# Assessment of Biocement Production Capability of Urease Producing Isolates

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***Abstract:*** Urease is known to catalyze the hydrolysis of urea present in the environment generating ammonia and carbonate. This carbonate in the presence of calcium ion precipitates as calcium carbonate ($CaCO_3$), the deposition of $CaCO_3$ precipitates is known as Biocement. Biocement is the binding material, which bind sand particles together to increase the shear strength which is the base for sand consolidation. The aim of this study was to assess the ability of urease producing microorganisms to produce biocement. For this study urease producing microorganisms were isolated from six different samples, tested for urease production & urea tolerance capacity and selected isolates were assessed for the biocement production. From six different samples total 14 isolates were obtained, from which only 4 isolates ($U_1$, $U_2$, $U_3$ and $U_4$) were selected on the basis of urease production within 48 hrs. From the selected 4 isolates 2 isolates ($U_1$&$U_3$) were selected on the basis of urea tolerance capacity and assessed for the biocement production and ability for sand consolidation. Study was also carried out with 2 known urease producing laboratory available bacteria *Proteus vulgaris* & *Klebsiella pneumoniae*. Selected isolates and known bacteria were able to produce biocement in hard water. $U_1$, $U_3$, *P.vulgaris & K.pneumoniae* produced 0.88g/l, 0.76g/l, 0.60g/l & 0.72g/l biocement respectively. For the sand consolidation test isolates, cementation solution and sand were mixed in plastic cup and incubated for 15-20 days and were capable to produce biocementation between sand particles.

***Index Terms*** **-**Biocement, $CaCO_3$ precipitation, Sand consolidation, Urease

**1.Introduction:**

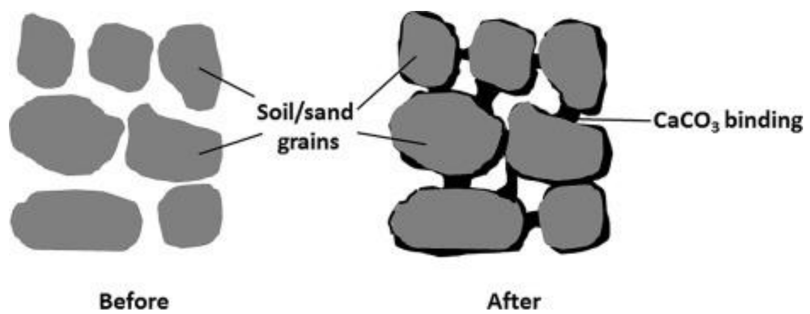
Cement is the most widely used man made construction material, which maintains the durability of building structures. These building materials are porous, this porosity of material will increase with moisture and other chemicals and affect the stability of material and due to low stability buildings were deteriorate. Constant development in the field of civil engineering and growth of industrial activity has created growing demand of new construction material. We need a material with self healing capability which can overcome the short coming of conventional sealing agent. Use of urease producing organisms addresses this problem effectively as they continue to survive and grow within the concrete and ability to produce biocement (Varalakshmi et al., 2014).

**1.1 Urease**

Urease or Urea aminohydrolase (E.C.3.5.1.5) is the nickel metalloenzyme produce by bacteria, fungi, algae, and higher plants. One of the robust ureolytic bacteria is *Sporosarcina pasteurii* formerly known as *Bacillus pasteurii*. Urease help in the biomineralization of Calcium carbonate by using urea as energy source and hydrolyzed the urea into ammonia and carbonate. This carbonate spontaneously decomposes to produce another molecule of ammonia and carbonic acid. Two molecules of ammonia and carbonic acid subsequently equilibrated in water resulting in increase in the pH causing $Ca^{+2}$ and $CO_3^{-2}$ precipitate as $CaCO_3$ in presence of calcium ions (Mobely&Hausinger, 1989).

**1.2 Microbial induce calcium carbonate precipitation (MICP)**

Calcium carbonate ($CaCO_3$) is one of the most common mineral found on earth. Calcium carbonate is precipitated through chemical precipitation and microbial precipitation. Precipitation of calcium carbonate through urea hydrolysis by microbial urease is known as microbial induce calcite precipitation (MICP). The main groups of microorganisms involved in $CaCO_3$ are photosynthetic organisms such as algae &cynobacteria, sulfate reducing organisms and organisms involved in nitrogen cycle. The $CaCO_3$ deposition that formed due to microbial urease activity is known as biocement. As shown in figure.1 generation of $CaCO_3$will act as binding medium and bind the sand or soil particles together thus help in the consolidation of sand.




