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Characterization of Two Urease-Producing and Calcifying *Bacillus* spp. Isolated from Cement

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Two bacterial strains designated as CT2 and CT5 were isolated from highly alkaline cement samples using the enrichment culture technique. On the basis of various physiological tests and 16S rRNA sequence analysis, the bacteria were identified as *Bacillus* species. The urease production was 575.87 U/ml and 670.71 U/ml for CT2 and CT5, respectively. Calcite constituted 27.6% and 31% of the total weight of sand samples plugged by CT2 and CT5, respectively. Scanning electron micrography analysis revealed the direct involvement of these isolates in calcite precipitation. This is the first report of the isolation and identification of *Bacillus* species from cement. Based on the ability of these bacteria to tolerate the extreme environment of cement, they have potential to be used in remediating the cracks and fissures in various building or concrete structures.

Keywords: *Bacillus*, cement, 16S rRNA, urease, calcite precipitation, crack remediation

Microorganisms are active in a wide range of mineralization processes and have been involved in the deposition of minerals throughout the history of the Earth [14]. Bacteria from soils, freshwater, and saline habitats have frequently been reported to be able to precipitate calcium carbonate both in natural and in laboratory conditions. This capability has been related to the formation of marine calcareous skeletons, carbonate sediments, and soil carbonate deposits [26, 29]. According to Boquet *et al*. [11], calcium carbonate precipitation is a general phenomenon in the bacterial world, and under suitable conditions, most bacteria are able to precipitate calcite crystals.

Microbiologically induced calcium carbonate precipitation (MICCP) is composed of a series of complex biochemical reactions, including concomitant participations of microbial urease (urea amidohydrolase; E.C. 3.5.1.5), and high pH. The microbial urease hydrolyzes urea to produce ammonia and carbon dioxide, and the ammonia released into the surroundings subsequently increases the pH, leading to accumulation of insoluble calcium carbonate [33].

The biomediated production of calcite crystals by calcifying bacteria has great applicable value for the restoration of deteriorated calcareous monuments and remediation of cracks and fissures in structures. Species of the *Bacillus* group are able to precipitate calcite on their cell constituents and in their microenvironment by conversion of urea into ammonia and carbon dioxide. Microbial mineral precipitation technologies have already been used for consolidation of sand columns [3, 33], for repair of limestones [35], and to a small extent for remediation of cracks in concrete [8, 10, 28].

Despite the importance of these bacteria in remediation of cracks and fissures, very few bacteria have been exploited. Microorganisms inhabit all possible locations including extremes, and exhibit growth and reproduction in such environments [31]. There is a need to explore extreme alkaline environments to isolate indigenous bacteria that can survive in concrete structures for effective biocalcification. In this investigation, two bacterial strains have been isolated from cement samples and their abilities to produce urease and calcite tested. Furthermore, these strains have been identified based on physiological and molecular characteristics.

Materials and Methods

Sample Collection

Cement samples were collected from the commercial bags and placed in radiation-sterilized polypropylene bottles (HiMedia, Mumbai). The physicochemical parameters of the cement, such as pH, specific gravity, silica content, and alumina content, were analyzed using standard methods (Table 1).

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Table 1. Physicochemical properties of cement.

Parameter	Value
Physical Analysis	
pH	12.5–12.8
Specific gravity (g/cm)	3.15
Chemical Analysis	
Lime (CaO)	63.5%
Silica (SiO_2)	19.1%
Alumina (Al_2O_3)	4%
Iron oxide (Fe_2O_3)	2.9%
Magnesia (MgO)	2.8%
Sulfur trioxide (SO_3)	2.6%
Soda and/or potash (Na_2O+K_2O)	0.8%
Loss on ignition	2.3%

Enrichment of Sample and Bacteria Isolation

To enrich the cement samples for urease-producing bacteria, 1 g of cement was inoculated into 50 ml of nutrient broth (HiMedia, India) (pH 8.0) containing 2% urea, and incubated at 37°C for 120 h under shaking condition (130 rpm). Bacteria were enumerated using the serial dilution technique by total plate count method on nutrient agar plates. The plates were incubated at 37°C overnight. Subsequently, the colonies were transferred onto urea agar base (HiMedia, India), a urease selective medium, to check the production of urease. Two isolates, designated as CT2 and CT5, were selected for further studies based on their abilities to produce urease qualitatively.

