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Calcium Carbonate Precipitation with Growth Profile of Isolated Ureolytic Strains

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Abstract: ***Natural sources, number of diverse microbial species participate in the carbonate precipitation in different natural environment as in soils, in geological formation, saline caco3 and ocean. Ureolytic bacterial strains play vital role in calcium carbonate precipitation for various applications including industrial, health and environmental aspects. Based on the ureolytic behaviour, four isolated strains are selected for this study. The presented works on bacterial calcium carbonate precipitation for determine the actual calcium precipitation and ammonium concentration with their growth profile of isolated strains.***

Keywords: Bacteria, Urease activity, Growth profile, Calcium carbonate, EDTA

1. Introduction

Bacteria are present everywhere on Earth can grow on soil, acidic hot springs, radioactive waste water and deep in earth's crust, as well as organic matter and the live bodies of plants and animals. Bacteria have numerous different types of shapes ranging from rod to spheres and spirals. The main role of bacteria in the precipitation, can ability to create an alkaline environment under the influence of various physiological activities. Negatively charged nature of bacterial cell can bind with divalent cations as ca2+ and mg2+ and made ideal crystal nucleation site. Specific proteins present in the cell cause the formation of different crystal polymorphs. Various microorganisms involve microbial mineral precipitation under different pathways and environments. Now days, research on calcium carbonate precipitation by ureolytic bacteria are most considerable. These ureolytic bacteria are able to precipitate calcium by influence of urease enzyme production. This enzyme catalyze the hydrolysis of urease to give ammonia and carbon dioxide, can increase the pH and carbonate concentration in the bacterial environment. Precipitation is a general process under favourable conditions of microbes through photosynthesis, ammonification, sulphate reduction and anaerobic sulphide oxidation. The best calcite precipitating bacteria was characterised high ureolytic efficiency, homogenous calcite deposition on limestone cubes and a very negative f-potential. Bio-mediated production of calcite crystals by calcinogenic bacteria has great applicable value, because of its high purity and coherency. In natural environments exists bio-mineralization, the precipitation of minerals by living organisms. This can be observed in both eukaryotic organisms like plants producing cystolith inclusion in leaves and animal forming bones, teeth and shells and prokaryotic organisms that can precipitate minerals like calcites, carbonates, silicates etc., In nature three groups of organisms can induce calcium precipitation: (i) Photosynthetic organisms such as cyanobacteria and algae that remove co2, (ii) Sulphate reducing bacteria – that are responsible for dissimilatory reduction of sulphates and (iii) Organisms that are involved in the nitrogen cycle – either ammonification of aminoacids or nitrate, reduction or hydrolysis of urea (Navneeth Chahal et al, 2011).

1.1 Various microbes involving MCP

Bacillus pasteurii produces intracellular urease. Bio-deposition of a calcium carbonate layer on degraded limestone by five different strains of the *Bacillus sphaericus* group and one strain on *Bacillus lentus* was studied (Diek et al;2006). It was found that Bacillus strains are capable of depositing calcium carbonate, but different in amount. Bio-mediated production of calcite crystals by calcinogenic bacteria has great applicable value for the restoration of deteriorated calcareous monuments, because of its high purity and coherency. Modern dolomite precipitation is often associated with dissimilatory sulfate reducing bacteria that remove sulfate produce alkalinity and presumably drive dolomite formation. Fresh water *cyanobacteria syncococcus*, *Scytonema*, Unicellular green algae *Chlorella*, marine *cyanobacteria syncchococcus* and *Synechocystis*, Unicellular green algae *Nannochloris atomus* potential for the precipitation of caco3. (Navneeth Chahal et al., 2011).

2. Materials and Methods

2.1 Isolation

Calcium carbonate precipitating bacteria were isolated from garden soil and landfill soil. The samples were suspended in a sterile solution (0.85%NaCl), diluted properly and plated on precipitation agar medium containing Nutrient broth (3 g/l), Urea 333mM (20 g/l), $NaHCO_3$ 25.0mM (2.12 g/l), NH_4Cl 187mM (10 g/l), $CaCl_2.2H_2O$ 25.2mM (25 g/l). Incubation was done at 32^0C. Positive isolates were purified through repetitive dilution and plating.

