Original Research Paper

Volume -10 | Issue - 4 | April - 2020 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Microbiology

OPTIMIZATION OF UREASE PRODUCTION BY BACILLUS MEGATERIUM TARA26 ISOLATED FROM MARBLE QUARRY SAMPLE AND ITS APPLICATION IN REDUCTION OF WATER HARDNESS

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ABSTRACT Urease enzyme produced by bacteria, hydrolyses urea in calcium rich soil to ammonia and carbonates thereby increasing the pH resulting in precipitation of $CaCO_3$. The optimum urease producing organism was isolated from marble quarry sample which was identified as *Bacillus megaterium* tara26 on the basis of morphological, cultural, biochemical and 16s rRNA sequence analysis. The promising isolate was tested for $CaCO_3$ precipitation. The isolate exhibited maximum urease (0.48 U/ml) production in an optimized medium containing urea (0.8 M), Meat extract (0.1%), Xylose (0.2%), pH 7.5 inoculated with 2% (v/v) of culture 0.8 O.D 540 nm kept on shaker (125 rpm) at 37°C for 36 hrs. The morphology of precipitated $CaCO_3$ crystals was studied using Scanning Electron Microscope. The potential application of reducing water hardness with the help of urease positive *Bacillus megaterium* tara26 was also demonstrated and it was observed that it was capable of reducing 60% total hardness (g/L) of water in 14 days.

KEYWORDS : Urease, Calcium carbonate, phenol hypochlorite assay, *Bacillus megaterium* tara26.

INTRODUCTION

Calcium carbonate ($CaCO_3$), a widely distributed mineral can form loose crystals by rapid chemical reaction of carbonate and calcium ions. However, its precipitation can also be triggered by microorganisms (Boquet *et al*., 1973). Numerous microbial species participate in the precipitation of mineral carbonates in various natural environments, these microorganisms use urea as sole nitrogen source , producing ammonia which increases the pH in the proximal environment causing Ca^{2+} to precipitate as $CaCO_3$ (Hammes *et al*., 2003). Urea is chiefly used as nitrogen fertilizer in agriculture, it is short-lived since it is easily metabolized by microbial activities of ureolytic bacteria which possess the enzyme urease that catalyses the hydrolysis of urea to ammonia and carbon dioxide (Seshabala and Mukkanti, 2013).

Ureases are a group of enzymes widely present in plants, bacteria, fungi, algae and invertebrates that although with different protein structures, exercise a single catalytic function, that is the hydrolysis of urea (H_2N-CO-NH_2), its end products are ammonia and carbonic acid (Khanafari *et al*., 2013). Microbial sources of urease that are involved in precipitation of calcium carbonate include gram positive bacteria such as *Bacillus licheniformis*, *Bacillus flexus*, *Bacillus subtilis*, *Sporosarcina pasteurii, Bacillus lentus, Bacillus pumilus, Bacillus sphaericus, Bacillus megaterium, Lactobacillus ruminis, Lactobacillus fermentum and Lactobacillus reuteri,* gram negative bacteria for instance *Pseudomonas calcis, Pseudomonas denitrificans* (Boquet *et al*., 1973; Kim *et.al*., 2005; Chu *et al*., 2012)*, Pseudomonas aeruginosa, Proteus vulgaris* and *Klebsiella aerogenes* (Helmi *et al*., 2016; Kumar *et al*., 2013; Afifudin *et al*., 2011; Kakimoto *et al*., 1989; Jones and Mobley, 1987). Filamentous fungi for example *Aspergillus niger*, *Aspergillus nidulans, Rhizopus oryzae* and yeast *Candida tropicalis* are also known to produce urease and precipitate calcium carbonate (Ghasemi *et al*., 2004; Mackay and Pateman, 1982; Farley and Sugiarto, 2003; Bharathi and Meyyappan, 2015).

Calcium carbonate precipitation has varied applications which take account of removal of heavy metals and radionuclide from groundwater, protection, restoration of limestone monuments, statuary and creation of biological mortars, reducing hardness of water, sequestration of carbon dioxide, removal of calcium ions and polychlorinated biphenyls, remediation of cracks in concrete, precipitated calcium carbonate can also be used as fillers for rubber, plastic and ink (Chunxiang *et al.*, 2009; Tiano *et al.*, 1999; Herzog and Drake, 1996; Sharma and Bhattacharya, 2010; Hammes *et al*., 2003; Fujita *et al*., 2008; Bang *et al.*, 2001).

The main aim of this research was to isolate and characterize a suitable bacterial strain from marble quarry samples, which is proficient of precipitating calcium carbonate. The prospective bacterium with maximum urease production was optimized and its role as catalyst in hard water samples (rich in calcium) for reduction of hardness by precipitation of calcium as calcium carbonate was also studied.

MATERIALS AND METHODS:

Sample collection

Eleven quarry samples were collected from Santacruz area in Mumbai city. All samples were collected in sterile containers and transferred to laboratory for processing.

Enrichment, isolation and screening:

As part of the screening programme, 1g of sample was suspended in 10ml of sterile PBS (pH 7.2) 1ml of the aliquot was inoculated in 50ml of sterile Nutrient broth (pH 7.2) and incubated at 30°C for 24 hrs under shaker conditions (125 rpm). Bacteria were enumerated by serially diluting up to 10^{-16} and 0.1 ml of sample was surface spread on sterile Nutrient agar plates which were incubated at 30°C for 24 hrs. 75 isolated colonies with different morphology were maintained on sterile Nutrient agar slants at 4°C. Each isolate was spot inoculated for qualitative urease test on Christensen's urea agar plates (pH 6.8) and incubated at 30°C for 48 hrs. Urease positive isolates were further subjected to quantitative estimation of urease enzyme. The isolates obtained from primary screening were grown in 100ml of sterile Nutrient broth supplemented with 2% (0.33M) urea and incubated at 30°C for 24 hrs under shaker conditions (125 rpm). The enriched broth was centrifuged at 5000 rpm for 20 minutes and the supernatant was used to assay urease production. The urease production was estimated using modified method of phenol hypochlorite assay (Chahal *et al*., 2011).

Enzyme assay to estimate urease production:

The assay was carried out using 0.5ml of supernatant, 3.5ml of Potassium Phosphate buffer (pH 8.0), 0.12ml of phenol solution (20g in 100 ml of 95% ethyl alcohol), 0.12ml of freshly prepared sodium nitroprusside solution (5g of sodium nitroprusside in 10 ml distilled water. Add 25 ml of 95% ethyl alcohol. Freeze for 24 hrs to reform crystals. Dissolve 1g of recrystallized sodium nitroprusside in 200 ml of distilled water), 0.3ml of oxidising reagent (solution A - 20g of Sodium citrate + 1g NaOH + 100ml of distilled water , solution B - Sodium hypochlorite. Mix 100 ml of solutions A and 25 ml of B) (Strickland and Parsons, 1972). The reaction mixture was incubated at 30°C in dark for 45 minutes. The absorbance of blue coloured complex formed was recorded spectrophotometrically at 640 nm against distilled water blank. The absorbance reading of the test supernatant was plotted on the standard graph of Ammonium chloride (5- 40µM). One unit of urease enzyme is equal to the amount of one micro mole of product formed per minute under standard conditions (Achal and Pan, 2011). The isolate showing maximum production of urease was used for further studies.

Detection of Calcium carbonate precipitation on agar:

The potential isolate with maximum urease production was tested for its ability to precipitate calcium carbonate through urea hydrolysis

(Stocks-Fischer *et al*., 1999). The medium composed of 100 ml of Nutrient agar supplemented with 2.85g calcium chloride and 10ml of 0.33 M urea. All the media components were autoclaved except urea, which was filter sterilised using membrane filter (0.2-0.45µm) under cold conditions and then added to 100 ml of medium. Loopful of 18 hrs old culture was spot inoculated on calcium carbonate precipitation agar plates and incubated at 30°C for 5 days. The plates were examined periodically to monitor the calcium carbonate crystals formed surrounding the colony with the help of a light microscope under 10X (Zoheir *et al*., 2013).