Phenotypic Characterization

Morphological characterizations of colony, cell, and spore morphology; Gram reaction; motility; catalase, oxidase, citrate, and malonate utilization; nitrate reduction; phenylalanine deamination; and starch, casein, gelatin, and esculin hydrolysis were performed by standard methods. A total of 35 carbohydrate fermentation tests were performed according to the manufacturer's instructions (HiMedia, India).

The isolates were also inoculated on nutrient media with different sodium chloride concentrations (0%, 2%, 5%, 7%, 10%, 12%, and 15%) to determine the survival in saline conditions. Temperatures between 25°C and 55°C at the interval of 5°C were chosen to test the growth pattern of bacterial isolates. The ability of bacterial isolates to grow in nutrient plates at different pH (*i.e.*, 4.0–13.0, with an interval of 0.5 units up to pH 12 and 0.1 unit from pH 12 to 13) was also determined after incubating at 37°C for different time intervals.

Molecular Characterization

Genomic DNA was extracted from overnight grown bacterial cells by alkaline lysis. The 16S rRNA gene from the genomic DNA was amplified by PCR using the following primers: 5'-AGAGTTTG ATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3', corresponding to the forward and reverse primers of 16S rDNA, respectively. PCR amplification was performed using a GeneAmp PCR system (Applied Biosystem, USA) as described by Krishna *et al*. [22]. The 16S rRNA amplicon was gel eluted and ligated into the pTZ57R/T vector as per the manufacturer's instructions (Fermentas, USA). The sequences were generated by the chain termination method using an Applied Biosystem automated sequencer (Delhi University, South Campus, Delhi, India). The 16S rRNA gene sequence was compared with the Ribosomal Database Project-II [15] and with those from GenBank using the BLASTN program [5]. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 4.0 software [34]. The 16S rRNA gene sequences for CT2 and CT5 determined in this study were deposited in the GenBank of the NCBI under the accession numbers FJ973471 and FJ973470, respectively.

Urease Activity

The urease activity was determined for bacterial isolates in nutrient broth media containing filter-sterilized 2% urea and 25 mM $CaCl_2$, by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method [27] at different time intervals, as described in Achal *et al*. [3]. One unit of urease is defined as the amount of enzyme hydrolyzing one μmole of urea per minute.

Protease Activity

Alkaline protease activity was determined according to the method of Fukumoto *et al*. [17]. One ml of the culture was added to 5 ml of 0.6% casein and incubated at 37°C for 10 min. The reaction was stopped by adding 5 ml of a TCA mixture [36 ml of 50% (w/v) TCA, 220 ml of 1 M sodium acetate, and 330 ml of 1 M acetic acid in a total volume of 1,000 ml]. The unreacted casein was precipitated, the resulting precipitate was filtered, and the optical density of the filtrate was measured at 275 nm against a blank. A standard graph was generated using 5–50 μg/ml of tyrosine. One unit of protease activity was defined as the amount of enzyme that liberated one microgram of tyrosine per minute at 37°C.

Extracellular Polymeric Substances and Biofilm Production

Extracellular polymeric substances (EPS) production from CT2 and CT5 was determined according to the procedure described by Friedman *et al*. [16]. Briefly, the dye Congo red (CR) was mixed in a bacterial culture (10^8 CFU/ml) at a concentration of 3.5 mg/l in order to stain EPS-producing strains, and the aliquots were incubated for 30 min. To remove the cells, the dyed culture was centrifuged for 5 min at 8,000 rpm. Optical density was measured at 430 nm. The quantity (nmol) of EPS production was calculated by CR bound per 10^8 CFU/ml.

