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EFFECT OF CARBONIC ANHYDRASE AND UREASE ON BACTERIAL CALCIUM CARBONATE PRECIPITATION

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ABSTRACT

There are number of diverse microbial species complementing the carbonate precipitation in different natural environment like soil, ocean and in geological formation. Even some common soil bacteria can precipitate calcium carbonate under appropriate conditions. Bacteria can serve as a nucleus of carbonate precipitation under the influence of two essential enzymes such as Urease and Carbonic anhydrase. Urease helps in mineralization of calcium carbonate, by hydrolysing urea into ammonium and carbonate in the environment. Carbonic anhydrase is an enzyme that catalyses reversible hydration of co_2 in different microorganisms and plays an important role in carbonate precipitation. In this present study, four strains of *Bacillus* and five strains of *Pseudomonas* were tested for its urease and carbonic anhydrase activity that significantly showed the capability of these bacterial strains for calcium carbonate precipitation.

KEYWORDS: *Carbonic anhydrase, Urease, Calcium Carbonate Precipitation.*

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INTRODUCTION

Bacteria are omnipresent in soil at high concentrations, regardless of saturation, minerology, pH and other environmental factors. In a wide range of ancient and geological environments such as caves, soils, fresh and marine water and hot springs, the precipitation of carbonate minerals is influenced by the microorganisms. They play an important role in promoting calcium carbonate precipitation which comprises of a series of complex biochemical reactions in the medium and precipitate at the cell surface that act as the nucleation site. Each bacterium has different types of cell surface that results in different cell surface charge. These cell surface charges are influenced by pH, ionic strength and ionic make-up of the water. Urea is the chief nitrogenous waste produced by vertebrates and is a major nitrogen resource in aquatic and soil ecosystems.[1] In response to the widespread availability of urea in the environment and the universal requirement for nitrogen, a diverse section of the biota has evolved with the ability to hydrolyse urea through the action of urease. Urease occurs in many bacteria and number of higher plants including jack beans (*Canavalia ensiformis*), soya bean (*Glycine max*) leaf and seed.[2] Urease was the first enzyme to be crystallized and first enzyme shown to contain Nickel. The ammonium ions were produced as a by-product of urea hydrolysis is toxic and its rate of production and removal are important for the utilization of urea in artificial kidney devices. Thus kinetic study of urease-catalyzed hydrolysis of urea is primary and fundamental search towards the development of artificial kidney.[3] Carbon-di-oxide, bicarbonate, carbonic acid and carbonates are key metabolites in all living system and its equilibrium of these different forms of living cell is important for proper physiological functioning. Carbonic anhydrases are zinc-containing enzymes that catalyses the reversible conversion of carbon dioxide to bicarbonates and protons with very high efficiency. There are five main classes (α, β, δ, ζ) of carbonic anhydrase enzyme found in the nature.[4] They can be found in animals and plants and even in the human erythrocyte. They exist in different forms with different structures and molecular weights and their activities vary from one to another. The enzyme was found to be in bacteria as well.[5] Mineralizing activities of bacteria can be harnessed positively, making them essential to the existence of ecology of Earth. The use of microbially induced carbonate biomineral is becoming popular day by day. From the removal of heavy metals and radio nucleotides, removal of calcium from waste water and biodegradation of pollutants, atmospheric co_2 sequestration, modifying the properties of soil and filler in rubber and plastics to fluorescent markers in stationery ink and remediation of building materials, bacterial calcium carbonates are serving many fields.[6] The objective of this study was to investigate enzymes involved in calcium carbonate precipitation and to determine the enzyme activity of urease and carbonic anhydrase quantitatively.