Detection of Calcium carbonate precipitation in broth:
The ability of bacteria to precipitate calcium carbonate was studied using 100 ml of sterile Nutrient broth supplemented with 2.85g of calcium chloride and 0.33 M of filter sterilised urea. 100ml of the above medium was inoculated with 2% inoculum of 0.8 $O.D_{540nm}$ and incubated at 30°C for 7 days under shaker conditions (125 rpm). The bacterial cells and calcium carbonate precipitate were separated by filtration using Whatman filter paper (Grade 1: 11µm). The precipitate which retained on the filter was dried in hot air oven at 60°C for 15 minutes (Zoheir *et al.,* 2013).

Qualitative test for calcium carbonate:
1. **Using 2N Hcl**
0.2g of dried powder was taken on a cavity slide followed by the addition of 3 drops of 2N HCl, which produced effervescence of carbon dioxide after reaction (Al-Omari *et al*., 2016).

2. Using 2N ammonium oxalate.
0.2g of powder was dissolved in 2N HCl and mixed well. Later a few drops of 2N ammonium oxalate solution were added. The tube was then checked for visible precipitation (Sinha and Gupta, 2016).

Identification of the promising isolate:
The promising isolate (ISL-7) was identified up to genus level by studying its morphological, cultural and biochemical characteristics using Bergy's Manual of Determinative Bacteriology, 8th edition. Further confirmation of the species was done using 16S rRNA analysis, which was outsourced to Sai Biosystems Private Limited, Nagpur, India

Optimisation of physio-chemical parameters for maximum urease production by the isolate:
Effect of various media on urease production by the isolate:
The maximum yield of urease by the isolate was studied using 7 different media. The media used were: Yeast extract broth (Elmanama and Alhour, 2013), Nutrient broth (Kahani *et al.,* 2019), Luria Bertani, King's B (Jokyani and Chouhan, 2018), Brain heart infusion broth, Tryptic soy broth and Beef extract broth (Williams *et al.,* 2016).

Effect of various carbon sources on urease production by the isolate:
The effect of carbon sources on urease production was studied by adding 0.5% of different carbon sources such as Dextrose, Sorbitol, Maltose, Sucrose, Mannitol, Lactose, Galactose, Xylose and Fructose in the optimized production medium. The flasks were incubated at 30°C for 24 hrs under shaker conditions (Elmanama and Alhour, 2013).

Effect of various concentration of optimized carbon source on urease production by the isolate:
The effect of different concentrations of optimized carbon source on urease production was studied. Optimized carbon concentrations viz. 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.7%, 0.8%, 0.9% and 1.0% were used in the optimized medium selected for urease production (Balan *et al*., 2012).

Effect of various nitrogen sources on urease production by the isolate:
The effect of nitrogen source on urease production was studied by adding 0.3% of different organic and inorganic nitrogen sources like Potassium nitrite, Glycine, Yeast extract, Sodium nitrite, Meat extract and Peptone in the optimized medium (Balan *et al*., 2012).

Effect of different concentration of optimized nitrogen source on urease production by the isolate:
Concentration of the optimised nitrogen source for urease production was studied. 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.7%, 0.8%, 0.9% and 1.0% concentrations of the best suited nitrogen source were used in the selected medium (Balan *et al*., 2012).

Effect of variable pH on urease production by the isolate:
To determine the optimum pH for maximum urease production, the pH range of 5 – 9 were used with the interval of 0.5. The pH of the optimized production medium was adjusted using 1N HCl and 1N NaOH (Smith *et al*., 1993; Kumar *et al*., 2013).

Effect of incubation period for maximum urease production by the isolate:
The time required for maximum urease production in the optimized medium was studied over a period of 6, 12, 18, 24, 30, 36, 42 and 48 hrs. The incubation period giving maximum urease yield was selected for further studies (Lehinger, 2002; Seshabala and Mukkanti, 2013).

Effect of aeration on urease production by the isolate:
The effect of aeration on urease production was studied by incubating one of the inoculated flasks under shaker conditions (125 rpm) and other at static condition at 30°C for 36 hrs (in the optimized medium) respectively (Kouhounde *et al*., 2015).

Effect of variable temperature on urease production by the isolate:
Effect of various temperatures on optimum yield on urease production in the optimized medium by the isolate was carried out using different incubation temperatures. The temperatures used for the study were 30°C, 37°C, 45°C and 55°C (Khanafari *et al.,* 2013; Ramanathan *et al*., 2016).

Effect of optical density of the culture on urease production:
The effect of optical density of the isolate on the production of urease in the optimized medium was determined by using different O.D at 540 nm ranging from 0.1 to 1 with an interval of 0.1 (Khanafari *et al.,* 2011).

Effect of various inoculum size of the isolate on urease production:
The effect of various inoculum size of the isolate such as 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1% (v/v) of 0.8 O.D. at 540 nm was checked for maximum urease production in the optimized medium (Seshabala and Mukkanti, 2013; Ramanathan *et al*., 2016).

Effect of urea concentration on urease production by the isolate:
The effect of different concentrations of urea in the optimized medium was studied on urease production by the isolate. The concentrations of urea used were 0.2M, 0.4M, 0.6M, 0.8M, 1.0M, 1.5M, 2.0M, 2.5M, 3.0M, 3.5M and 4.0M (Varalakshmi and Anchana, 2014).

Scanning electron microscopic observation of calcium carbonate crystals:
The morphology of bacteria and the precipitated calcium carbonate crystals were analysed using Field Emission Gun-Scanning Electron Microscopy (FEG-SEM), which was outsourced to Sophisticated Analytical Instrument Facility at IIT, Bombay. The sample was completely dried and then examined at accelerating voltage of 10kV and the imaging was done at a magnification of 100000X. During the analysis, the pressure maintained inside the vacuum chamber was 9.6 x 10^{-5} Pascals.

Application of *Bacillus megaterium* tara26 for remediation of hard water:
To determine total water hardness, different bore well water samples were collected from Palghar (SP-01), Dahanu (SD-02), Chembur (SC-03), Asangaon (SA-04) and Kalauli (SK-05) nearby places in Mumbai, Maharashtra. 20 ml of the water sample was taken into 250 ml Erlenmeyer's flask, to which the reagents were added in the following order, 1ml of liquor ammonia, a pinch of Eriochrome black-T indicator and titrated against 0.01M EDTA, till the endpoint i.e. colour change from wine red to blue is reached. The hardness of water sample was calculated by using the following formula
Hardness (mg/L) = ml of EDTA used X 1000 ÷ ml of sample
After determining the total water hardness, 100ml of respective water sample (SP-01, SD-02, SC-03, SA-04, SK-05) were inoculated with 20ml of 0.8 $O.D_{540nm}$ of *Bacillus megaterium* tara 26 and 10ml of 0.33 M urea followed by incubation for 14 days under shaker conditions at 30°C to check reduction in hardness of water samples by the isolate (Khanafari *et al.*, 2011). All experiments were run in triplicates and the average of three readings was used to plot the graphs and calculate the standard deviations.

RESULT AND DISCUSSION:
Enrichment, isolation and screening of urease producing bacteria:
In the present study, 11 quarry samples were screened for urease

producing bacteria.

However, Balan et al. (2012), Elmanama and Alhour (2013), Khanafari et al. (2013) and Varalakshmi1 and Anchana (2014) isolated urease positive bacterial strains from water and sediment samples of coastal area, urea rich soil, nursery garden soil and sea water samples respectively. The samples were enriched in 100ml Nutrient broth with 10ml of 0.33 M filter sterilized urea for 1 week at 30°C under shaker conditions at 125 rpm. 75 organisms were isolated from the enriched broth and maintained on sterile Nutrient agar (NA) slants. Isolates from these slants were spot inoculated on Christensen's urea agar plates. The colour of the medium changed from yellow to pink around the spot inoculated culture which indicated the production of urease (figure 1).

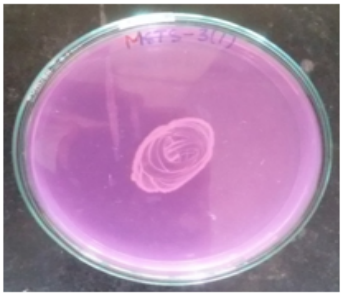

Figure 1: Bacterium showing urea hydrolysis on Christensen's urea agar.

