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Investigation on Isolation and Purification of Calcium Carbonate from Soil Sample

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Abstract
Biomineralization of calcium in the form of carbonates finds broad range of scientific and technical applications. The concept of $CaCO_3$ precipitation was chosen, because it has multi functions both in industry and in human life. The present investigation has focused on the isolation of $CaCO_3$ from soil sample. The organism which possessed the ability to produce urease, solubilize $CaCO_3$ and crystallize $CaCO_3$ was identified as *Brachybacterium paraconglomeratum* using rRNA sequencing analysis. After the period of incubation for 15 days and 30 days by *Brachybacterium paraconglomeratum*, the yield of the $CaCO_3$ was identified to be 0.38 mg/ml and 10.12 mg/ml. The isolated $CaCO_3$ was confirmed using qualitative assay and the different sized $CaCO_3$ was viewed under the microscopic analysis. Thus, the present investigation has put forth the result that soil bacteria, *Brachybacterium paraconglomeratum* possess the ability to precipitate $CaCO_3$.

Keywords
Brachybacterium paraconglomeratum, determination of yield, Precipitating bacteria, production of $CaCO_3$, qualitative analysis, rRNA sequencing, screening for $CaCO_3$ crystals, solubility test, Urease test.

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INTRODUCTION:
Mineralization or mineral precipitation is a general property found among many microorganisms (Dupraz *et* al., 2009a; Lowenstam and Weiner 1989). Wide text exists on microbial contributions in carbonate precipitation in natural environments and laboratory experiments (Sanchez-Roman *et* al., 2007). The precipitation of calcium carbonate is originated to be the one of the most appropriate illustration of mineralization that can be sketched back to the Precambrian period.
Calcium carbonate or $CaCO_3$ is found to be the most recognizable minerals and comprises more than 4% on the earth's crust. It is found throughout the world that is usually found in the natural form as chalk, limestone and marble. This natural form of calcium carbonate is found to be produced by the sedimentation of the shells of little fossilized snails and shellfish. (Castanier, Le Metayer-Levrel & Perthuisot *et* al., 1999). Ground calcium carbonate (GCC) and precipitated calcium carbonate (PCC) are found to be the 2 sources of calcium carbonate, in the world. GCC is obtained from the earth and is available in varying quantities in the appearance of calcite, aragonite, vaterite, limestone, chalk, marble of travertine. (Kilic, 2015). Depending on the response conditions and impurities in the process, PCC can be found in three main crystal polymorphs: Calcite (rhombohedral), aragonite (orthorhombic), and vaterite (hexagonal).
The mechanism in which bacteria precipitate calcium carbonate can occur through a number of different pathways. Calcium ion concentration, pH and the occurrence of nucleation sites control the chemical

precipitation of calcium carbonate. In microbial calcium carbonate precipitation, bacteria themselves act as nucleation sites in which calcium ions bind to microbial cell surfaces. When these external become saturated with calcium ions, the ions commence to aggregate, thus starts the first urease crystalline nuclei which then cultivate from these structures. (M. Singh, 2019)

MICP as a new technique is found to be better than the conventional chemical methods, which are environmentally harmful, often toxic and expensive. MICP method is very effective in increasing the strength parameters in the ground such as shear strength and decreasing the permeability of gravelly and sandy soil where the same was confirmed in many literatures. (Katarzyna *et* al*,* 2018). The amount of calcite precipitation by MICP is increased by the high concentrations of bacterial cells (from 10^6 to 10^8 cells), which increases the urease concentration for urea hydrolysis (Okwadha and Li 2010). Hence it is noted that the urea hydrolysis has a direct relationship with bacterial cell concentrations (Ng *et* al*.* 2012).

Calcium carbonate, which is most commonly known as calcitic lime or garden lime, has long been a staple in backyard, flied and lawn management. Calcium carbonate is a great source of calcium. Calcium is an important nutrient that strengthens a plant's cellular walls, and it is found to be vital in new cell development.

MATERIALS AND METHODS:

In the present study, for isolation and purification of calcium carbonate from soil bacteria, the following materials and method were followed.

Isolation of $CaCO_3$ precipitating strain – Serial dilution method.

1 gm of soil sample collected from banana farm at Sithalapakkam, Chennai was suspended in 100 ml of 0.9% saline. It was subjected to serial dilution. 0.1ml of serially diluted sample was taken from 1, 3, 5, 7 and 9 tubes which were then inoculated in the petridish containing calcium urea agar medium. It was incubated at 37^0 C for 10 days.