MATERIALS AND METHODS

Isolation of Calcium Carbonate Precipitating Strains

Soil samples from different areas in and around Chennai were collected. Organisms were isolated and identified by preliminary and biochemical tests by previous study.[7,8] The obtained isolates were further screened for calcium carbonate precipitation by inoculating it in B4 medium (calcium acetate 2.5g, Yeast extract 4g, Glucose 10g, Agar 18g/1000ml of distilled water, pH 7.2) in triplicates and incubated at 37ºC for 28 days and checked periodically for the presence or absence of crystals using optical microscopy. After 28 days, the precipitates were removed by cutting out pieces of the media, which were placed in boiling water to dissolve the agar. The sediments were re-suspended and washed in distilled water to free of impurities. The washed crystals were finally air-dried at 37 ºC. Controls were included in all experiments.[9]

Preparation of crude enzyme

The calcium carbonate precipitating strains were inoculated into 50ml of nutrient broth containing 1g of urea and incubated at 30^{o}C for 48 hrs in shaker. Then the bacterial cells were harvested by centrifugation at 8000 rpm for 30 minutes. Then the pellets were washed with the extraction buffer (100mM NaH_2P0_4, 1mM EDTA) at pH 7 and centrifuged at 15000 rpm for 10 minutes. The extracted pellets were stored in the extraction buffer for further assay.[10]

Urease Assay

The urease activity was determined for all the calcium carbonate precipitating strains by measuring the amount of ammonia released from urea by Phenol hypochlorite assay method.[3] It is based on Berthelot's reaction. The ammonium ions were released as a result of urea hydrolysis which then reacts with phenol in the presence of hypochlorite to give the blue dye, indophenol. Ammonium chloride (50-100 µM) was used as standard. 250µl of culture filtrates were added to the mixture containing 1ml of 0.1 M potassium phosphate buffer (pH 8) and 2.5 ml of urea (0.1 M) and incubated at 37^{o}C for 5 minutes followed by addition of 1ml of phenol nitroprusside and alkaline hypochlorite and incubated at 37^{o}C for 25 minutes. The absorbance was measured at 626 nm and recorded. One unit of urease activity is defined as the amount of enzyme that would hydrolyze 1 µmol urea per minute.

Carbonic anhydrase Assay

The carbonic anhydrase activity was determined for all the calcium carbonate strains by the measurement of the amount of p-nitrophenol produced by spectrophotometric assay of carbonic anhydrase.[11,12] Bacterial isolates were grown in the basal medium. 1% of overnight grown cultures of OD 0.5 was inoculated into media and incubated at 37^{o}C in a shaker (130 rpm). After an interval of 24, 48, 72 and 96 hours the cultures were centrifuged at 8000 rpm for 5 minutes. 200µl of culture filtrates were added to a mixture of 1.8 ml 100mM phosphate buffer at pH 7 and 1 ml of 3mM p-nitrophenyl acetate solution. The increase in absorbance at 348nm was recorded for 5 minutes. One unit of carbonic anhydrase activity is defined as the amount of enzyme required to form 1 µmole of p-nitrophenol per minute.

RESULTS

The isolates obtained from the soil sample after preliminary and biochemical tests were identified as *Bacillus* and *Pseudomonas*. These isolates were screened for calcium carbonate precipitation in the B4 medium. There were four strains of *Bacillus* such as B1, B2, B3, B4 and five strains of *Pseudomonas* such as P1, P2, P3, P4, P5 precipitated calcium carbonate in an *in vitro* condition. Urease activity for four strains of *Bacillus* and five strains of *Pseudomonas* were determined by phenol hypochlorite assay method in different time intervals of 24hrs, 48hrs, 72hrs and 96hrs respectively. The urease activity values ranged from 1.10U/ml by *Bacillus* (B4) strain to the highest urease activity of 7.05U/ml by *Bacillus* (B1) strain (fig 1) after 48hrs of incubation. Among *Pseudomonas* strains the highest urease activity of 4.59U/ml was recorded by *Pseudomonas* (P3) strain (fig 2) after 24hrs of incubation.