Out of 75 isolates, 11 cultures showed urease production on Christensen's urea agar plates indicated by pink coloured zone surrounding the growth. Researcher Zoheir et al. (2013) also used Christensen's urea agar base for studying qualitative urease production. In addition, urea agar base medium has been used in several studies for isolating ureolytic microorganisms for the purpose of carbonate precipitation (Achal and Pan, 2011; Hammes *et al.*, 2003; Burbank *et al*., 2012; Achal and Pan, 2010; Chahal *et al*., 2011; Balan *et al*., 2012 and Khanafari *et.al.*, 2011). The urease positive isolates were grown in 100ml Nutrient broths supplemented with 10ml 0.33 M of filter sterilized urea. After incubation at 30ºC for 24 hrs under shaker conditions (125rpm), the broth was centrifuged and the supernatant was used for enzyme assay by phenol hypochlorite method. Amongst the 11 isolates, ISL-7 was selected for further studies as it showed maximum urease production (0.48 U/ml) in comparison to ISL-6 (0.24 U/ml), ISL-3 (0.18 U/ml) and ISL-4 (0.12 U/ml) as shown in figure 2. The gram positive isolate giving maximum urease production was maintained on NA slants (figure 3) and used for further optimization study.

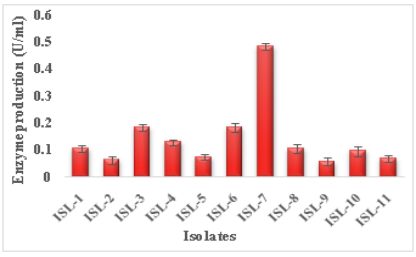

Figure 2: Urease assay of 11 different isolates.

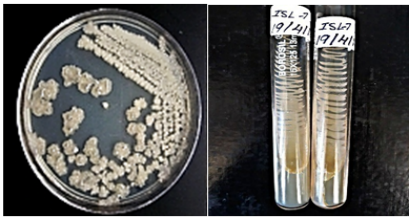

Figure 3: Promising isolate on Nutrient agar plate and slants.

Enzyme assay for estimation of urease production:
Standard graph of NH_4Cl was plotted to calculate the amount of ammonia (micro moles) released during the chemical reaction by phenol hypochlorite method (Balan *et al*., 2012). Ammonium chloride dissociates as ammonium ion and chloride ion in an aqueous solution. Hence, urease production can be indirectly correlated with ammonium chloride concentration at 640nm since urea hydrolysis yields two moles of ammonia and one mole of carbon dioxide where ammonia ionizes to ammonium ion in the medium (Burbank *et.al*., 2012). Most of the researchers used phenol hypochlorite method to measure the amount of ammonia released as a result of urea hydrolysis (Balan *et al.*, 2012; Smith *et al*., 1993; Dhami *et al*., 2013; Kumar *et al*., 2013; Ramanathan *et al*., 2016; Priya and Kannan, 2017). Whereas Hammes et al. (2003) used Nessler assay method to determine the ammonia in the medium. Kantzas et al. (1992) isolated urease from *Bacillus pasturii* and the enzyme was detected in the production medium, signifying that the enzyme is extracellular. In contrast, author Priya and Kannan (2017) recorded urease production of 1.10 U/ml for Bacillus sp and 4.59 U/ml for Pseudomonas strain. Whereas Balan et al. (2012) testified urease production of 1.75 U/ml for Klebsiella sp.

Detection of Calcium carbonate precipitation on agar plate:
Calcium carbonate precipitation agar (CPA) was used to study the calcium carbonate precipitating ability of the isolate. Figure 4 shows precipitation on CPA plates after 7 days of incubation at 30°C, which appeared as distinct circular zones around the growth and were found to be irregularly shaped (figure 5) when observed under compound microscope with 10X objective (Zoheir *et al*., 2013; Stocks-Fischer *et al*., 1999; Kumar *et al.*, 2013). Similar medium was used by many researchers for $CaCO_3$ precipitation with some modifications (Rivadeneyra *et al*., 1991; Chahal *et al*., 2011; Canakci *et al*., 2015).

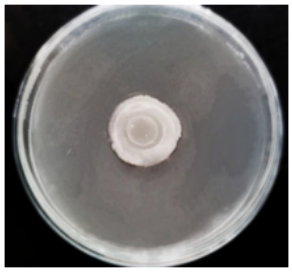

Figure 4: $CaCO_3$ precipitation on agar plate after 7 days of incubation showen by the isolate

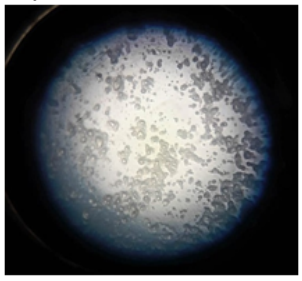

Figure 5: Observation of $CaCO_3$ crystals under compound microscope with 10X objective

Detection of Calcium carbonate precipitation in broth:
After inoculation of the isolate in Nutrient broth with urea and $CaCl_2$, white precipitate appeared in the medium after an incubation period of 7 days at 30°C under shaker conditions (Zoheir *et al*., 2013). After the incubation period, $CaCO_3$ precipitate was collected by filtration (figure 6). However, authors Hammes et al. (2003) and Perito et al. (2014) used urea and $CaCl_2$ to study $CaCO_3$ precipitation in liquid medium.

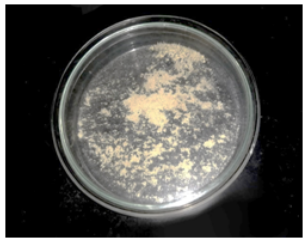

Figure 6: $CaCO_3$ powder obtained after filtration

Formation of calcium carbonate in the medium occurred as a result of urea hydrolysis to give ammonium ion and carbonates. The release of ammonium ion raised the pH of the medium which favoured the precipitation of carbonates as $CaCO_3$. The carbonates binds to calcium ions present in the medium leading to the formation of $CaCO_3$ that gets

deposited in broth. The reaction can be written as follows (De Muynck *et al*., 2010).

$CO(NH_2)_2 + 2H_2O \rightarrow 2NH_4^{++} CO_3^{2-}$

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

Qualitative test for calcium carbonate:

Using 2N HCl: When $CaCO_3$ powder reacted with 2N HCl it produced carbon-dioxide showing visible effervescence (figure 7) indicating a positive test for carbonate ions (Al-Omari *et al*., 2016). This is a preliminary test for carbonate ions.

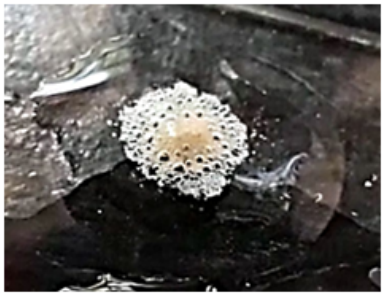

Figure 7: Effervescence seen after treating the obtained precipitate with 2N Hcl.

Using 2N ammonium oxalate: When dried $CaCO_3$ was dissolved in dilute 2N HCl, it formed soluble salt of $CaCl_2$. Thick white precipitate (figure 8) of calcium oxalate was observed when dissolved $CaCO_3$ was treated with 2N ammonium oxalate (Sinha and Gupta, 2016). This is a confirmatory test for determining the presence of calcium carbonate.

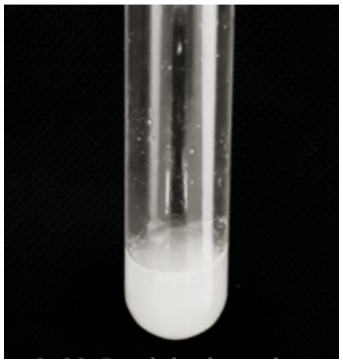

Figure 8: $CaCO_3$ precipitation using ammonium oxalate

Identification of isolate:

The potential isolate was characterized as aerobic, gram positive, rod shaped bacteria. From the biochemical test it was concluded that the isolate belong to the genus Bacillus (Bergy's Manual of Determinative Bacteriology, 8th edition, 1974). The isolate ISL-7 was identified as *Bacillus megaterium* tara 26 by 16 S rRNA gene sequence analysis and submitted to NCBI with accession no.LC333997.