Identification of bacterial strain

Colonies were primarily classified based on morphology and further characterized based on their shape and color. The positive 2 individual colonies were finally chosen based on their visual crystal formation and purified by a frequent streaking on the calcium urea agar media. The isolated strains were analyzed using gram staining and confirmed using rRNA studies.

Screening for $CaCO_3$ crystal formation

Urease activity assay

Two strains isolated from 5th plate was subjected for their urease activity on the urea agar media containing Peptone (1.0 g/l), Dextrose (1.0 g/l), Sodium chloride (5.0 g/l), and Monosodium phosphate (2.0 g/l), Urea (20 g/l), Phenol red (12.0 mg/l), Agar (15 g/l) pH was adjusted to 6.8. 5µL cell suspension of each candidate strain was inoculated on the urea agar media and the plates were incubated for 1-2 days at 28^0 C. The urease activity was determined on the media to the extent of the indication of the pink-red color, which specifically indicates the generation of alkaline conditions that are attributed to the production of ammonia via urease activity on urea.

Calcium carbonate solubilization test

2 strains isolated from the calcium carbonate precipitation agar plates were tested for their solubilization capability of calcium carbonate for the structure of clear halo in the region of a colony. Hence the calcium carbonate media was prepared with the Yeast extract (0.5 g/l), Dextrose (10 g/l), $CaCl_2$ (5 g/l), $(NH_4)_2SO_4$ (0.5 g/l), $Ca_3(PO_4)_2$ of (5 g/l), KCl (0.2 g/l), $MgSO_4$ (0.1 g/l), $MnSO_4$ (0.0001 g/l), $FeSO_4$ (0.0001 g/l), Agar (20 g/l), pH 7.0. The strains were inoculated in the media prepared in the petri dish plate by making a small hole with the help of the well cutter and then the colonies were grown at 28^0 C for 5-10 days. The solubilization capability of calcium carbonate was quantified by measuring the diameter of the clear halo.

Organism identification using rRNA sequencing.

Colonies were primarily classified based on morphology and further characterized based on their shape and color. The positive 2 individual colonies were finally chosen based on their visual crystal formation and purified by a frequent streaking on the calcium urea agar media. The isolated strains were analyzed using gram staining and confirmed using rRNA studies. According to morphological and biochemical identification result, potential isolates were selected for molecular and phylogentic identification. The isolated genomic DNA was further used as a template in PCR amplification of 16S rRNA. The Universal primers used for amplification of 16S rRNA are 16S-RS-F (forward) 5'CAGGCCTAACACATGCAAGTC-3' and 16S-RS-R (reverse) 5'GGGCGGWGTGTACAAGGC-3'. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1 X PCR buffer (100mM Tris HCl, pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM MgCl2, one unit of AmpliTaq Gold DNA polymerase enzyme catalyst, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward

and reverse primers and model DNA. The amplification reactions was performed in a PCR thermal cycler (GeneAmpPCR System 9700, Applied Biosystems) that was programmed as: Initial denaturation at 95˚C for 5 mins, Denaturation for 35 cycles at 95˚C for 30 sec, Annealing at 60˚C for 40 sec, Extension at 75˚C for 60 sec and Final extension at 72˚C for 7 mins. The PCR products were stored at -4˚C for further use. The amplified products were quantified in 1.2% agarose gel electrophoresis. PCR product (5 L) and 1 L loading dye was loaded along with 5 µl of 1 kb ladder into the separate wells. The gel was run at 100 volts for 1 hour and amplified products were visualized under UV trans illuminator.

Production of $CaCO_3$

For the production of $CaCO_3$, only the B3 positive strain were inoculated in 250 ml of urea broth containing nutrient broth (3 g/l), Urea (20 g/l), $NaHCO_3$ (2.12 g/l), NH_4Cl (10 g/l) and $CaCl_2.2H_2O$ (25 g/l). The inoculated culture in the broth is kept in the shaker at 50^0 C for the cell to grow and spread evenly in the conical flask and also incubated for 15 and 30 days.

Determination of yield

To quantify the yield, after the period of incubation at 15 days and 30 days the calcium carbonate crystals formed in the conical flask were filtered separately with the help of what's man filter paper. Also, the crystals settled at the bottom of the flask were removed with the help of spatula and the same was washed with the distilled water. Post filtering the calcium carbonate crystals, the same was allowed to dry for 2 days. Finally, the dried samples were weighed at a periodic interval of 15 days and 30 days accordingly.