Biofilm production from both isolates was established aseptically in nutrient broth containing urea-$CaCl_2$ on glass plates (25×75 mm). Solid-surface-associated biofilm formation was estimated by the crystal violet (CV) staining method as described in Achal *et al*. [4]. The CV attached to the biofilm was quantified by measuring its absorbance at 570 nm.

Microbial Calcification and Calcite Estimation

Calcification was studied by microbial sand plugging. In this method, overnight grown bacterial cells of OD_{600} 1.0 in nutrient broth medium containing filter-sterilized 2% urea and 25 mM $CaCl_2$ were mixed with autoclaved river sand and plugged in a plastic column (diameter=4 cm, height=12 cm), and the bottom side of the column was blocked using Whatman filter paper. A control reaction was packed in the column in which sterile sand was mixed with media only (without cells). The column was fed with the above-mentioned medium for 10 days at an interval of 24 h. After complete sand consolidation, the microbial sand column was divided into three

layers (upper, middle and lower layer) and each layer was individually ground and sieved through a 45-μm-diameter mesh prior to calcite estimation. Precipitated calcite from each layer was measured by the EDTA titration method [7].

Scanning Electron Microscope (SEM) Examination

Microbial sand columns were broken by applying mechanical force. The samples were then dried under vacuum in a desiccator at room temperature. SEM micrographs were obtained using a Zeiss EVO 50 at accelerating voltages ranging from 30 to 35 kV. Samples were gold coated with a sputter coating Emitech K575 prior to examination.

RESULTS

Isolation, Characterization, and Identification of Bacteria

Among the different bacteria isolated, only two bacterial isolates were selected based on higher urease and calcite production. Both isolates (CT2 and CT5) were Gram positive, rod shaped with opaque creamy appearance on agar plate, nonmotile, and catalase and oxidase positive. No pigments were produced by these isolates. CT5 was able to hydrolyze starch, casein, gelatin, and esculin, whereas CT2 hydrolyzed starch only. Acid production was noted with lactose, xylose, fructose, dextrose, raffinose, trehalose, arabinose, glycerol, sorbitol, mannitol, sucrose, ribose, and ONPG in the case of CT5, whereas only with sucrose, lactose, and ribose in the case of CT2. Citrate was utilized by CT5 only. The CT2 and CT5 isolates showed growth in a pH range of 6.5–10 and 6–11, respectively, after incubating the plates for 12–16 h, but these isolates tolerated pH up to 12.6 and 12.9, respectively, when incubated for 72 h. When subjected to salinity and temperature tests, CT2 and CT5 were able to survive 0–8% and 0–10% NaCl concentrations and a temperature range of 25–50°C and 25–55°C, respectively (Table 2). These physiological features show marked differences between the isolates.

The nucleotide BLAST and RDP-II analyses showed that both isolates belong to phylum Firmicutes and family Bacillaceae. Phylogenetic analysis revealed that CT2 and CT5 showed less than 98% similarity with different species of *Bacillus*. The phylogenetic analysis grouped CT5 into *B. cereus*, *B. anthracis*, and *B. mycoides*, and CT2 into *B. fusiformis* and *Lysinibacillus sphaericus*.

Urease and Protease Activities

Both isolates showed maximum urease productivity at 120 h. The CT2 isolate was able to produce 575.87 U/ml of urease, whereas the CT5 produced 670.71 U/ml of urease in nutrient broth containing urea-$CaCl_2$ (Fig. 2A). After 120 h, the urease production was decreased. The alkaline protease activity increased with the increase in time. The maximum protease activity was observed with CT5 (63.67 U/ml) followed by CT2 (54.39 U/ml) at the end of 168 h (Fig. 2B).

Table 2. Physiological characteristics of cement isolates CT2 and CT5.