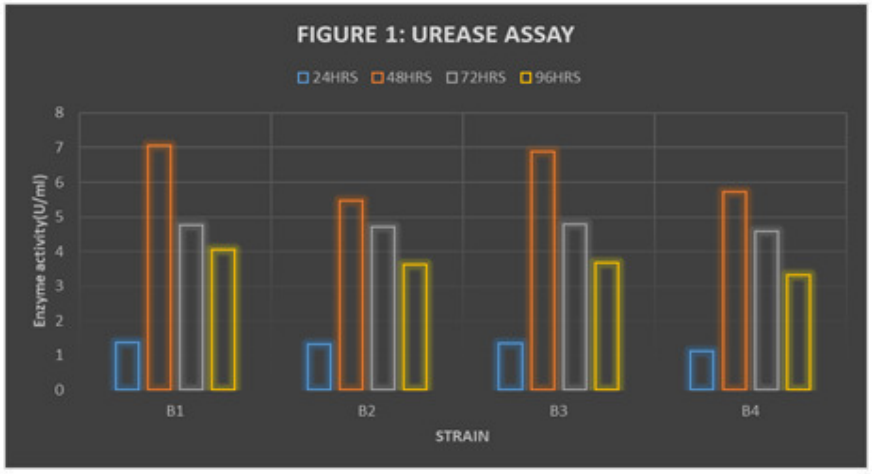

Figure 1
Urease assay of *Bacillus* strains

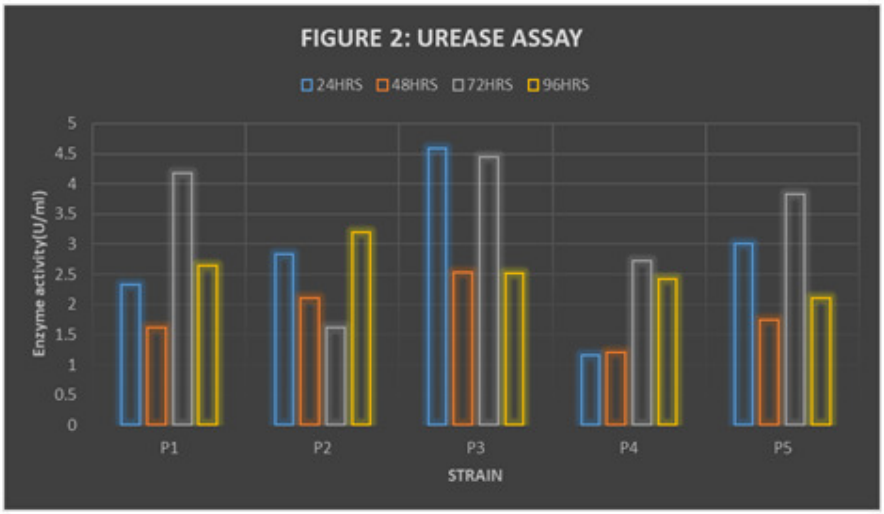

Figure 2
Urease assay of Pseudomonas strains

Carbonic anhydrases activity for four *Bacillus* strains and five *Pseudomonas* strains were determined in different time intervals 24hrs, 48hrs, 72hrs and 96hrs respectively. The carbonic anhydrase activity values ranged from 1.18U/ml by *Pseudomonas* (P4) strain to the highest carbonic anhydrase activity of 9.4U/ml was recorded by *Bacillus* (B1) strain (fig 3) after 24hrs of incubation. Among *Pseudomonas* strains the highest carbonic anhydrase activity of 5.9U/ml was recorded by *Pseudomonas* (P3) strain (fig 4) after 24hrs of incubation.