Qualitative Analysis of isolated $CaCO_3$:

The qualitative analysis performed in this research was to confirm that to the isolated crystal dilute H_2SO_4 (0.03 ml H_2SO_4 diluted to 10 ml with distilled water) was added drop by drop to the dried $CaCO_3$ crystals taken in the test tube. Formation of effervescence indicates the presence of $CaCO_3$.

Microscopic observation of $CaCO_3$ crystals

The isolated $CaCO_3$ crystals were placed in the microscopic slide and reviewed under 10x and 100x magnification.

RESULTS AND DISCUSSION:

In the current study "Isolation of $CaCO_3$ from the soil bacteria", the following results were obtained and further interpreted.

Isolation of $CaCO_3$ precipitating bacteria - Serial dilution.

Biomineralization of calcium in the form of carbonates finds broad range of scientific and technical applications. The concept of $CaCO_3$ precipitation was chosen in this present study because it has multi functions both in industry and in human life. For isolating the Calcium carbonate precipitating strains from soil sample, the soil sample were suspended in a sterile saline solution (0.9% NaCl) and subjected to serial dilution which is picturized in Fig no: 1

Fig no: 1. Serial dilution in saline solution

Identification of $CaCO_3$ precipitating bacteria.

Fig no: 2 depict the serially diluted sample inoculated plates. By analyzing the plates, it was evident that in the plate no: 5, the growth of colonies was seen. The growth of the organism in calcium agar medium indicated that the strains were able to mineralize $CaCO_3$ by utilizing the nutrient supplied by the calcium urea media. The current study was also supported by Navneet Chahal *et* al., 2011 and Hyun Jung Kim *et* al, 2016

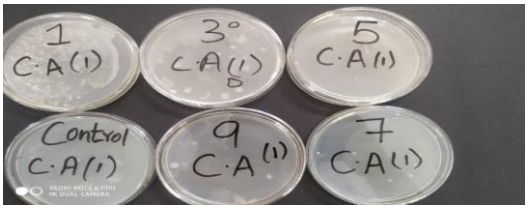

Fig no: 2. Isolation of bacteria in the calcium urea media

Selection of $CaCO_3$ precipitating strain

The two colonies grown in the 5th plate was further used for various studies. It was denoted as B2 and B3. Both the strains were individually inoculated in Calcium agar plates for pure culture isolation which is depicted in Fig no: 3

Fig no: 3 Selection of calcium carbonate precipitating strain

Screening for $CaCO_3$ crystal formation

After the period of incubation, it was noted that in the B3 strain inoculated plate, crystal formation was observed which is picturized in Fig no: 4 Hence, it is also evident that only the B3 strain possess the characteristics of crystal formation.

Fig no: 4 Screening for $CaCO_3$ crystal formation

Screening for Urease production

As a confirmative, the isolated 2 strains were then assayed for urease production which is depicted in Fig no: 5.

The results revealed that, only B3 strain inoculated plate developed pink coloured colony. The appearance of pink color specifically indicates the generation of alkaline conditions that are attributed to the production of ammonia via urease activity on urea. The phenol red which is present in the media at alkaline condition appeared as pink in colour. Also, this alkaline environment helps the bacteria to mineralize the $CaCO_3$ crystals and hence the result proves that the urease producing bacteria were capable of precipitating $CaCO_3$.

Thus, the result concludes that only B3 strain possess the ability to precipitate $CaCO_3$ and not the B2 strain.

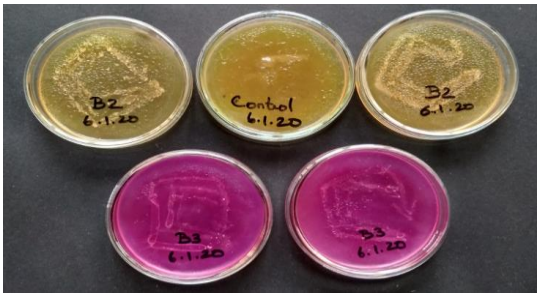

Fig no: 5. Screening for Urease production

This result was further confirmed by Naveneet Chahal ***et* al**, 2011 and Frederik Hammes *et* al, 2003.

Calcium carbonate solubilization test

The 2 strains (B2 and B3) isolated are assayed for solubilization test. As a result of the solubilization test, a clear halo was formed around a colony and the same was shown in the Fig no: 6 .

