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# Isolation and characterization of urease producing bacteria from different soil types.

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**Abstract:**

Urea is hydrolyzed by means of urease enzyme with production of ammonia and carbonic acid. It provides an alkaline pH, and leads to $CaCO_3$precipitation.This are one of the mechanism for microbially induced calcium carbonate precipitation (MICP).For this study, ureolytic bacteria capable of precipitating calcium carbonate were isolated from different types of soil samples and were tested for their urease activity. Each isolate were identified morphologically and using basic biochemical tests.Isolates were also further tested for their ability to grow in extreme environment such as high pH, high salt concentration and low nutrient availability.16 out of 28 isolates showed positive results for urease production. Hydrolysis of urea was examined using Christensen's urea agar base. Conductivity method was used to assay urease activity and it was in the range of 0.009-0.051 mS/min.Calcium carbonate precipitation via urea hydrolysis was investigated in both solid and broth media. $CaCO_3$crystals in the precipitation agar media were imaged.The present experimental approach can be potentially useful for a variety of environmental bioremediation applications such as heavy metal removal, self-healing concrete etc.

***Keywords:*** Urease, calcium carbonate precipitation, electric conductivity, soil bacteria.

## INTRODUCTION:

Soil is loose surface material and contains inorganic matter, organic matter, gases and different kinds of micro-organisms. These microorganisms present in soil play a very vital role in different biogeochemical cycles, in degradation of various pollutants, biomineralization process etc.

Urease (urea amidohydrolase: EC3.5.1.5) is an enzyme that hydrolyzes urea into one mole of carbonate and two moles of ammonia per mole of urea, resulting in an increase of the pH and carbonate concentration in the bacterial

environment, which induces the formation of calcium carbonate. This enzyme is widespread in plants, bacteria algae , fungi etc.

Calcium carbonate ($CaCO_3$) is a widely distributed mineral in nature. Production of calcite by bacteriais general phenomenon which occurs under appropriate conditions.(Bouquet etal, 1973).Calcium carbonate is widely spread in nature and has major applications in construction industry. Bio mineralization is the science of precipitation of minerals by living organisms. Both eukaryotic and prokaryotic organisms deposit minerals.Most crystals formed by biomineralization consisted of inorganic minerals, which may contain trace organicelements, that can regulate the process of biomineralization. (Yoshida *et al*. 2010).This property was explored by various scientists worldwide and its application in bioengineering field.(Dhami, etal (2013d)).There are different parameters that govern the induction of calcium carbonate precipitation which are; (a) calcium concentration, (b) carbonate concentration, (c) pH of the environment, and (d) presence of nucleation sites.(Hammes and Verstraete (2002)).

The microbial induction mechanism for the precipitation of calcium carbonate is called Microbially Induced Calcite Precipitation (**MICP**). Biologically controlled or biologically inducedis some mechanism by which CaCO3 is precipitated(Lowenstan and Weiner, 1988).

In this study the isolation of ureolytic bacteria is carried out from different soil samples.Urease activity of the isolates is done using conductivity method. Further isolates are tested for calcium carbonate precipitation in both broth and agar state.

## MATERIALS AND METHODS:

### *Sample collection and preparation:*

Soil samples from alkaline locations were used for this study. A total of 3samples were aseptically collected randomly using sterile spatula and put in a sterile zipper bag. The samples were transported to the laboratory and stored at $4^0$C till use.

### *Study area/ location:*

The samples were collected from various locations as follows:

1. Alkaline soil: Niphad
2. Tulsi plant rhizosperesoil:Dindori
3. Cementous soil: Mhasrul, Nashik

### *Enrichment and Isolation of microorganisms:*

All soil samples were enriched by inoculating samples in 50ml sterile nutrient broth pH-9 containing 2% urea and incubated at $37^0$c.For isolation and enumeration of cultivable bacteria, all the samples were serially diluted in saline (NaCl, w/v 0.85%) and plated on alkaline nutrient agar having pH 9), and the plates were incubated at 37°C. Viable bacterial count: Viable bacterial count was done and TVC(cfu/ml) was calculated by usin formula as follows:

TVC (cfu/ml) =Avg. no. of colonies ×dilution factor

Volume plated

The grown bacterial colonies from the plates were sub cultured several times on the same medium from where it was picked.