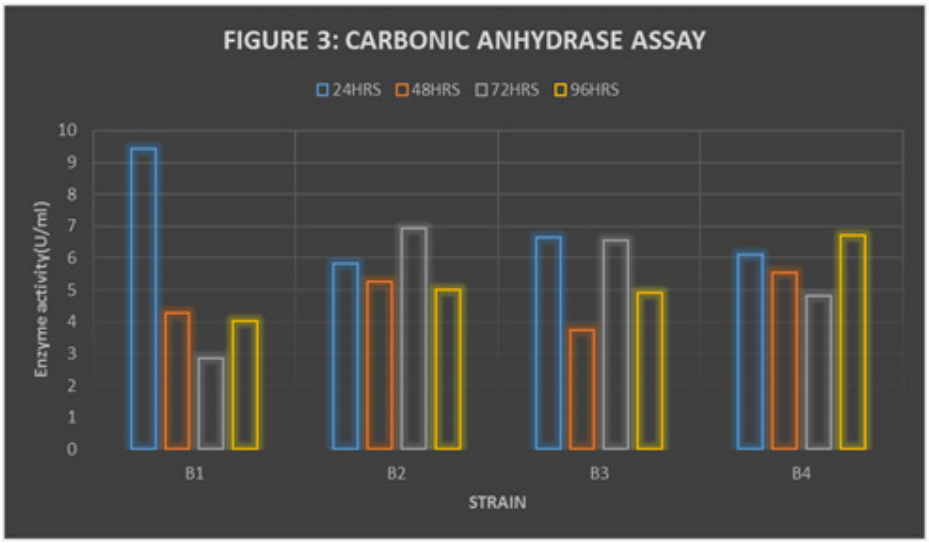

Figure 3
Carbonic anhydrase assay of Bacillus strains

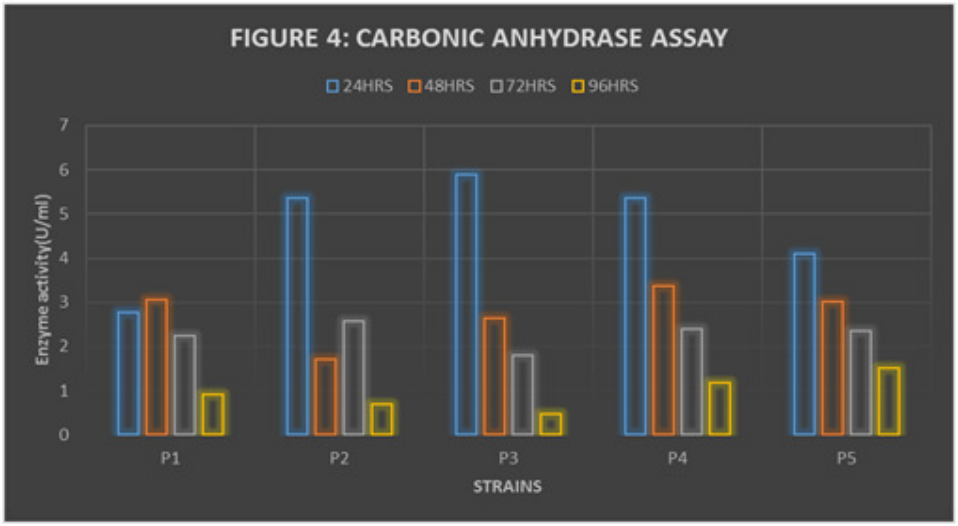

Figure 4
Carbonic anhydrase assay of Pseudomonas strains

DISCUSSION

Considerable research on carbonate precipitation by bacteria have been done by ureolytic bacterial strains, its influence in the precipitation of calcium carbonate was due to the production of urease enzyme. It catalyses in the hydrolysis of urea to carbon dioxide to ammonia, resulting in increased pH and the carbonate concentration in the bacterial environment. It emphasizes such broad distribution of urease activity and its possible alternative functions in ruminal and gastrointestinal microecology and in environmental nitrogen cycling.[13] The bacterial isolates showed increased urease activity by Phenol hypochlorite assay method. It was estimated that three strains showed maximum urease activity of 598U/ml in 120 h. After 120 h, urease production was decreased in the biomineralisation media. It was proposed due to calcite precipitation and survival in high pH. Hence it is used in the remediation of cracks in the building materials.[14] However, the obtained strains in this study did not show such higher urease activity. BioGrout is an *in situ* soil strengthening technique involving microbial-induced carbonate precipitation (MICP). It describes a methodology based on a two-phase injection procedure: a bacterial suspension is injected into the sand body, immediately followed by a fixation fluid to distribute and fix bacteria relatively homogeneously in a sand bed, before supplying cementation reagents. It was demonstrated that bacteria are retarded by adsorption and filtration processes and are permanently adsorbed to the sand grains when overtaken by the fixation fluid. An estimate of urease activity was reported from 84mM/hr to 300mM/hr. [15] The experimental report showed lesser activity obtained by a much simpler method which did not use any sand columns. Ureolysis was examined for the bacterium, *Sporosarcina pasteurii* (NCIMB 8841) on the urea agar base medium (UAB). Urease activity rate was assayed by conductivity