Characteristics	CT2	CT5
Shape	Rod	Rod
Catalase	+	+
Oxidase	+	+
Citrate utilization	-	+
Nitrate reduction	-	+
Temperature tolerance (°C)	25–50	25–55
Alkalinity (pH tolerance)	6.5–10.0 (at 16 h)	6–11.0 (at 16 h)
	6.0–11.0 (at 24 h)	6–11.0 (at 24 h)
	6.0–12.1 (at 48 h)	5–12.5 (at 48 h)
	6.0–12.6 (at 72 h)	5–12.9 (at 72 h)
NaCl tolerance (%)	0–8	0–10
Phenylalanine deamination	-	+
Starch hydrolysis	+	+
Casein hydrolysis	-	+
Gelatin hydrolysis	-	+
Esculin hydrolysis	-	+
Acid production		
Xylose, fructose, dextrose, raffinose, trehalose, arabinose, glycerol, sorbitol, mannitol, ONPG	-	+
Sucrose, lactose, ribose	+	+

+, Positive; -, Negative

Extracellular Polymeric Substances and Biofilm Production

EPS production was more in the case of CT5 (46 nmol/ml) compared with CT2 (35.4 nmol/ml). A mature sessile bacterial community was observed in the case of both isolates as a result of biofilm production; however, CT5

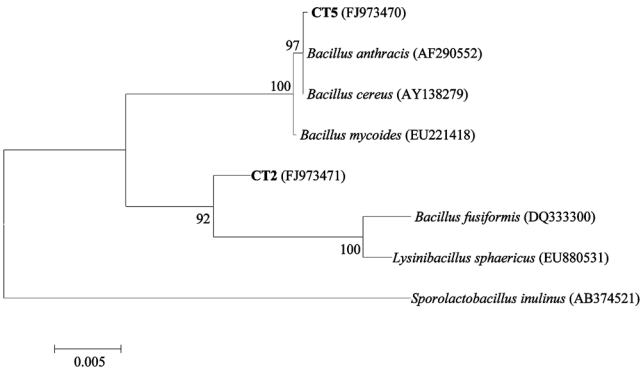

Fig. 1. 16S rRNA gene sequence-based dendrogram showing the phylogenetic position of the cement isolates CT2 and CT5 along with sequences available in the GenBank database.

The tree was constructed using the neighbor-joining method and rooted using *Sporolactobacillus inulinus* as an outgroup taxa. GenBank accession numbers are given in parentheses. Numerical values indicate bootstrap percentile from 1,000 replicates. Bar, 0.005 substitution per nucleotide.

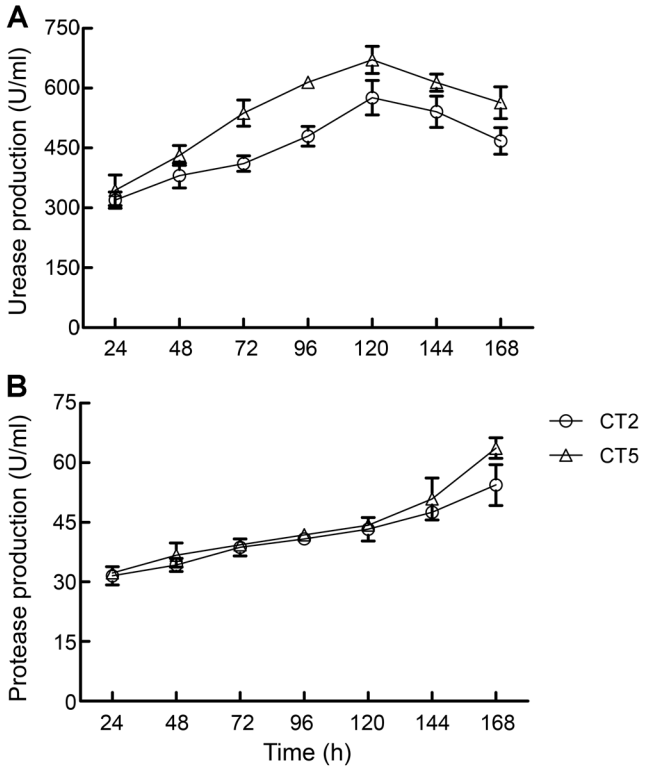

Fig. 2. The production of (**A**) urease and (**B**) protease by CT2 and CT5 isolates.
Error bars show standard deviation (n=3).

was highly effective in covering the surface compared with CT2. Monoxenic biofilms of CT2 and CT5 contained 330 and 370 CFU/mm, respectively.