***Preliminary Screening:***

This screening was mainly based on tolerance of organism against high pH levels and high salt concentrations and growth at minimum nutrient availability. Nutrient broth with varying pH concentrations (pH 8, 9, 10, 11) was prepared. Nutrient broth with varying salt concentrations (NaCl 0.5%, 1%, 1.5%, 2% per 100 ml) was prepared. Each isolate were aseptically inoculated incubated at $37^0$C for 24 – 48 hrs. Growth of organisms was monitored. Half strength Nutrient broth was prepared (i.e. keeping the volume same adding half the amount of component that are added normally). Isolates were aseptically inoculated and incubated at $37^0$C for 24 – 48 hrs. Tubes were monitored for the growth of the isolates.

***Morphological and Biochemical analysis for identification of the isolate:***

All isolates were identified using following tests:

Gram staining, motility test , Indole test Catalase test, Oxidasetest, MR-VP test, urease test ,Starch hydrolysis test, Sugar fermentation test.

***Qualitative urease assay:***

All the isolates were tested for urease activity. This was done by streaking the purified cultures on Christensen's Urea Agar Base which is used for rapid screening of urease enzyme(Himedia)(Dhami et al., 2013d)It composed from (g/l); urea , 20.0; NaCl, 5.0; peptone, 1.0; glucose, 1.0; KH2PO4, 2.0; phenol red, 0.012 and agar, 15.0; (pH 6.5). All components of media were autoclaved except urea which filter-sterilized then added after autoclaving. CUAB were inoculated with isolates, and then kept at 37°C for incubation and were examined continually to record the pink color development.

***Quantitative urease assay by electric conductivity:***

Conductivity method for urease activity assay in the absence of calcium ionswas used in thestudy((Al-Thawadi, 2008, Whiffin, 2004)). For enzyme assay, 1.0ml of bacterial broth culture (NB-U) was added to 9.0 ml of 1.11 M urea solution (Harkes et al., 2010). The urease reaction involves the hydrolysis of non-ionic substrate urea to ionic products thus generating a proportionate increase in conductivity under standard conditions. Final conductivity record could be taken after 5 minutes of incubation at 20ºC by electric conductivity meter (EQUIP-TRONICS NO. EQ-660A).

Urease activity is presented by the rate of conductivity increase as mS/min.

***Calcium carbonate precipitation test:***

B4 medium(Yeast extract-0.4 gm., glucose 0.5gm, calcium acetate-0.25 gm) (Marvasi.M et al,2012)was used for test.Both broth and agar medium were used for the study. Broth was for tube test were the broth wasaseptically inoculated with test isolates and incubated at $37^0$C for 2-3 weeks and was monitored for formation of precipitates of calcium carbonate (calcite) at the bottom of the tube. While the test isolates were aseptically spot inoculated on the medium plates and incubated at $37^0$C for 2 weeks plates were monitored for observation of accumulation crystals on the colonies of the organism.

***Confirmatory test for Calcite:***

"Acid test" was used as a confirmatory test for acid production which involves use of 5% of concentrated acid solution which is placed on the surface of the colony present on B4 medium plates. Later the colonies of organism submerged in the drop of 5% acid were monitored for observation of emission of $CO_2$ in the form of effervescences. Colonies showing effervescences showed positive test.

## RESULTS AND DISCUSSION:

***Preliminary Screening:***

All isolates showed growth in the nutrient broth having pH range from 8-10 and were able to grow in low nutrient availability.

In **sample 1**, 4 out of 7, in **sample 2** , 4 out 0f 8 and in **sample 3** all isolates showed growth in nutrient broth having salt concentration from 0.5% -5%.