2.2 Gram staining

The procedure for Gram staining was discovered by Christian Gram in 1884. The Gram stain is a differential stain which allows most bacteria to be divided into two groups, Gram-positive bacteria and Gram-negative bacteria. The technique is based on the fact that the Gram positive cell wall has a stronger attraction for crystal violet when Gram's iodine is applied than does the Gram negative cell wall. Gram's iodine is known as a Mordant. It is able to form a complex with the crystal violet that is attached more

tightly to the Gram-positive cell wall than to the Gram-negative cell wall. This complex can easily be washed away from the Gram-negative cell wall with ethyl alcohol. Gram-positive bacteria, however, are able to retain the crystal violet and therefore will remain purple after decolorizing with alcohol. Since Gram-negative bacteria will be colourless after decolorizing with alcohol, counterstaining with safranin will make them appear pink. For the confirmation of gram positive strains, streaking on EMB (Eosin methylene blue) agar medium not showed growth of isolated strains. EMB medium partially inhibit the growth of gram positive bacteria. Growth of gram negative is most abundant.

2.3 Urease activity

Urease, Urea amidohydrolase E C 3.5.1.5 is a nickel-containing enzyme that hydrolysis of urea to give ammonia and carbomate then decomposes spontaneously to generate a second molecule of ammonia and carbon dioxide. The urease from Jack bean was the first enzyme to be crystallized. Urease is found in plants, algae, yeast and fungi. Urease enzyme is a diagnostic kit for measuring urea, in alcoholic beverages as a reducing agent of urea. Isolates were tested for urease activity, which was done by tryptic soy broth culture containing urea (20g/l), Na_2HPO_4 (9.5g/l), KH_2PO_4 (9.1g/l), Yeast extract (0.1g/l) and 0.01g phenol. pH was made to 6.8+/- 2. This test detects the ability of organism to produce urease enzyme. This enzyme converts urea to ammonia and co_2.

2.4 Analytical methods

Calcium ion concentration was measured by ethylene diamine tetracidic acid (EDTA) titration. DisodiumEDTA is commonly used to standardize aqueous solutions of transition metal cations. DisodiumEDTA only forms four coordinate covalent bonds to metal cations at pH values ≤12. The main reason that EDTA is used so extensively in the standardization of metal ions solutions is that the formation constant for most metal cation-EDTA complexes is very high, measuring that the equilibrium for the reaction. The completion of the reaction detects from the formation of the metal cation-EDTA complex and it is chiefly used in titrations or standardization. Eriochrome Black T is used as a complexometric indicator to determine the end point. EBT is an organic dye which displaces the metal cation and reached the end point with as metallic blue. So the free indicator is serves as the end point indicator. The concentration of ammonia as ammonium hydroxide was determined by titration with sulphuric acid. Ammonium hydroxide is a weak base and titration of bases is done with acid depending upon the strength of acid. Methyl orange is used as an indicator. Number of equalent of base=Number of equalent of acid

$H_2SO_4 + 2NH_4OH \rightarrow 2H_2O + (NH_4)_2SO_4$

Previously standardised 0.05M sulfuric acid was used. The presence calcium carbonate was confirmed by the addition of 0.05n sulphuric acid.

3. Results

3.1 Isolation of calcium carbonate producing bacteria

Soil samples were collected from various sites of garden soil and landfill soils. Strains were isolated using Blank's media containing Glucose (1g/l), Nacl (1g/l), Mgso4 (0.8g/l), KH2po4 (0.2g/l), Na2s2o3 (1g/l) and Urea (6g/l). Sterilized urea was added into the media at the time of inoculation. The isolated strains were allowed to grow for further studies. Urea is an important nitrogen compound found in natural environment, is a fairly inexpensive substrate. Also the use of bacteria to raise the pH in the environment is preferable to the direct injection of the base because the gradual hydrolysis of urea is likely to promote a wider spatial distribution of calcite, whereas the direct addition of base is likely to cause immediate precipitation at the injection site.

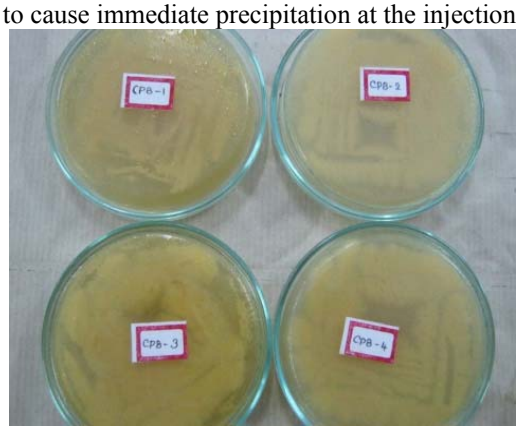

Figure 1: Isolated strains

3.2 Growth profile of strains

The growth profiles of isolated strains were studied up to 5 days (120 hrs). It was observed that the growths of four strains were decreased in sixth day simultaneously. The maximum growth observed in 1.95 optical densities at 600nm of strain 1. The other three strains are approximately same growth. All the isolates have high urea affinities. Urease production was decreased in the calcite precipitating media at the beginning of 6th day. The presence of calcium amounts in all the strains was determined at 120th hr.