Fig No: 6 Halo formations in Calcium carbonate solubilization test

The results suggest that some acidic compounds could be produced by the bacteria which enhanced the solubilization of calcium carbonate.

This study was also supported by Gopinath Rana *et* al, 2015.

rRNA sequencing of B3 strain

Since B3 strain showed the ability to precipitate $CaCO_3$, it was further subjected to identification studies. B3 strain was identified to be gram positive and coccid shape using the preliminary identification examination (Gram staining). Then it was subjected to rRNA sequencing.

Initially the genomic DNA was isolated and then subjected to PCR amplification and finally to DNA sequencing. The sequence obtained was subjected to BLAST analysis. The online program BLAST (NCBI-2012) was used in identifying the related sequence with known taxonomic information available at the data bank of national centre for biotechnology information.

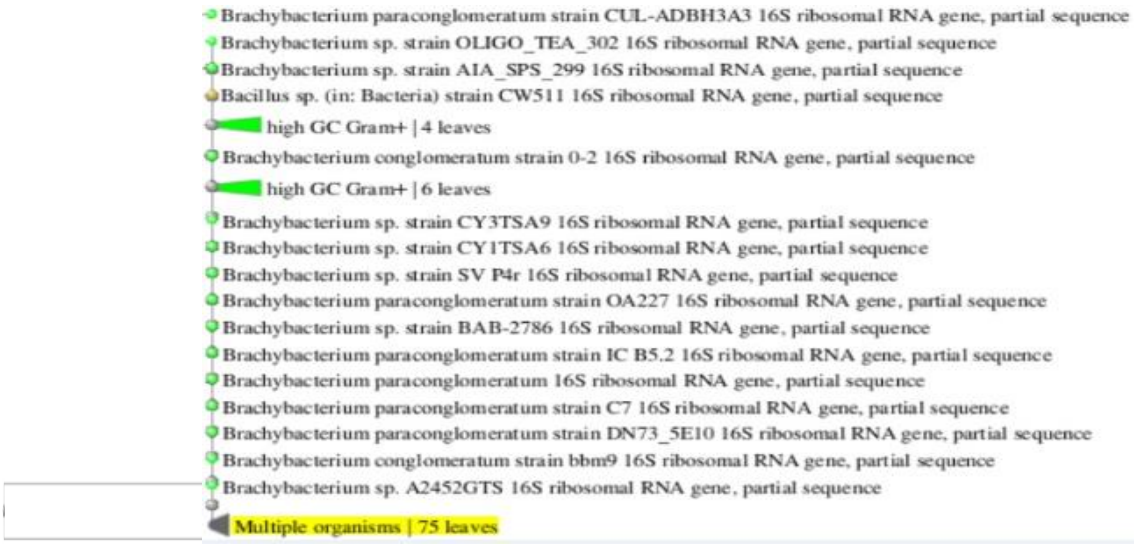

Fig no: 7 rRNA sequencing of B3 strain

The result of rRNA sequencing is represented as phylogenetic tree and is depicted in fig no: 7. from the result, it is evident that the isolated B3 strain was confirmed to be *Brachybacterium paraconglomeratum* with a sequence similarity of >98.65%

Production of $CaCO_3$

For the production of $CaCO_3$, B3 strain is inoculated in the Nutrient broth containing Calcium-Urea media. At different intervals of time (15 days and 30 days), the yield of Calcium Carbonate precipitated were recorded in comparison with control and is depicted in Fig no: 8.

Fig no: 8. Production of $CaCO_3$

By visualizing the culture flask, the $CaCO_3$ precipitated at the bottom of the flask were identified. The carbonate crystals precipitated were colored and in the current research it was found to be in brown colour. This was confirmed by comparing with the control. The mechanism of precipitation is predicted as a complex mechanism which is a function of cell concentration and pH of the medium including ionic strength. During this mechanism, it has noted that the urea gets degraded which gets converted further as carbonic acid and ammonia. Carbonic acid formation is an intermediate product and the bicarbonate increase pH level in the environment around the bacterial cell wall. Due to this mechanism, the carbonate concentration increases which reacts with the calcium ions present in the medium and can precipitate calcium carbonate. The current study was also supported by Shipping Wei ***et* al**, 2014.