***Morphological and Biochemical analysis for identification of the isolate:***

Table No.1: Morphological and biochemical identification of isolates.

| Sample | Code no. | Gram character | Catalase | Oxidase | Indole | MR | Citrate | Urease | glucose | Lactose | Maltose | Nitrate | VP | Starch hydrolysis | CaCO3pptn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | A1 | +ve rods | + | + | + | + | + | ++ | + | + | + | - | - | + | ++ |
| | A2 | +ve rods | + | + | + | + | + | ++ | + | + | + | + | - | + | ++ |
| | A3 | +ve rods | + | + | + | + | + | ++ | + | + | + | + | - | + | ++ |
| | A4 | +ve rods | + | + | + | + | + | ++ | + | + | + | + | - | + | ++ |
| | A5 | -ve rod | + | + | + | + | - | - | + | + | - | - | - | - | - |
| | A6 | +vecocci | + | + | - | + | + | - | + | + | + | - | - | + | - |
| | A7 | +vecocci | + | + | - | + | + | - | + | + | + | - | - | + | - |
| | | | | | | | | | | | | | | | |
| 2. | B1 | +ve rods | + | + | - | + | - | ++ | + | + | + | - | - | | + |
| | B2 | +ve rods | + | + | - | + | - | ++ | + | + | + | + | - | + | + |
| | B3 | +ve rods | + | + | - | + | + | ++ | + | + | + | - | - | + | + |
| | B4 | +ve rods | + | + | - | + | + | ++ | - | + | + | + | - | + | - |
| | B5 | +vecocci | + | + | - | + | - | - | + | + | - | - | - | + | - |
| | B6 | +vecocci | + | + | - | + | - | - | + | + | - | - | - | + | - |
| | B7 | -ve rod | + | + | - | + | - | - | + | + | - | - | - | + | - |
| | B8 | -ve rod | + | + | + | + | - | - | + | + | - | + | - | + | - |
| | | | | | | | | | | | | | | | |
| 3. | H1 | +ve rods | + | + | - | + | - | + | +/- | - | - | | - | + | ++ |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | H2 | +ve rods | + | + | - | + | - | + | +/- | + | - | + | - | + | ++ |
| | H3 | +ve rods | + | + | - | + | - | + | +/- | - | - | + | - | + | ++ |
| | H4 | +ve rods | + | + | - | + | - | + | +/- | + | + | + | - | - | + |
| | H5 | +vecocci | + | + | + | + | - | + | - | - | - | + | - | + | ++ |
| | H6 | +vecocci | + | + | + | + | - | + | + | + | + | + | - | + | + |
| | H7 | +vecocci | + | + | - | + | - | + | - | - | - | + | - | + | ++ |
| | H8 | +vecocci | + | + | - | + | - | + | - | - | - | + | - | +/- | + |

Key:+ = positive,- = negative

***Qualitative urease activity:***

The capability of isolates to hydrolyze urea by urease was tested onChristensen's Urea Agar medium. Fig.1 shows qualitative urease activity on plate after 24h of incubation. Positive urease activity appeared in plates which its color turned from yellow to pink in comparison with control negative (un-inoculated medium) which still yellow. Out of 28 isolates 16 showed positive results.

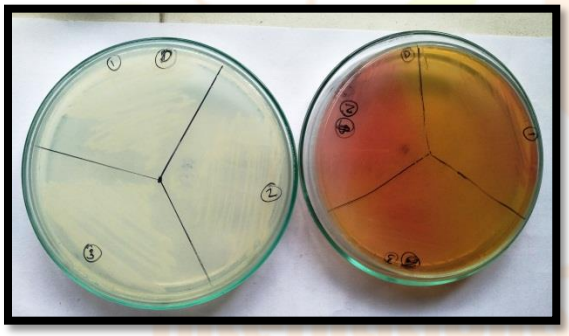

Fig 1: Urease positive test.