Microbial Calcification

In the experiment with a plastic column that was packed with a mixture of sand and bacterial isolates and fed with nutrient broth containing urea-$CaCl_2$ medium, complete consolidation occurred within 10 days and the columns were tightly packed, whereas the control column lost its form when removed from the plastic. Calcite constituted 27.6% and 31% of the total weight of the sand samples plugged by CT2 and CT5, respectively (Fig. 3).

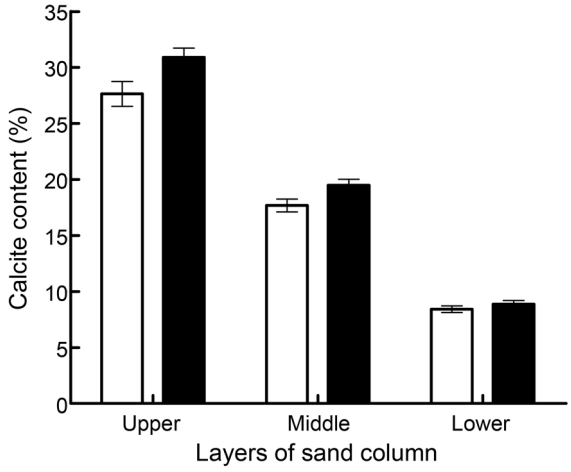

Fig. 3. Calcite content in different layers of sand columns consolidated with CT2 (empty bar) and CT5 (filled bar) isolates.

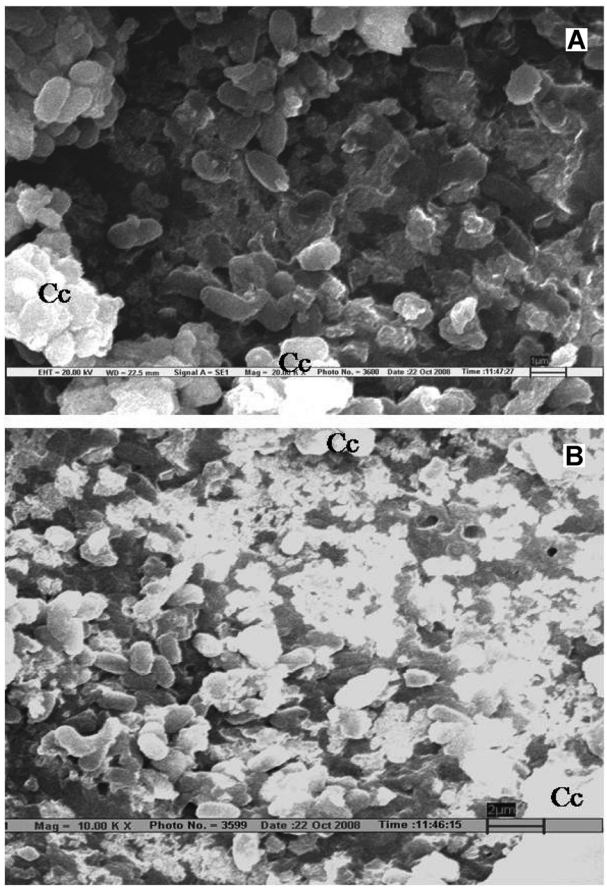

Fig. 4. Scanning electron micrographs of microbiologically induced calcite precipitation.
The distribution of rod-shaped (**A**) CT2 and (**B**) CT5 isolates along with calcite crystals (Cc, calcite crystals) can be seen.

