formation

Phenol hyphochlorite assay method

The urease positive isolates were further tested for the urease activity. Optical density was measured at 626 nm and one unit of urease is defined as the amount of enzyme hydrolyzing 1 µmol urea/min [14].The growth profile studied up to 24, 48, 72, 96 and 120 hours. It was observed from graph that in *Bacillus* the optical density has increased up till 48 h which is 0.72 respectively which keep on decreasing up till 120 hours (0.54) linearly.(Fig.11)

Crystal nucleation site development at Biocement

To 500 ml of water samples, 100ml (3M) concentrations of filtered urea and 100ml inoculums were added and incubated for one week at 37°C. After incubation the deposits of calcium carbonate was filtered using normal filter paper. After filtration the paper containing

the deposits is kept inside the hot air oven at 35°C for 6hours. The strain like urease positive organism *Bacillus* precipitated at similar rates but produced whitish and transparent crystal aggregates.(Fig.12)

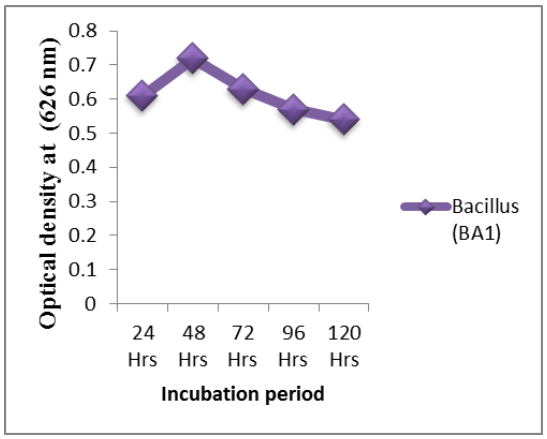

Fig.11 & 12: Phenol hyphochlorite assay and Biocement formation

DISCUSSION

Cement dumping soil samples were taken from R.R.Nagar 10 Km near to Virudhunagar town, Tamilnadu. Among four isolates two strains were enumerated for pure culture. They were designated as namely BA1 and PS1. Selected strains were inoculated into silicate bacteria medium for cement degradation at different time intervals (24, 48, 72 and 96 hours). The maximum growth rate was observed in BA1 at 96 hours and the maximum growth rate was observed in PS1 at 96 hours. Further the selected bacterium BA1 and PS1 were identified by various morphological and biochemical analysis. The strain BA1 was Gram positive, rod shaped and motile bacterium and the strain PS1 was Gram negative, rod shaped bacterium. In biochemical characterization according to Bergey's manual of determinative bacteriology, the selected bacteria were confirmed as *Bacillus and Pseudomonas.*

Similarly,[12] stated that the culture used for this study was enriched from soil taken adjacent to a concrete pavement brought up in one of two medias, one with acetate as a carbon source and other with glucose as a carbon source. One gram of soil was added to 10ml sterilized milli-Q water and then vortex. Two ml of this solution were used to inoculate the two different media. Both enrichments tolerated an alkali pH of 11 to 12, and were isolated at 30°C. The glucose media is published as Horikoshi media and consists of the following 10g D-dextrose;5g peptone; 1g yeast extract;1g KH_2PO_4; 0.2g $MgSo_4.7H_2O$; 10g Na_2CO_3 (Horikoshi, 1998). The acetate media is alkaline mineral base media consisting of the following 1g K_2HPO_4; 1g $(NH)_4H_2PO_4$; 0.1g $MgSo_4.7H_2O$; 10g Na_2CO_3 with 20mM sodium acetate. Both media were mixed in milli-Q-water. In both culture types the media turned turbid within 1-2 days; this was accompanished the appearance of a sticky pinkish mass suspended in a liquid presumed to be biomass. Both Gram negative and Gram positive organisms were in both cultures.

In the present study, we developed a simple procedure to accelerate biodegradation of cement pastes by incubating bacteria such as *Bacillus* (BA1) and *Pseudomonas* (PS1) in semicontinuous culture. The biodegradation kinetics of the cement was evaluated by

monitoring the concentrations of elements leached from the cementitious mixture and by measuring the gravimetric weight loss of the cement samples [13].

If microorganisms colonize and destroy specific feldspars to release limiting nutrients, then feldspar weathering is not always controlled by simple abiotic kinetics. In this microbial weathering scenario, colonized feldspars containing trace nutrients may weather very quickly and early, leaving behind only a clay residuum and those feldspars without nutritive value. Alternatively, the weathering rate of Ca-containing feldspars through geologictime, a potential part of the global carbon dioxide balance [3], maybe partly controlled by the trace nutrient potential of each mineral and the local microbial ecology.

The Article [13] reported that commercially, 2-ketogluconic acid serves as an intermediate in the production of isoascorbicacid. *Serratia marcescens* NRRL B-486 was chosen from the10 strains examined because of the high yields of2-ketogluconic acid produced in a short fermentation time. In the present study reported that 2-Ketogluconic acid was analyzed by Paper chromatography and HPLC analysis method.

Urease positive organisms were involved in Biocement formation. The mechanism of urease in which an organism creates a local microenvironment with conditions that allows optimal chemical precipitation of mineral phases [14]. Bacterial community has very limited diversity that can withstand extreme alkaline condition. Urease producing microorganisms were selected on the basis of their survival in alkaline environments.

In the present study demonstrated that urease positive organisms were isolated and then 500ml of hard water samples were taken.100ml of filtered urea and 100ml inoculums were added. These are incubated at one week for 37°C. After one week incubation the calcium carbonates were deposited in a medium. These calcium carbonates were dried in a hot air oven at 65°C and get a powder form. These are shown in white crystalline in nature such as these are known as calcium carbonate or Biocement.

CONCLUSION

Now a days, cement and concrete industries are huge. These industries can make many environmental issues such as Air pollution, Water pollution, Energy consumption and CO_2 emissions. The purpose of the work has to expand the current knowledge of microbiological activity on the mineralogical properties on the concrete. It was hypothesized that perhaps certain strains of bacteria might find the unique environment within concrete suitable habitation, and through metabolic activity, damage the concrete either directly or indirectly. The goal of this study to expand the knowledge regarding the evidence of microbiological activity in concrete from two bacteria cultures such as *Bacillus* (BA1) and *Pseudomonas* (PS1). Some bacteria play an important role in mobilization of silica and silicates in nature. Some bacteria can induce precipitation of calcium carbonate extracellularly through a number of processes that include photosynthesis, ammonification, denitrification, sulfate reduction and anaerobic sulphide oxidation.*Bacillus pasteurii* produces intracellular urease constitutes close to 1% of the cell dry weight. Biocementation through microbial carbonate precipitation is a new branch of microbial geotechnology that deals with the applications of microbiological methods to produce cemented materials. The applications of biocementation would require an interdisciplinary research at the confluence of microbiology, ecology, geochemistry, civil and environmental engineering. This new field has the potential to meet society's expanding needs for innovative treatment processes that improve soil engineering properties.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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