***Quantitative urease assay by electric conductivity:***

Conductometery is considered to be accurate method for urease assay.After addition of culture into substrate, conductivity was recorded at zero time and at 1, 2, 3, 4, and 5 minutes. As showed in Table no. 2, the conductivity continuously increased with time in a positive proportional relation with urease activity. It shows stability and consistency in the rate of conductivity increase in the first 5 minutes of reaction (i.e. conductivity increase per minute). The rate of enzyme activity was in the range of 0.009-0.051.

Table No. 2 Electric conductivity (EC (mS/S) of urease assay mixture at different time intervals (min).

| **Isolate** | **Electric conductivity (mS/min)** | | **Isolate** | **Electric conductivity (mS/min)** | |
|---|---|---|---|---|---|
| | 0 min | 5 min | | 0 min | 5 min |
| A1 | 0. 023 | 0.029 | H1 | 0.010 | 0.018 |
| A2 | 0.032 | 0.043 | H2 | 0.012 | 0.016 |
| A3 | 0.029 | 0.030 | H3 | 0.010 | 0.011 |
| A4 | 0.028 | 0.040 | H4 | 0.031 | 0.039 |
| B1 | 0.036 | 0.041 | H5 | 0.033 | 0.034 |
| B2 | 0.042 | 0.047 | H6 | 0.010 | 0.012 |
| B3 | 0.047 | 0.051 | H7 | 0.014 | 0.017 |
| B4 | 0.028 | 0.030 | H8 | 0.007 | 0.009 |

***Calcium carbonate precipitation test:*** B4 media was used for $CaCO_3$ precipitation test. Calcium carbonate crystals formed in agar appeared as white precipitant within andaround the growth area. Fig.2 and Fig.3 shows the $CaCO_3$ precipitation in broth and in plates respectively after 7 days of incubation.

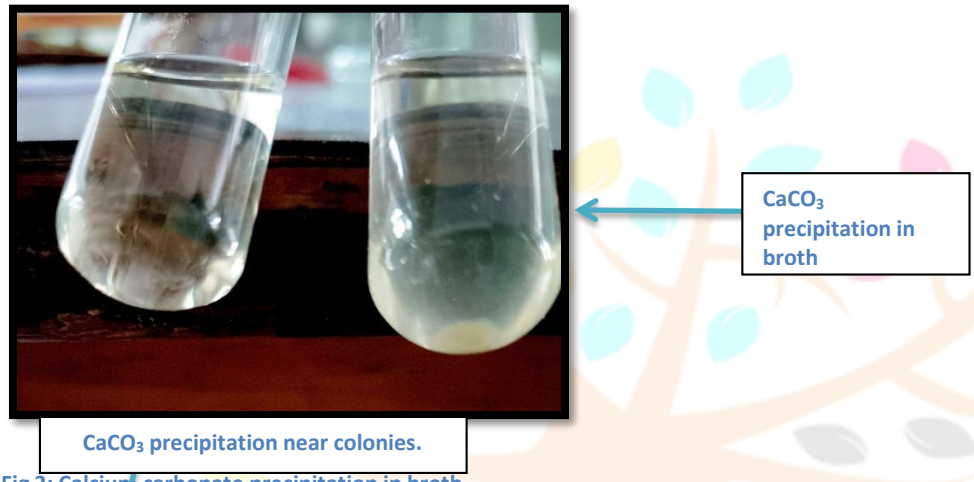


Fig 2: Calcium carbonate precipitation in broth.

Fig 3: Calcium carbonate precipitation in agar medium within and around growth area.

## CONCLUSION:

In this study, isolation of bacteria was done from different soil samples. All isolates were tested for the growth at high temperature, high pH and high salt concentration. For qualitative analysis of urease activity, Christensen's Urea Agarwas used and for quantitative assay conductivity method was done.In solid and broth media effective $CaCO_3$ precipitation was tested. The study resulted in 5 efficient isolates capable of producing carbonate precipitates *in vitro* (A2, A4, B1, B2& B3).

Further studies on these isolates is to be carried out to exploit there use in the self-healing approach of concrete.

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