Optimisation of physio-chemical parameters for maximum urease production by the isolate:

Effect of various media on urease production by the isolate:

Seven different crude media viz King's B, Tryptic soy broth, Luria Bertani, Brain heart infusion broth, Nutrient broth, Beef extract broth and Yeast extract broth were tested for maximum urease production. *Bacillus megaterium* tara26 gave maximum urease production (0.71 U/ml) in 50ml of Yeast extract broth supplemented with 5 ml of 0.33 M of filter sterilized urea which consisted of Peptone and Yeast extract which serve as organic carbon and nitrogen source respectively (figure 9). Yeast extract individually gave an increased enzyme production (0.57 U/ml) as compared to that of Peptone (0.40 U/ml), however when two were used in conjunction the production was enhanced (0.73 U/ml), indicating that Yeast extract supplemented with Peptone is a suitable medium for enhanced urease production. Yeast extract provides the organism with the essential growth factors and vitamins which are necessary for their growth. Williams et al. (2016) remarked that Meat extract supplemented with sodium acetate was an appropriate growth medium for *Sporosarcina pasteurii* in urease production. Whereas, Achal and Pan (2010) who carried out study of urease enzyme commented that Nutrient broth was found to be the best medium for urease production by *Bacillus megaterium* EU256395.

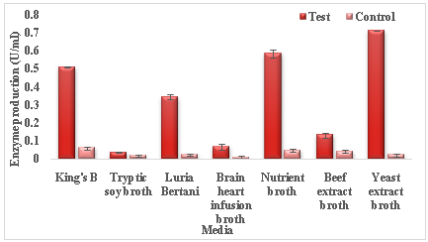

Figure 9: Effect of various media on urease production by the isolate

Similarly, *Sporosacrcina pastuerii* NCIMB 8841 and marine bacteria NCIMB also showed maximum urease production when they were grown in Nutrient broth supplemented with 2% urea (Zoheir *et al.,* 2013). In another study conducted by Elmanama and Alhour (2013) on *Bacillus mycoides*, the results revealed that the maximum urease production was seen when the organism was grown in Rabbit feed. Thus, crude media of different origin and composition were found to be supportive for urease production.

Effect of various carbon sources on urease production by the isolate:

The effect of different carbon source on production of urease by the isolate was analysed using the optimised medium i.e. Yeast extract broth. Since peptone alone contributed to lower urease production, various carbon sources i.e. 0.5% of Glucose, Fructose, Maltose, Mannitol, Sucrose, Xylose, Galactose, Lactose, Sorbitol and were used in place of Peptone. Among the tested carbon sources Xylose showed maximum urease yield (0.94 U/ml) followed by Lactose (0.91 U/ml), Fructose (0.82 U/ml), Sorbitol (0.75 U/ml), Sucrose (0.72 U/ml), Glucose (0.67 U/ml). Maltose (0.66 U/ml), Galactose (0.66 U/ml) and Mannitol (0.54 U/ml) as shown in figure 10. Hence, Xylose was used as a carbon source in the medium for further optimization studies.

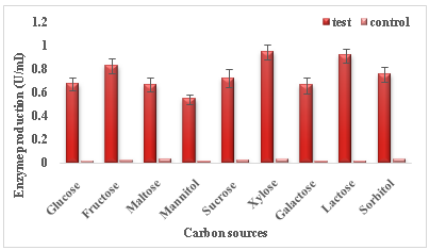

Figure 10: Effect of various carbon sources on urease production by the isolate

When varying concentrations of Xylose were used, *Bacillus megaterium* strain tara 26 showed maximum urease production (0.66 U/ml) in the presence of 0.2 % Xylose along with 0.33 M of filter sterilized urea. After reaching maxima, there was gradual decrease in urease production (figure 11). High concentration of Xylose might have led to high osmotic pressure which inhibited the organism growth and enzyme production. Also, when Xylose was readily available in sufficient concentration, the urease enzyme produced by *Bacillus megaterium* tara26 remained uninduced to utilize urea as it is a secondary carbon source and hence there was a marked decrease in urease production.

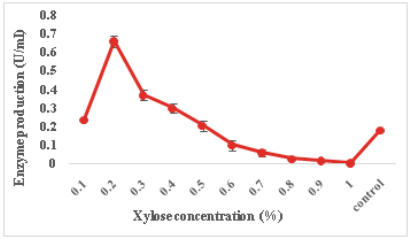

Figure 11: Effect of various concentrations of optimized carbon source on urease production by the isolate

In a study conducted by Ruth et al. (1998) 2% Glucose gave maximum urease production (0.93 U/ml) from urease producing *Corynebacterium glutamicum*. Marine Klebsiella sp showed

maximum urease production (1.72 U/ml) in the presence of 0.7% Glucose (Balan *et al*., 2012). Enterobacter sp showed maximum urease production (1.07 U/ml) in presence of 2% Glucose (Yang *et al.,* 2008). *Bacillus mycoides* showed maximum urease production in presence of rabbit feed (Elmanama and Alhour, 2013). *Sporosarcina pastuerii* showed maximum urease production (0.73 U/ml) in the presence of 1% lactose mother liquor (Williams *et al.,* 2016) this value is close to our obtained values. To the best of our knowledge, this is the first study which shows that Xylose is the preferred carbon source for maximum urease production.

Effect of various nitrogen sources on urease production by the isolate:

Besides the carbon source, the type of nitrogen source in the medium also influences the urease yield in the production broth. The effect of different nitrogen sources in Yeast extract broth with 0.2 % Xylose were analysed for maximum urease production using different nitrogen sources (0.3%) such as KNO_3, Glycine, Yeast extract, Sodium nitrite, Meat extract and Peptone. Maximum urease production (1.76 U/ml) by *Bacillus megaterium* strain tara 26 was seen with Meat extract followed by Yeast extract (1.39 U/ml), Peptone (0.91 U/ml), Glycine (0.81 U/ml), Sodium nitrite (0.52 U/ml) and Potassium nitrate (0.50 U/ml) as shown in figure 12.

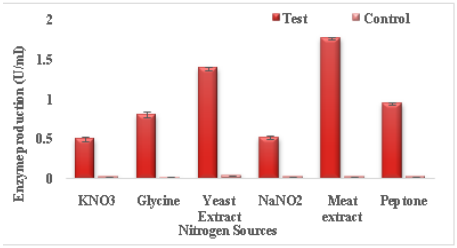

Figure 12: Effect of various nitrogen sources on urease production by the isolate

The present findings indicated that the supply of organic nitrogen sources like Meat extract, Yeast extract and Glycine resulted in high urease production as compared to the inorganic nitrogen sources such as Potassium nitrate and sodium nitrite. The organic nitrogen sources provided the organism with vitamins and co-factors which play a vital role in enzyme production. From the above result it could be interpreted that Meat extract may contain co-factors in considerable amount as compared to Yeast extract which contributed to increase in the enzyme production. However, Ghasemi et al. (2004) and Balan et al. (2012) stated Yeast extract and Peptone as the significant nitrogen source for maximum production of urease by Klebsiella spp and *Aspegillus niger* respectively. When different concentrations of Meat extract were used, *Bacillus megaterium* tara26 showed maximum urease production (0.70 U/ml) in the presence of 0.1% Meat extract along with 0.33 M urea while 0.3% meat extract (0.57 U/ml) was used as a control (figure13).