Scanning Electron Microscope Examination

Microbial calcite precipitation was visualized by SEM. The SEM analysis on microbial involvement by CT2 and CT5 isolates in sand consolidation is depicted in Fig. 4, where calcite crystals embedded with rod-shaped microorganisms can be seen between and on the surface of sand grains.

Discussion

The limited diversity of the bacterial community in cement is not surprising; because of its extreme alkaline condition, only organisms capable of growing in these conditions can survive at this environment. Urea agar base is used

for selection of urease-producing microorganisms. When organisms utilize urea, ammonia is formed during incubation that makes the reaction of these media alkaline, producing a red-pink color due to the presence of phenol red, a pH indicator. Based on the qualitative urease productions, two isolates (CT2 and CT5) were selected. The traditional definition for an alkalophile is an organism capable of growing above pH 9.5 [23]. Both isolates were able to survive at highly alkaline ranges and could be considered alkalophiles. One of the reasons for the survival of these indigenous cement isolates at high pH might be their highly alkaline habitat. Reportedly, certain structural components of the cell wall of some alkalophiles, such as teichuronopeptide, may contribute to pH homeostasis at alkaline pH and aid bacteria to survive in alkaline environments [6]. Owing to the ability to tolerate high pH, CT5 enhanced the compressive strength of cement mortar cubes significantly and is regarded as a microbial concrete producer [2]. The phenotypic and physiological properties of these isolates resemble those of *Bacillus* species reported previously [19, 33], which was further confirmed by 16S rRNA gene analysis. The genus *Bacillus* seems to be well suited for long-term survival in extreme environments. This may explain its isolation from cement samples. It is noteworthy that CT2 and CT5 16S rRNAs did not show high identity with sequences of existing databases. On this basis, the cement isolates described here probably represents a new member of *Bacillus*. The predominance of this genus led us to hypothesize, according to Rivadeneyra *et al*. [30], that *Bacillus* species play a major role in the carbonate deposition in natural habitats.

Both isolates were found to produce significant amounts of urease. Bacteria are known to hydrolyze urea by urease for the purposes of (1) increasing the ambient pH [13], (2) utilizing it as a nitrogen source [12], and (3) using it as a source of energy [25]. Earlier, Kantzas *et al*. [20] reported that the urease from *B. pasteurii* that was detected in the culture medium was extracellular, and offered an option of using the urease rather than the whole cell to consolidate sand with $CaCO_3$. In biological systems, many calcareous organisms couple calcification to their metabolic assimilation processes to scavenge protons [24]. The subsequent increase of pH in the surrounding medium due to the presence of ammonia ions and the additional release of CO_2 from the enzymatic urea hydrolysis further accelerate the rate of the urease-induced calcite precipitation. Thus, an active participation of urease is of essence in biochemical calcite precipitation. The urease activity was significantly decreased and the protease activity increased after 120 h in this study. Although protease activity has not been reported to inhibit urease production, it can be hypothesized that owing to breakdown of proteins, urease activity declines [1, 4]. A high level of protease after 120 h indicates a high protein breakdown and hence the turnover after these hours, suggesting the possibility of degradation of urease at this stage.

The matrix of extracellular polymeric substances (EPS) secretions has been described to influence the calcium carbonate precipitation in a positive way [21]. EPS appear to play a role in the coverage of the surface by biofilms, cell adhesion [36], and possibly the capturing of the produced calcium carbonate, which might result in a homogeneous layer of calcium carbonate. The calcite content was found to be maximum in the case of the upper layer of microbial sand column as compared with the other two layers in both isolates. Similar kinds of observations were reported by previous researchers [3, 9], when they plugged sand columns with *B. pasteurii*. Calcite precipitation occurred predominantly in the areas close to the surface of the sand column. It was mainly due to the fact that facultatively anaerobic bacterial cells grow at a higher rate in the presence of oxygen, and consequently induce active precipitation of $CaCO_3$ around the surface area.