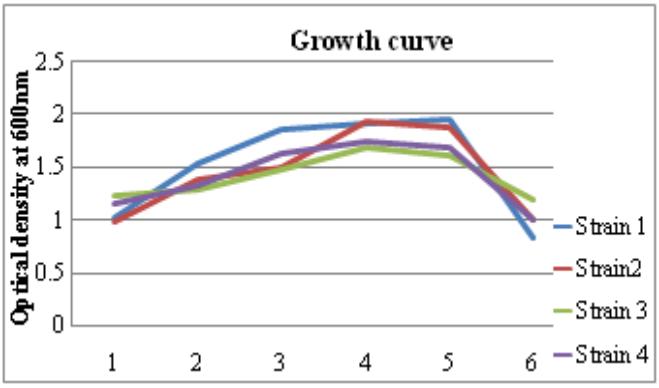

Figure 2: Growth profile of different bacterial strains

The 48 hrs grown selected strains were further inoculated in to the Calcite mineralizing media containing (Nutrient broth (3 g/l), Urea 333mM (20 g/l), NaHCO3 25.0mM (2.12 g/l), NH4Cl 187mM (10 g/l), CaCl2.2H2O 25.2mM (25 g/l))

containing urea as the nitrogen source of bacteria get hydrolyze due to the presence of urease enzyme which increases pH. Addition of calcium chloride in the medium supports the microbial growth. In this study, the actual Calcite precipitation of isolated strains along with their growth profile was studied.

3.3 Urease activity

Hydrolysis of one mole of urea gives two moles of ammonia and one mole of carbon dioxide. Urease test media contains 2% urea and phenol red indicator. An increase in pH due to the production of ammonia results in a color change from yellow to pink Stuart's urea broth is a highly buffered medium requiring large quantities of ammonia to raise the pH resulting in a colour change.

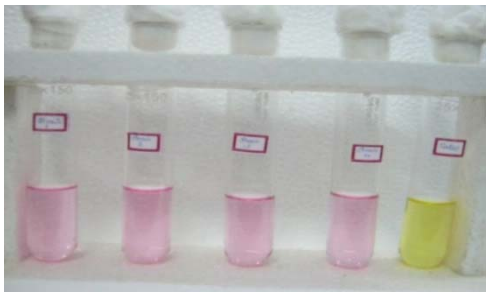

Figure 3: Positive result of urease activity

3.4 Calcium carbonate precipitation

The amount of calcium carbonate precipitation was determined and calculated in percentage along with the growth profile of isolated strains. The maximum calcium precipitation was observed 29.5% in strain 1 and the minimum precipitation was 25.2% in strain 3. Due to lack of urease enzyme the precipitation was decreased. Excess of calcium amounts present in the isolated bacterial samples confirmed that the calcite was present as calcium carbonate. A simple biochemical test with 0.05m sulphuric acid which was added to the centrifuged and dried bacterial samples, effervescence was found.

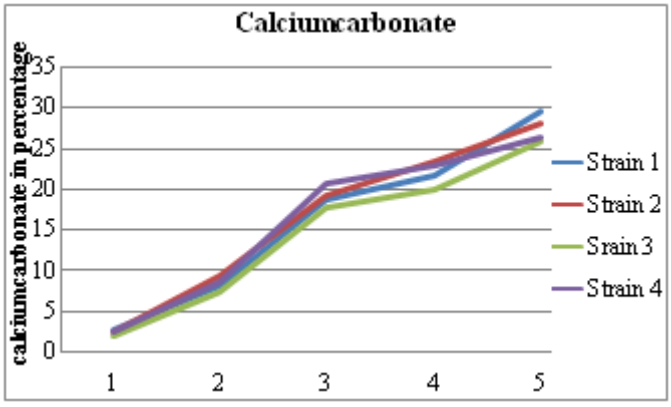

Figure 4: Ammount of calcium carbonate

Figure 5: Calcium presents with EDTA with metallic blue end point.