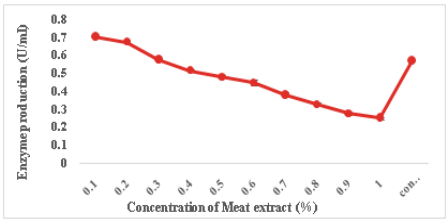

Figure 13: Effect of various concentrations optimized nitrogen source on urease production by the isolate

The urease production decreased with increase in the concentration of Meat extract. This was because *Bacillus megaterium* tara26 produces an inducible urease enzyme and in presence of readily available nitrogen sources it would never invest upon its ATP to regulate urease production. However, Joshi et al. (2016) reported maximum urease production in the presence of 1.5% yeast extract using *Bacillus subtilis*. Whereas, Kakelar and Ebrahimi (2016) testified that 0.5 % yeast extract was optimum for urease production by *Sporosarcina pasteurii.*

Effect of variable pH on urease production by the isolate:

The production of urease was analysed at different pH with a control (0.83 U/ml) maintained at pH 7. From the above results, *Bacillus megaterium* tara26 grown at pH 7.5 resulted in maximum urease production (0.93 U/ml) as shown in figure 14.

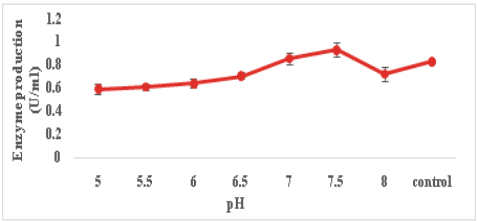

Figure 14: Effect of variable pH on urease production by the isolate:

Urease production gradually decreased at pH 8.0 due to the ionization of the side chains of which constitute the tertiary structure of urease enzyme. This result was in accordance to Seshabala and Mukkanti (2013) who reported maximum production at pH 7.0. Urease production is favoured under alkaline conditions which was also reported by other researchers for example Suzuki *et al.,* 1979, showed that maximum urease production was at pH 8.0 for *Bacillus multiacidus*. Other researchers (Balan *et al*., 2012; Mobley and Hausinger, 1989) stated that maximum urease production was seen at pH 7.0 and pH 8.2 in Klebsiella sp and *Campylobacter pylori* respectively.

Effect of incubation period on maximum urease production by the isolate:

The production of urease was analysed for 6, 12, 18, 24, 30, 36, 42 and 48hrs at shaker (125 rpm). The control was run in parallel at an incubation period of 24 hrs. In our study, *Bacillus megaterium* tara26 initially showed no urease production until 12 hrs. The production increased after 18 hrs and was found to be maximum (0.96 U/ml) at the time period of 36 hrs after which the production decreased (figure 15). The drop may be due to the saturation of active sites of the enzymes by the substrate molecules and was no longer involved in breakdown of it (Lehinger, 2002). Balan et al. (2012) reported 36 hrs as an ideal period for maximum urease production (1.7 U/ml) by Klebsiella sp which is similar to our result. A similar study by Yang et al. (2008) on Enterobacter sp, maximum urease production was seen at an incubation period of 36 hrs.

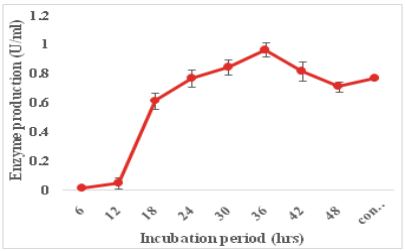

Figure 15: Effect of incubation period on maximum urease production by the isolate

Urease is an inducible enzyme and though the medium was supplemented with 0.33 M urea, the organism utilised the readily available nutrients and the urease enzyme remained uninduced. As the time period increased, the nutrients depleted and the organism switched to urea as its secondary carbon and energy source.

Effect of aeration on urease production by the isolate:

As shown in figure 16, the shaker culture of *Bacillus megaterium* strain tara 26 gave maximum urease production (0.41 U/ml) than culture incubated at static condition (0.011 U/ml). Hence, urease production was carried out under shaker conditions for further optimization studies.

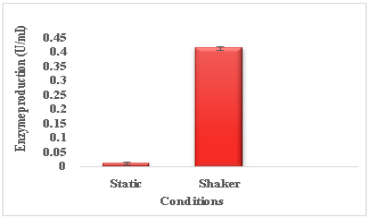

Figure 16: Effect of aeration on urease production by the isolate

Shaker conditions enhance the aeration rate and are preferred for extracellular enzymes production by aerobic microorganisms. In our study, aeration has been found essential for urease production as it leads to adequate supply of oxygen in culture medium. Other urease producing bacteria such as *Bacillus pasteurii* and *Bacillus subtilis* MBRL576 isolated by Navneet et al. (2011) and *Dhami* et al. (2013) also showed maximum production of urease under shaker conditions (180 rpm).

Effect of variable temperature on urease production by the isolate:
The urease production was analysed at different temperatures viz 4°C, 37°C, 45°C and 55°C. The urease production (0.79 U/ml) by B*acillus megaterium* strain tara 26 was optimum at 37°C which gave similar results with the control kept at 37°C (0.77 U/ml) (figure 17). The enzyme production decreased with higher temperature due to reduced growth rate. Balan et al. (2012) stated maximum urease production was seen at 37°C in marine bacterium Klebsiella. However, Seshabala and Mukkanti (2013) recorded increase in urease production with an increase in temperature and the highest reading for same was found at 35°C.

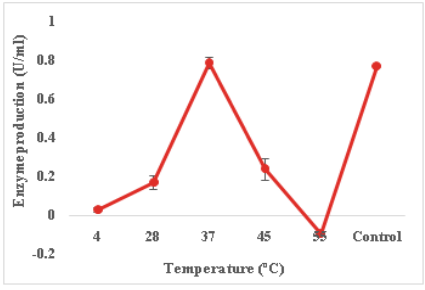

Figure 17: Effect of variable temperature on urease production by the isolate

However, further increase in temperature altered the enzyme structure and affected its catalytic property which resulted in decreased urease production (Akogal *et al.,* 2002). Enterobacter sp also showed maximum urease production (0.89 U/ml) at 35°C (Yang *et al.,* 2008).

Effect of optical density of the culture on urease production:
The production of urease was analysed using different O.D. ranging from 0.1 to 1 at 540 nm and a control (0.32 U/ml) was maintained at 0.5 O.D. As shown in figure 18, maximum urease production (0.66 U/ml) by *Bacillus megaterium* strain tara 26 was observed at high Optical density of 0.8.

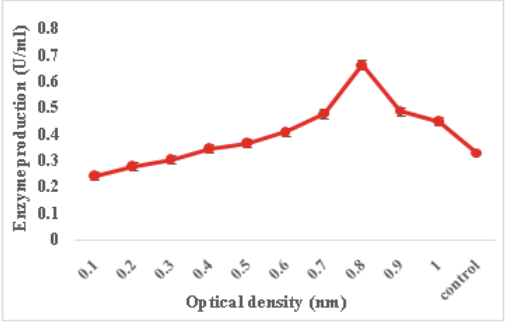

Figure 18: Effect of optical density of the culture on urease production

These results were supported by a study carried out by *Bacillus pastuerii* showed maximum urease production at OD of 0.6 (Achal *et al*., 2011). Our results were in accordance to author Varalakshmi and Anchana (2014) who reported increase in urease production in *Proteus vulgaris* within O.D. range of 0.8-1 indicating that use of higher optical density contributes to large amount of inoculum resulting in better urease production.

Effect of various inoculum size of the isolate on urease production:
Amongst all the inoculum sizes, urease production by *Bacillus megaterium* strain tara 26 was maximum (0.57 U/ml) with an inoculum volume of 2ml of 0.8 $O.D_{540nm}$. An inoculum size of 2ml was used as a control (0.54 U/ml). The enzyme production decreased with an increase in inoculum size (figure 19). An optimal inoculum level is essential to maintain a proper balance between proliferating biomass and nutrients available to obtain maximum enzyme yield.

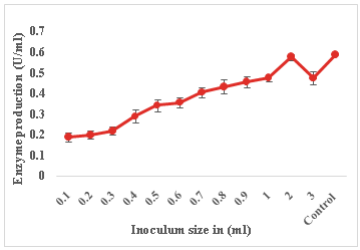

Figure 19: Effect of various inoculum size of the isolate on urease production

A lower enzyme yield at higher inoculum level could result from faster consumption of nutrients. Further, a large inoculum size could lead to formation of thick suspension and hence improper mixing of substrates. Similar results were obtained by Ramanathan et al. (2016) in Bacillus sp and Pseudomonas sp exhibiting maximum production with 2% inoculum. However, optimum inoculum size for *Proteus vulgaris* and *Aspergillus niger* was found to be with 3% and 1% respectively (Smith *et al*., 1993; Varalakshmi and Anchana, 2014).