method. It showed stability and consistency in the rate of conductivity increase in the first 5 minutes of reaction. The conductivity continuously increased with time in a positive proportional relation with urease activity. The rates of enzyme activity of the three tests were 0.056, 0.038 and 0.052 mS/min. Stability of urease activity in prolonged incubation of reaction mixture was investigated and it showed a stable rate of activity of 106 minutes.[16] The ureolysis was demonstrated in this experiment by bacterial isolates obtained from soil samples. thereby further studies are necessary using standard strains. Microbial weathering is a complex process includes bio-mechanical action, secretion of organic acids, chelation effects and redox reactions. Carbonic anhydrase (CA) gene expression from *Bacillus mucilaginosus* and its effects of recombination protein(PCA4) on wollastonite dissolution and carbonate formation under different conditions were explored. The impact of CO_2 on the role of CA in carbonate formation, regardless of whether the reaction system contains PCA4 or not, the CO_2 concentration is positively correlated with Calcium carbonate production. At any CO_2 concentration, the $CaCO_3$ content is significantly different due to the participation of PCA4. The proportion of the CaCO3 formed as a result of the recombinant protein(PCA4) was by 419%, largely due to the behaviour of the CA. Thus, PCA4 causes a much greater difference in the amount of $CaCO_3$ at lower CO_2 concentrations.[17] A comparison of titrimetric method with the traditional electrometric method of estimation of CA activity in the ten different tree species revealed. Titrimetric method was found to be better over the electrometric method. No significant variation was observed between the two methods used when different buffers were used for extraction. Among the species, highest CA activity was recorded in Casuarinas (64.98 units) while the least was recorded in teak (37.35 units) thus justified the use of both methods for measurement of the enzyme activity.[18] Calcite forming bacteria has been reported in various geological environment including limestone caves and soil. *Bacillus pumilus*, *Bacillus sphaericus* and *Bacillus cereus* were isolated and identified in lime stone areas of Malaysia. It described the extracellular carbonic anhydrase assay by esterase activity done on all isolates to detect carbonic anhydrase activity which measured about 0.006U/min.[19] The precise role of microbes in the carbonate precipitation process was suggested by mineralization occurs as a by-product of microbial metabolism involving either autotrophic or heterotrophic pathways. During these passive processes, reactions, such as enzymatic hydrolysis of urea or the dissimilatory reduction of nitrate and sulphate, cause an increase in pH that shifts the bicarbonate carbonate equilibrium toward the production of more CO_3^{2-} and ultimately leads to the precipitation of $CaCO_3$, if free Ca^{2+} is present. The nucleation of the carbonates take place on the cell wall, either due to ion exchange through the cell membrane or due to the support of negatively charged specific cell wall functional groups that adsorb divalent cations, such as Ca^{2+}.[6] Hence an attempt was made in this study to demonstrate biomineralisation process to precipitate calcium carbonate includes contribution of both the enzyme urease and carbonic anhydrase effectively.

CONCLUSION

This study was an experimental approach to use potential insights of both the enzyme urease and carbonic anhydrase in calcium carbonate precipitation brought out by soil bacterial strains of *Bacillus* and *Pseudomonas* in an *in vitro* conditions. It also demonstrated bacteria's common trait of utilizing such enzymes in the process of biomineralisation in the laboratory which was proved by both qualitative and quantitative analysis. It is evident that potential of this biomineral calcite has brought a new revolution in various industrial applications like paper, paint, plastics, medicines and also in bioremediation of building materials.

CONFLICT OF INTEREST

Conflict of interest declared none.

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