Table: Amount of calcium carbonate in percentage

Isolated Samples	*Calcium carbonate precipitate in percentage at 120hr (5 days)*
Strain 1	29.5
Strain 2	28
Strain 3	25.8
Strain 4	26.2

Ammonium concentration

Concentration of ammonia from the isolated bacterial samples was found as ammonium hydroxide which was determined by titration with sulphuric acid. The concentration of ammonia was gradually increased till the end of incubation time.

Figure 6: Titrate with Sulfuric acid gives pink colour endpoint.

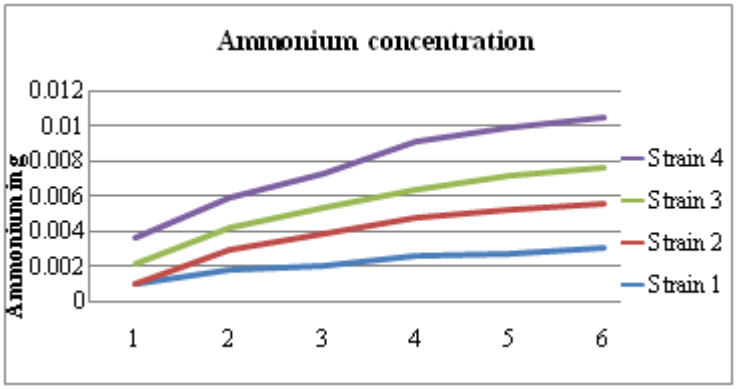

Figure 7: Ammonium concentration

Concentration of ammonia present in the isolated sample medium was calculated by this formula shown below. Concentration of ammonia=2*moles of sulphuric acid*titre value of sulphuric acid*molar mass of ammonia/ volume of sample taken.

4. Discussion

The mechanism of urease enzyme activity creates a local microenvironment that allows optimized extra cellular precipitation of minerals. Very low bacteria in their community can withstand extreme alkaline environment. Ureolytic bacteria were selected on the basis of urease producing ability which utilizes urea to produce ammonia in the reaction medium. That medium producing pink colour under the influence of phenol red indicator in general urease test broth was used for the bacterial strain selection. The calcium carbonate precipitation was complex mechanism which is a function of cell concentration and pH of the medium including ionic strength. During the mechanism, urea gets degradation which converts carbonic acid and ammonia. Immediately the carbonic acid gets hydrolyse into ammonia and bicarbonate.

$NH_2CONH_2+H_2O \rightarrow NH_2COOH+NH_3$
$NH_2COOH+H_2O \rightarrow NH_3+H_2CO_3$

Carbonic acid formation is an intermediate product and the bicarbonate increase pH level in the environment around the bacterial cell wall. Due to this continuous mechanism, the carbonate concentration increase which reacts with calcium ions present in the medium can precipitate calcium carbonate.

$H_2CO_3 \rightarrow HCO_3^- + H^+ \#$
$2NH_3+2H_2O \leftrightarrow 2NH_4^{++}+2OH^-$
$HCO_3^-+H^++2OH^- \rightarrow CO_3^{2-}+2H_2O$
$CO_3^{2-}+Ca^{2+} \rightarrow CaCO_3$
Overall process:
$NH_2CONH_2+2H_2O+Ca^{2+} \rightarrow 2NH^{4+}+CaCO_3$

The production of calcium carbonate achieved in the super saturation level of bicarbonates. The possible biochemical reaction in urea and calcium chloride medium on the cell structure of bacterial strains was shown. The growth of isolated strains was divided into four stages. Growth and reproduction will occur in the first stage of lag phase. In second log phase, cell multiplication at an exponential rate which leads to huge production of carbonate and hydrogen carbonate in the environment. In the third stationary phase, reproduction occurs not in the same speed as in second phase. The last death decline phase, the cell started dying and the production and the conversions also decreasing. This same general aspect was found in growth of these isolated strains indicate the growth decreased after 120 h at the same time the urease activity was also decreased. The maximum urease activity was found 248 and 342 in strain 1 and strain 2 respectively. The minimum activity was found 123 and 132 in strain4 and strain 3 respectively. The maximum percentage of calcium carbonate precipitation was observed 29.5% in strain 1. Besides ammonium concentration was also observed maximum 0.002g in strain 1 at 120h. The other 2 strains were observed minimum concentration.

In this study, we concluded clearly shown that the urease activity, actual amount of calcium carbonate precipitate and actual ammonium concentration along with their growth profile of isolated strains from soil. The work proved that the bacterial community plays vital role for mineral precipitation on environment everywhere possibly.

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