Effect of urea concentration on urease production by the isolate:
The substrate urea was optimised from 0.2 – 4M along with a control (0.47 U/ml) and 0.8M concentration of urea gave maximum urease production of (0.69 u/ml) and was able tolerate urea up to a concentration of 1M (figure 20). Eventually, as the substrate concentration increased, it exhibited a negative effect on urease production. Initial increase in the enzyme production was due the availability of active sites which were not bound by the substrate.

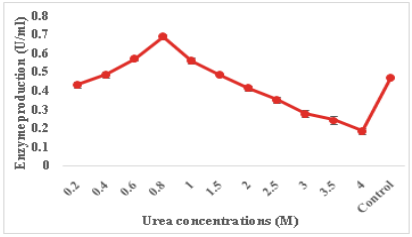

Figure 20: Effect of urea concentration on urease production by the isolate

However, an abrupt decrease in production with increasing concentration of substrate was because of saturation of the active sites by binding of the substrate (*Pozniak et al.,* 1995). Other studies have recorded maximum urease production at urea concentration of 2M and 3M using *Proteus vulgaris* and Bacillus sp respectively (Khanafari *et al*., 2011; Ramanathan *et al*., 2016). Other researchers, Balan et al. (2012) reported maximum urease production (2.25 U/ml) at urea concentration of 0.05M in Klebsiella sp and further when the substrate concentration was increased the growth of the organism was inhibited and the production of urease decreased as observed in present study.

Scanning electron microscopic observation of calcium carbonate crystals:
Ureolytic bacteria in higher concentrations of urea and calcium usually produce two types of calcium carbonate crystals viz. rhombohedral and spherical (Chunxiang *et al*., 2009). The SEM images of calcium carbonate crystals precipitated by *Bacillus megaterium* tara 26 were spherical and the bacteria were found to be in close association with the crystals as shown in (figure 21) this indicates that bacilli provide favourable conditions by serving as a nucleation site during precipitation reaction (Stock-Fisher *et al*., 1999). This result was in consistent with that of Zamarreno et al. (2009) observed from the strain of *Pseudomonas putida* F2.

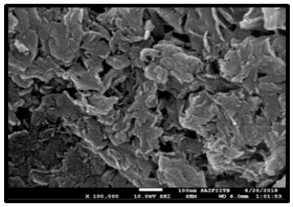

Figure 21: Scanning electron microscopic observation of calcium carbonate crystals

In a study conducted by Al-Thawadi and Cord-Ruwisch (2012) and Chu and Ivanov (2012) both rhombohedral as well as spherical deposits were observed whereas in another study by (Bang *et al*., 2001) rhombohedral crystals were seen. Li et al. (2010) reported that the crystal morphologies of precipitates induced by Bacillus sp. dominantly showed polyhedral and cubic crystals. Many factors could affect the type and morphology crystals such as concentrations of urea and calcium ions (De Muynck *et al*., 2013), urease production, accessibility of nucleation sites (De Muynck et al., 2010), production of extracellular polymeric substances (Braissant *et al*., 2003), pH and temperature (De Muynck *et al*., 2010). However, SEM provides only the morphology of crystals, further characterization and confirmation of the calcium carbonate polymorph can be done by Energy Dispersive X-Ray spectra analysis and X-ray Diffraction method (Navneet *et al*., 2011; Dhami *et al.*, 2013; Chunxiang *et al*., 2009; Kumar e*t al.*, 2013).

Application of *Bacillus megaterium* tara26 for remediation of hard water:

The total water hardness by EDTA titration for six water samples were tested (figure 21). The bacterium was capable of reducing water hardness, the percent reduction observed was more than 60% for SP-01, SD-02 and SK-05 water samples (figure 21). SP-01, SD-02 and SK-05 water samples having an initial total hardness of 296, 292 and 294 mg/L was reduced in period of 14 days and percentage reduction was found to be 62, 68 and 66% respectively. Whereas, municipal water samples SC-03 and SA-04 collected from different localities in Mumbai city, had a total water hardness of 56 and 188 mg/L respectively were reduced to soft water in a period of 14 days with the application of *Bacillus megaterium* tara26 and percent reduction was found to be 41 and 56 % respectively.

Figure 21: Titration of hard water samples against 0.01M EDTA

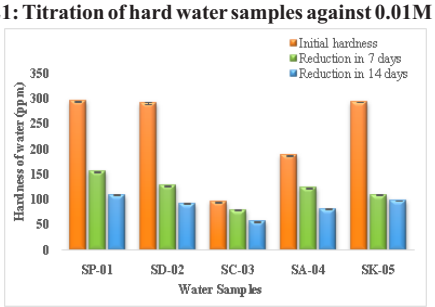

Figure 22: Reduction of hardness of different water samples using *Bacillus megaterium* tara26.

Khanafari *et al*. (2011) used *Proteus vulgaris* PTCC 1079 to determine the reduction of water hardness by EDTA titration for two natural sources (Fashk village and Kobar dam). The reduction of hard water to soft water was achieved when the organism could break down urea to create alkaline conditions enabling the calcium ions in water to precipitate as calcium carbonate thereby, reducing the calcium concentration present in water and making it soft. Chemical methods for reducing water hardness include Ion Exchange resins which can reduce water hardness up to 2 ppm however, the equipment is costly making it less economical. Conversely, lime soda process is inexpensive but large amount of insoluble precipitate generated, poses disposal problems (Richards and Reynolds, 1995). Exploiting microorganisms to treat hard water could be a suitable replacement for chemical methods as it makes the process cost effective and environmental friendly.

CONCLUSION:

In this study, urease producer *Bacillus megaterium* tara26 was isolated from marble quarry, which has the ability to produce the enzyme urease. The enzyme is catalytically active (1.49 U/ml) in an optimised medium supplemented with 0.8M urea at 37^{o}C and pH 7.5. Application of urease in bioremediation of hard water was studied and the hardness for five water samples i.e. SP-01, SD-02, SA-04 and SK-05 were found to be more than 60%. Whereas, with SC-03, the reduction was only production 41%. Further, implementation of strain improvement program can enhance the enzyme at lower concentration of substrate urea. Urease enzyme have been used as fillers in rubber and plastic industries. Moreover, urease enzyme also plays a significant role in wine industry, the stored wine is treated with acid urease to remove urea thereby preventing a formation of ethyl carbamate (carcinogen) from urea and ethanol. The potential of calcium carbonate precipitate by bacteria has brought a new revolution for solving environmental problems like removal of chemical pollutants such as heavy metals from industrial waste water by precipitating them as carbonates of heavy metals.