The presence of crystalline calcite associated with bacteria indicates that bacteria served as nucleation sites during the mineralization process [33]. Our observations from the SEM have added credence to previous reports [4, 18], providing conclusive evidence of direct participation of a few *Bacillus* species such as *B. pasteurii* in calcite formation. Findings from this study provide additional support to the notion that *in situ* implementation of microbial mineral plugging might provide a practical means for bioremediation of porous media.

The two bacterial strains CT2 and CT5 isolated from the cement samples were found to produce urease, which has been implicated in calcite precipitation. Both bacteria were identified as different species of *Bacillus*, on the basis of phenotypic and molecular characteristics. The ability of cement isolates to tolerate highly alkaline environments may have important implications for remediation of the cracks and fissures in various concrete structures.

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REFERENCES

1. Achal, V., A. Mukherjee, and M. S. Reddy. 2010. Biocalcification by *Sporosarcina pasteurii* using corn steep liquor as nutrient source. *Industr. Biotechnol.* **6:** 170–174.

2. Achal, V., A. Mukherjee, and M. S. Reddy. 2010. Microbial concrete: A way to enhance the durability of building structures. *J. Mater. Civil Eng.* DOI: 10.1061/(ASCE)MT.1943-5533.0000159
3. Achal, V., A. Mukherjee, P. C. Basu, and M. S. Reddy. 2009. Lactose mother liquor as an alternative nutrient source for microbial concrete production by *Sporosarcina pasteurii*. *J. Ind. Microbiol. Biotechnol.* **36:** 433–438.
4. Achal, V., A. Mukherjee, P. C. Basu, and M. S. Reddy. 2009. Strain improvement of *Sporosarcina pasteurii* for enhanced urease and calcite production. *J. Ind. Microbiol. Biotechnol.* **36:** 981–988.
5. Altschul, S. F., T. L. Madden, A. A. Schaffer, S. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25:** 3389–3402.
6. Aono, R., M. Ito, and T. Machida. 1999. Contribution of cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile *Bacillus lentus* C-125. *J. Bacteriol.* **181:** 6600–6606.
7. APHA. 1989. *Standard Methods for the Examination of Water and Wastewater*, 17th Ed. American Public Health Association, Washington.
8. Bachmeier, K. L., A. E. Williams, J. R. Warmington, and S. S. Bang. 2002. Urease activity in microbiologically induced calcite precipitation. *J. Biotechnol*. **93:** 171–181.
9. Bang, S. S. and V. Ramakrishnan. 2001. Microbiologically enhanced crack remediation (MECR), pp. 3–13. *Proceedings of the International Symposium on Industrial Application of Microbial Genomes*, Daegu, Korea.
10. Bang, S. S., J. K. Galinat, and V. Ramakrishnan. 2001. Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*. *Enz. Microb. Technol.* **28:** 404–409.
11. Boquet, E., A. Boronat, and A. Ramos-Cormenzana. 1973. Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature* **246:** 527–529.
12. Burne, R. A. and R. E. Chen. 2001. Bacterial ureases in infectious diseases. *Microbes Infect.* **2:** 533–542.
13. Burne, R. A. and R. E. Marquis. 2000. Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiol. Lett.* **193:** 1–6.
14. Chafetz, H. S. and R. L. Folk. 1984. Travertines: Depositional morphology and the bacterially constructed constituents. *J. Sed. Petrol*. **54:** 289–316.
15. Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, *et al.* 2003. The ribosomal database project (RDP-II): Previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.* **31:** 442–443.