Determination of the yield

After 15 days and 30 days of incubation, the $CaCO_3$ crystals were isolated from the culture flask and are subjected to weight determination. The yield obtained is shown in the Table no: 1 and also represented in the bar diagram which is shown in the Fig no: 9

Period	Mean (mg/ml)
15 days	0.38
30 days	10.12

Table No: 1. Determination of yield.

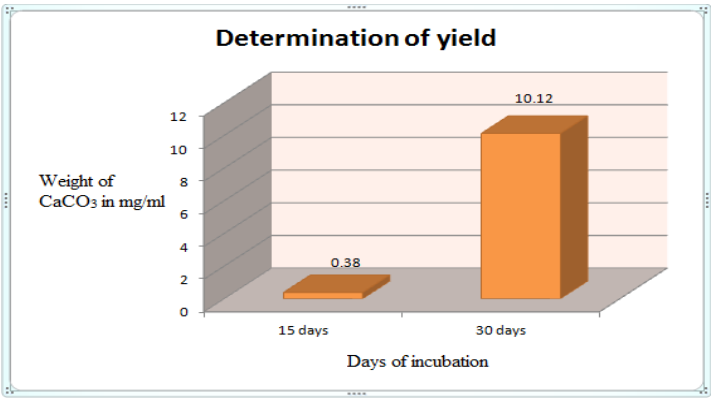

Fig no: 9 Determination of yield (F value < 0.05% significance)

Fig no: 10. Isolated $CaCO_3$ after 15 days

Fig no: 11. Isolated $CaCO_3$ after 30 days

By analyzing the yield of $CaCO_3$, it is evident that the yield has increased from 0.38 mg/ml (15 days) to 10.12 mg/ml (30 days). This highlights us that the production of $CaCO_3$ has taken place continuously to a remarkable quantity and hence B3 strain can be considered to be one of the good strains in production of $CaCO_3$ which is depicted in fig no: 10 and 11.

Qualitative Analysis of isolated $CaCO_3$:

The qualitative analysis was performed to confirm the isolated crystal to be calcium carbonate. Fig no: 12 shows the formation of the white effervescence, when the sulpuric acid is added with the crystals. Hence this test positively confirms that the isolated crystal is $CaCO_3$. When the calcium carbonate reacts with the sulphuric acid, it forms water, carbon dioxide and calcium sulphate. The formation of CO_2 indicates the formation of effervescence.

Fig no: 12 Qualitative Analysis of isolated $CaCO_3$

This present research was also supported by Bharathi N, *et* al, 2014.

Microscopic observation of $CaCO_3$ crystals

The isolated crystals were further examined under the microscopy in both 10x and 100x and is presented in fig no: 13 and 14

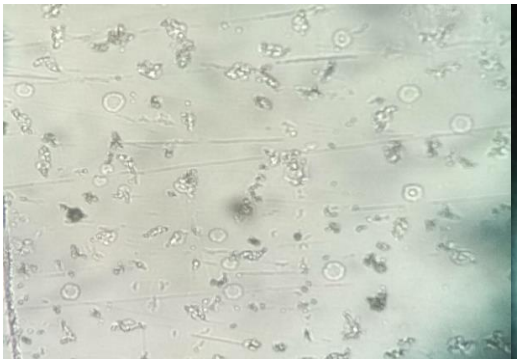

Fig no: 13 Crystal image under 10x – B3

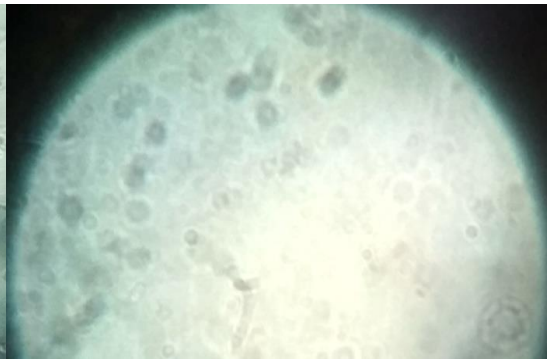

Fig no: 14 Crystal image under 100x – B3

In the current research, difference sized crystals were viewed under the microscope. Multiple factors affect the type and shape of the carbonate crystals. The crystal morphology depends on the functional characteristics of the precipitating bacteria. High calcium amounts in all the bacterial samples proved that calcite was found to be in the form of calcium carbonate. The occurrence of crystalline calcium carbonate associated with bacteria shows that bacteria served as nucleation sites during mineralization process. This process was supported by Achal *et* al*,* 2009.

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