REFERENCES

1. Achal, V., and Pan, X. (2011), "Characterization of urease and carbonic anhydrase producing bacteria and their role in calcite precipitation." Current Microbiology, 62, 894–902. https://doi.org/10.1007/s00284-010-9801-4.
2. Achal, V., Pan, X., and Ozyurt, N. (2011), "Improved strength and durability of fly ash-amended concrete by microbial calcite precipitation." Ecological Engineering, 37(4), 554–559. https://doi.org/10.1016/j.ecoleng.2010.11.009.
3. Achal, Varenyam & Pan, Xiangliang. (2010),"Characterization of Urease and Carbonic Anhydrase Producing Bacteria and Their Role in Calcite Precipitation." Current microbiology. 62. 894-902. 10.1007/s00284-010-9801-4.
4. Afifudin, H., Hamidah, M. S., Noor Hana, H., and Kamaruddin, K. (2011), "Microorganism Precipitation in Enhancing Concrete Properties." Applied Mechanics and Materials, 99–100, 1157–1165. https://doi.org/10.4028/www.scientific.net/amm.99-100.1157.
5. Akogal, S., Yagmur, Y., Gulay, B., Adil, D., Yakuparica, M. (2002),"Reversible immobilization of urease on to Procion Brown MX-5BR-Ni (II) attached polyamide hollow fibre membranes." Process Biochemistry, 38, 675-683 (9 pages).
6. Al Omari, M.M.H., Rashid, I.S., Qinna, N.A., Jaber, A.M., and Badwan, A.A. (2016), "Calcium Carbonate." Profiles of Drug Substances, Excipients and Related Methodology, 41, 31-132. https://doi.org/10.1016/bs.podrm.2015.11.003.
7. Al-Thawadi, S., and Cord-Ruwisch, R. (2012), "Calcium carbonate crystals formation by ureolytic bacteria isolated from australian soil and sludge." Journal of Advanced Science and Engineering Research, (2), 12-26.
8. Balan, S., Fathima, F. and Jayalakshmi, S. (2012), "Characterization of urease enzyme from marine bacterium Klebsiella species." African Journal of Microbiology Research, 6(30), 5914–5923. doi: 10.5897/AJMR12.218.
9. Bang, S. S., Galinat, J. K., and Ramakrishnan, V. (2001), "Calcite precipitation induced by polyurethane immobilized Bacillus pasteurii." Enzyme and Microbial Technology, 28, 404–409.
10. Bauerfeind, P., Garner, R., Dunn, B. E., & Mobley, H. L. (1997), "Synthesis and activity of Helicobacter pylori urease and catalase at low pH." Gut, 40(1), 25–30. https://doi.org/10.1136/gut.40.1.25
11. Bharathi, N., and Meyyappan, R. M. (2015), "Potentiality of yeast strain on cement concrete specimen." International Journal of Science, Engineering and Technology Research, 3:12.
12. Boquet, E., Boronat, A., and Ramos-Cormenzana, A. (1973), "Production of calcite (calcium carbonate) crystals by soil bacteria is a common phenomenon." Nature, (246), 527–529. doi: 10.1038/246527a0.
13. Braissant, O., Cailleau, G., Dupraz, C., & Verrecchia, E. P. (2003),"Bacterially induced mineralization of calcium carbonate in terrestrial environments: the role of exopolysaccharides and amino acids." Journal of Sedimentary Research, 73, 485–490.
14. Burbank, M.B., Weaver, T.J., Williams, B.C., and Crawford, R.L. (2012), "Urease Activity of Ureolytic Bacteria Isolated from Six Soils in which Calcite was precipitated by Indigenous Bacteria." Geomicrobiology journal, 29:4, 389-395. https://doi.org/10.1080/01490451.2011.575913.
15. Burne, R.A. and Chen, Y.Y. (2000), "Bacterial ureases in infectious diseases." Microbes andInfection, (2): 533-542.
16. Canakci, H., Sidik, W., and Halil K. I. (2015), "Effect of bacterial calcium carbonate precipitation on compressibility and shear strength of organic soil." Soils and Foundations, 55(5), 1211–1221. doi: 10.1016/j.sandf.2015.09.020.
17. Chahal, N., Rajor, A and Siddique, R. (2011), "Calcium carbonate precipitation by different bacterial strains." African Journal of Biotechnology, 10(42), 8359-8372. https://doi.org/10.5897/AJB11.345.
18. Chu, J., & Ivanov, V. (2012), "Microbially induced calcium carbonate precipitation on surface or in the bulk of soil." Geomicrobiology Journal, 29:6, 544-549. https://doi.org/10.1080/01490451.2011.592929.
19. Chunxiang, Q., Jianyun, W., Ruixing, W., and Liang C. (2009), "Corrosion protection of cement-based building materials by surface deposition of CaCO3 by Bacillus pasteurii." Materials Science and Engineering C, 29(4), 1273–1280. doi:10.1016/j.msec.2008.10.025.
20. De Muynck, W., Belie, N. De., and Verstraete, W. (2010), "Microbial carbonate precipitation in construction materials." Ecological Engineering, 36, 118–136. https://doi.org/10.1016/j.ecoleng.2009.02.006.
21. De Muynck, W., K. Verbeken, N. De Belie and W. Verstraete, (2013). "Influence of temperature on the effectiveness of a biogenic carbonate surface treatment for limestone conservation." Applied Microbiology and Biotechnology, 97: 1335-47.
22. Devi, A., and Varalakshmi. (2014), "Isolation and characterization of urease utilizing bacteria to produce biocement." IOSR journal of environmental science, toxicology and food technology,8(4), 2319–2399. https://doi.org/10.9790/2402-08425257.
23. Dhami, N. K., Reddy, M. S. and Mukherjee, M. S. (2013), "Biomineralization of calcium carbonates and their engineered applications: A review." Frontiers in Microbiology, 1–13. doi: 10.3389/fmicb.2013.00314.
24. Elmanama, A. A., and Alhour, M. T. (2013), "Isolation, characterization and application of calcite producing bacteria from urea rich soils." Journal of Advanced Science and Engineering Research, 3(4), 388–399.
25. Farley, P. C., and Sugiarto, S. (2003), "Regulation of expression of the Rhizopus oryzae uricase and urease enzymes." Canadian journal of microbiology, 48, 1104-8. doi:10.1139/w02-103.
26. Fujita,Y., Taylor J.L., Gresham, T., Delwiche, M., Colwell, F., McLing, T., Petzke, L., and Smith, R. (2008), "Stimulation of microbial urea hydrolysis in groundwater to enhance calcite precipitation." Environmental Science & Technology, 42 (8), 3025-3032. DOI: 10.1021/es702643.
27. Ghasemi, M. F., Bakhtiari, M. R., Fallahpour, M., Noohi, A., Moazami, N., and Amidi, Z. (2004), "Screening of urease production by Aspergillus niger strains." IranianBiomedical Journal, 8(1), 47–50.
28. Hammes, F., Boon, N., Villiers, J., Verstraete, W., and Siciliano, S. D. (2003), "Strain-specific ureolytic microbial calcium carbonate precipitation." Applied and