16. Friedman, L. E., B. N. de Passerini Rossi, M. T. Messina, and M. A. Franco. 2001. Phenotype evaluation of *Bordetella bronchiseptica* cultures by urease activity and Congo red affinity. *Lett. Appl. Microbiol.* **33:** 285–290.
17. Fukumoto, J., T. Yamamoto, and D. Tsuru. 1971. Process for producing detergent resisting alkaline protease. *Canadian Patent* **910:** 214.
18. Gollapudi, U. K., C. L. Knutson, S. S. Bang, and M. R. Islam. 1995. A new method for controlling leaching through permeable channels. *Chemosphere* **30:** 695–705.
19. Gordon, R. E., W. C. Haynes, and C. H. Pang. 1973. *The Genus Bacillus*, pp. 427. US Department of Agriculture Handbook, Washington.
20. Kantzas, A., F. G. Ferris, L. Stehmeier, D. F. Marentette, K. N. Jha, and F. M. Mourits. 1992. A novel method of sand consolidation through bacteriogenic mineral plugging (CIM 92-46), pp. 1–15. *Proceedings of the CIM Annual Technical Conference*, Vol. 2. Petroleum Society of CIM, Calgary, Canada.
21. Kawaguchi, T. and A. W. Decho. 2002. A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing $CaCO_3$ polymorphism. *J. Cryst. Growth* **240:** 230–235.
22. Krishna, P., A. Arora, and M. S. Reddy. 2008. An alkaliphilic and xylanolytic strain of actinomycetes, *Kocuria* sp. RM1, isolated from extremely alkaline bauxite residue sites. *World J. Microbiol. Biotechnol.* **24:** 3079–3085.
23. Krulwich, T. A. and A. A. Guffanti. 1989. Alkalophilic bacteria. *Annu. Rev. Microbiol.* **43:** 435–463.
24. McConnaughey, T. A. and F. F. Whelan. 1997. Calcification generates protons for nutrient and bicarbonate uptake. *Earth Sci. Rev.* **42:** 95–117.
25. Mobley, H. L. T. and R. P. Hausinger. 1989. Microbial ureases: Significance, regulation and molecular characterisation. *Microbiol. Rev.* **53:** 85–108.
26. Morita, R. Y. 1980. Calcite precipitation by marine bacteria. *Geomicrobiol. J.* **2:** 63–82.
27. Natarajan, K. R. 1995. Kinetic study of the enzyme urease from *Dolichos biflorus*. *J. Chem. Educ.* **72:** 556–557.
28. Ramachandran, S. K., V. Ramakrishnan, and S. S. Bang. 2001. Remediation of concrete using microorganisms. *Am. Con. Inst. Mat. J.* **98:** 3–9.
29. Rivadeneyra, M. A., R. Delgado, A. Moral, M. R. Ferrer, and A. Ramos-Cormenzana. 1994. Precipitation of calcium carbonate by *Vibrio* spp. from an inland saltern. *FEMS Microbiol. Ecol.* **13:** 197–204.
30. Rivadeneyra, M. A., R. Delgado, G. Delgado, A. Moral, M. R. Ferrer, and A. Ramos-Cormenzana. 1993. Precipitation of carbonate by *Bacillus* sp. isolated from saline soils. *Geomicrobiol. J.* **11:** 175–184.
31. Sharma, A., A. Pandey, Y. S. Shouche, B. Kumar, and G. Kulkarni. 2009. Characterization and identification of *Geobacillus* spp. isolated from Soldhar hot spring site of Garhwal Himalaya, India. *J. Basic Microbiol*. **49:** 187–194.
32. Smith, N. R., R. E. Gordon, and F. E. Clark. 1952. *Aerobic Spore Forming Bacteria*, pp. 16. US Department of Agriculture Monograph, Washington.
33. Stocks-Fischer, S., J. K. Galinat, and S. S. Bang. 1999. Microbiological precipitation of $CaCO_3$. *Soil Biol. Biochem*. **31:** 1563–1571.
34. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24:** 1596–1599.
35. Tiano, P., L. Biagiotti, and G. Mastromei. 1999. Bacterial bio-mediated calcite precipitation for monumental stones conservation: Methods of evaluation. *J. Microbiol. Methods* **36:** 138–145.
36. Tsuneda, S., J. Jung, H. Hayashi, H. Aikawa, A. Hirata, and H. Sasaki. 2003. Influence of extracellular polymers on electrokinetic properties of heterotrophic bacterial cells examined by soft particle electrophoresis theory. *Colloid Surface B* **29:** 181–188.