Environmental Microbiology, 69(8), 4901–4909. https://doi.org/10.1128/AEM.69.8.4901
29. Helmi, F. M., Elnagdy, S. M., Hemdan, E.R., and El-Hagrassy, A. F. (2016), "Calcium carbonate precipitation induced by ureolytic bacteria Bacillus licheniformis." Ecological Engineering, 90, 367-371. https://doi.org/10.1016/j.ecoleng.2016.01.044
30. Herzog, H. J., and Drake, E. M. (1996), "Carbon Dioxide Recovery and Disposal from Large Energy Systems." Annual Review of Energy and the Environment, 21, 145-166. http://dx.doi.org/10.1146/annurev.energy.21.1.145
31. Jokyani, D. H., and Chouhan, D. (2018), "Isolation, Characterization, and Application of Calcite Producing Bacteria for Self-Healing Concrete Preparation." International Journal of Life-Sciences Scientific Research, 4. doi:10.21276/ijlssr.2018.4.5.10.
32. Jones, B. D., and Mobley, H. L. (1987), "Genetic and biochemical diversity of ureases of Proteus, Providencia, and Morganella species isolated from urinary tract infection," Infection and immunity, 55(9), 2198–2203.
33. Joshi, K.A., Kumthekar, M. B., and Ghodake, V. P. (2016), "Bacillus Subtilis Bacteria Impregnation in Concrete for Enhancement in Compressive Strength." International Research Journal of Engineering and Technology, 3, 1229-1234.
34. Kahani, M., Kalantary, F., Soudi, R., Pakdel, L., and Aghaalizadeh, S. (2019), "Optimization of cost effective culture medium for Sporosarcina pasteurii as biocementing agent using response surface methodology: Up cycling dairy waste and seawater." Journal of cleaner production, 253. https://doi.org/10.1016/j.jclepro.2020.120022
35. Kakelar, M. M., and Ebrahimi, S. (2016),"Up-scaling application of microbial carbonate precipitation: optimization of urease production using response surface methodology and injection modification." International journal of environmental science and technology, 13, 2619-2628. doi: 10.1007/s13762-016-1070-8.
36. Kakimoto, S., Yasuhiro, S., Shun-Ichi, A., and Yoshio, N. (1989), "Purification and characterization of acid urease from Lactobacillus reuteri." Agricultural and BiologicalChemistry, 53(4), 1119-1125. doi:10.1271/bbb1961.53.1119.
37. Kantzas A, Ferris FG, Stehmeier L, Marentette DF, Jha KN, Mourits FM. (1992),"A novel method of sand consolidation through bacteriogenic mineral plugging." (CIM 92-46). In: Proceedings of the CIM annual technical conference, vol 2. Petroleum Society of CIM, Calgary, Canada, 1–15
38. Kaur, N., Reddy, M. S. and Mukherjee, A. (2013), "Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites." Journal of Microbiology and Biotechnology, 23(5), 707–714. doi: 10.4014/jmb.1212.11087.
39. Khanafari, A., Khams, F., and Seddpahy, A. (2011), "An Investigation of Biocement Production from Hard Water." Middle-East Journal of Scientific Research, 7(6), 964-971.
40. Khanafari, A., Khams, F., Seddpahy, A., Park, K., Jun, S., Kim, D., Kumar, R. S. (2013), "Isolation and characterization of urease utilizing bacteria to produce biocement." Ecological Engineering, 8(4), 336–338. https://doi.org/10.4014/jmb.1212.11087.
41. Kim, J. K., Mulrooney, S. B., and Hausinger, R. P. (2005), "Biosynthesis of active Bacillus subtilis urease in the absence of known urease accessory proteins." Journal of bacteriology, 187(20), 7150–7154. https://doi.org/10.1128/JB.187.20.7150-7154.
42. Kouhounde, S. H., M. K., Somda M. K., Bokossa, Y. I., and Baba, L. M. (2015), "Screening of microorganisms producing polygalacturonase (PG) in microbiota of fermented cassava." International Journal of Biochemistry and Biotechnology, 4(2), 537-543,
43. Kumar, J., Prabhakara, R., and Pushpa (2013), "Bio Mineralisation of Calcium Carbonate by Different Bacterial Strains and Their Application in Concrete Crack Remediation." International Journal of Advances in Engineering & Technology, 6(1), 202–213.
44. Li, W., Liu, L., and Chen, W. (2010), "Calcium carbonate precipitation and crystal morphology induced by microbial carbonic anhydrase and other biological factors." Process Biochem, 45:1017-1021.
45. Mackay, E.M., and Pateman, J.A. (1982), "The regulation of urease activity in Aspergillus nidulans. Biochemical Genetics, 20, 763–776. https://doi.org/10.1007/BF00483972
46. Maleki, M., & Ebrahimi, S. (2016), "Up-scaling application of microbial carbonate precipitation: optimization of urease production using response surface methodology and injection modification."International Journal of Environmental Science and Technology, 13, 2619–2628. https://doi.org/10.1007/s13762-016-1070-8.
47. Mobley, H.T. and Hausinger, R. (1989),"Microbial ureases: Significance, regulation, and molecular characterization." Microbiological reviews, 53. 85-108. 10.1128/MMBR.53.1.85-108.1989.
48. Navneet, C., Anita, R., and Rafat, S. (2011), "Calcium carbonate precipitation by different bacterial strains." African Journal of Biotechnology, 10(42), 8359–8372. https://doi.org/10.5897/AJB11.345.
49. Nelson, D. L., Cox, M. M., & Lehninger, A. L. (2013), "Principles of Biochemistry, 3rd Ed." Published by Worth publishers. www.worthpublishers.com/lehninger
50. Perito, B., Marvasib, M., Barabesia, C., Mastromeia, G., Braccic, S., Vendrelld, M., Tiano, P. (2014), "A Bacillus subtilis cell fraction (BCF) inducing calcium carbonate precipitation: Biotechnological perspectives for monumental stone reinforcement." Journal of Cultural Heritage, 15(4), 345–351. doi: 10.1016/j.culher.2013.10.001.
51. Poznaik, G.,Korjewska, B., and Trochisczuk, W. (1995),"Urease immobilized on modified polysulphone membrane, preparation and properties." Biomaterials, 16, 129-134.
52. Priya, N.J., and Kannan, M. (2017), "Effect of carbonic anhydrase and urease on bacterial calcium carbonate precipitation." International Journal of Pharma and Bio Sciences, 8(3), 609–614.
53. Ramakrishnan, V., Panchalan, R.K., and Bang, S.S. (2005), "Microbial Participation in the Formation of Calcium Silicate Hydrated (CSH) from Bacillus subtilis." Procedia Engineeering, 20 (2011) 159 – 165.
54. Ramanathan, G., Kumar, T. V., Rama, R., and Vijayalalitha, R. (2016), "Isolation of cement degrading bacteria and screening of their efficacy for biocementation." Journal of Pharmaceutical, Chemical and Biological Sciences, 3(4): 518–527.
55. Richards, P.A., and Reynolds, T.D. (1995), "Unit Operations and Processes in Environmental Engineering." Second Edition, published by PWS publishing company.
56. Rivadeneyra, M.A., Delgado I. R., Quesada, E., and A. Ramos-Cormenzana (1991), "Precipitation of Calcium Carbonate by Deleya halophila in Media Containing NaC1 as Sole Salt." 22, 185–186.
57. Ruth, M. S., Weil B., Burkovski, A., Eggeling, L., Krämer R., and Jahns, T. (1998),"Urea uptake and urease activity in Corynebacterium glutamicum." Arch Microbiol, 169, 411–416.
58. Seshabala, P., and Mukkanti, K. (2013), "Isolation of urease rich bacteria and determination of its optimal conditions." Indian Journal of Applied Research, 3(7), 336–338.
59. Sharma, A., and Bhattacharya, A. (2010), "Enhanced biomimetic sequestration of CO2 into CaCO3 using purified carbonic anhydrase from indigenous bacterial strains." Journal ofMolecular Catalysis B Enzymatic, 67, 1-162. doi"10.1016/j.molcatb.2010.07.016.
60. Sinha, N., and Gupta, R. (2016), "Lab Manual chemistry." New Delhi, New Saraswati house (India) Pvt. Ltd.
61. Smith, P. T., King, A. D., and Goodman, N. (1993), "Isolation and characterization of urease from Aspergillus niger." Journal of General Microbiology, 139(5), 957–962. https://doi.org/10.1099/00221287-139-5-957.
62. Stocks-Fischer, S., Galinat, J. K., and Bang, S. S. (1999), "Microbiological precipitation of CaCO3." Soil Biol. Biochem, 31, 1563–1571.
63. Strickland, J. D. H., and Parson, T. R. (1972), "A practical handbook of sea water analysis, 2nd Ed Ottawa." published byFisheries Research Board of Canada.
64. Suzuki K, B., Mitsuoka, T., Takebe S., Kobashi, K., and Hase, J. (1979),"Urease-producing species of intestinal anaerobes and their activities." Applied and Environmental Microbiology, 37(3): 379–382.
65. Tiano, P., Biagiotti, L., and Mastromei, G. (1999), "Bacterial bio-mediated calcite precipitation for monumental stones conservation: Methods of evaluation." Journal of MicrobiologicalMethods, 36, 139-145.
66. Williams, S. L., Kirisits, M. J., and Ferron, R. D. (2016), "Optimization of growth medium for Sporosarcina pasteurii in bio-based cement pastes to mitigate delay in hydration kinetics." Journal of Industrial Microbiology and Biotechnology, 43(4), 567–575.
67. Yang, L., Wang S., and Tian, Y. (2008), "Purification, Properties, and Application of a Novel Acid Urease from Enterobacter sp." Applied Biochemistry and Biotechnology, 160, 303–313.doi:1007/s12010-008-8159-6.
68. Zamarreño, D.V., Inkpen, R. and May, E.(2009),"Carbonate Crystals Precipitated by Freshwater Bacteria and Their Use as a Limestone Consolidant." Applied and Environmental Microbiology, 75: 5981-5990.
69. Zoheir, A. E., Hammad, I. A., and Talkhan, F. N. (2013), "Urease activity and induction of calcium carbonate precipitation by Sporosarcina pasteurii NCIMB 8841." Journal of Applied Sciences Research, 9(3), 1525–1533.