Source: Krajewska, B. (2018)

**1.3 Biocement**

Biocement is product innovation from developing bioprocess technology called Biocementation. Biocement refers to a $CaCO_3$ deposit that formed due to microorganism activity in calcium rich system (Ariyanti et al., 2012). This biocement is known as Bacterial concrete which is a self-repairing biomaterial that can remediate cracks and fissures in concrete (T. Alhour, 2013). Biocement is a sand consolidation technology in which carbonate released from microbial urea hydrolysis and precipitates with excess of calcium ion to form in-situ calcite ($CaCO_3$) and under the right conditions this can result in soil solidification and bind the sand particles (Al-Thawadi, 2008). Biocement is applicable as biological construction material as well as crack healer, water purifier and also used for enhance the stability of building materials.

## 2. MATERIALS AND METHODS

**2.1 Sample collection:** Six different samples were collected from different areas of Khergam village like; Soil samples from agricultural field, agricultural field applied with urea fertilizer, animal resident, peat, rice-plant field and Sewage sample.

**2.2 Enrichment of samples:** Enrichment of sample was done by inoculating the samples into nutrient broth containing 2% urea (enrichment medium) & incubated on shaker for 3-5 days at 28°C- 30°C (Phang et al., 2017).

**2.3 Isolation & Screening of urease producing organisms:** Enriched samples were serially diluted and streaked on the sterile urea agar plates. Plates were then incubated at 28°C-30°C for 48 hrs. Positive results were indicated by color change from pale yellow to pink around the colonies. Form the isolates organisms which gave good urease production within 48 hrs were selected for further work.

**2.3.1 Phenotypic characterization:** Isolates were characterized phenotypically with respect to morphology and Gram's reaction.

**2.4 Standard Curve for Ammonium chloride:** Standard ammonia activity was performed using 1000μg/ml ammonium chloride ($NH_4Cl$) solution. Different aliquots of $NH_4Cl$ solution were taken and made up to 1ml by adding phosphate buffer (pH 7.0). Then 1 ml of Nessler's reagent was added and incubates it for 10 min, after incubation take the absorbance at 405 nm (Phang et al., 2017).

**2.5 Measurement of urease activity:** Urease activity was measured with Nessler's method described by Phang et al., (2017) with slight modification. For the measurement of urease activity loopful culture of selected isolates were first grown into 20 ml of urea broth for urease production and incubated on shaker for 24 hrs at 28-30°C. The reaction mixture contained 1ml of supernatant, 1 ml of 0.2M phosphate buffer (pH 7.5) and 1ml of 0.1M urea solution and was incubated at 37°C for 10 min for enzyme-substrate reaction. After incubation reaction was stopped by inactivating the enzyme with 1ml of 5% trichloroacetic acid (TCA). 1ml mixture was taken in another tube and 1ml of Nessler's reagent was added. Total volume was made 50 ml by adding 48ml distilled water. The yellow color produced was measured at 405 nm and the urease activity was calculated. One unit of urease activity was defined as the amount of urease that produces 1μmole of ammonia per minute under assay conditions. Isolates that showed high urease activity were selected for further work (Phang et al., 2017).

**2.6 Measurement of urea-tolerance level:** Urea tolerance level of organisms were measured by inoculating the isolates into 20ml of urea broth tubes having 1M, 1.5M, 2M, 2.5M, 3M, 3.5M & 4M urea and incubated at 37°C for 48hrs. Then the turbidity was determined at 600nm using spectrophotometer (Varalakshmi et al., 2014).

**2.7 Screening for calcium carbonate ($CaCO_3$) precipitation**: The ability of isolates to precipitate carbonate was screened by culturing the organisms on the calcium carbonate precipitation agar with calcium source. Agar plates were then incubated at 30-35°C for 4-5 days and the precipitation of crystals were observed under light microscopy (Phang et al., 2017).

**2.8 Production of biocement from hard water**

**a) Determination of water hardness:** Two water samples 1) laboratory tap water and 2) Ground water was taken and their hardness was measured. Water with high hardness was selected for the biocement production. Water hardness was determined by EDTA titramatric method. 20 ml of water sample was taken and pH was adjusted to 10 by adding 2ml of ammonia buffer solution (pH 7.0). Then few drops of Eriochrome black T indicator was added and titrated against 0.01M of Sodium Ethylenediamine tetra acetate (EDTA) till the color changes from wine red to metallic blue (Varalakshmi et al., 2014).Hardness of water was calculated in mg/L using following formula.

Water Hardness (mg/L) = ml of EDTA used × 1000/ml of water sample

**b) Production of biocement:** For the biocement production, in 250 ml water sample 100ml of 1.5M urea solution and 100ml of bacterial inoculums was added and incubated at 37°C for one week. After incubation the deposit of calcium carbonate was filtered using whatman filter paper. After filtration filter paper containing deposit was kept inside hot air oven for drying. The weight of filter paper was measured before and after filtration and amount of biocement was calculated (Varalakshmi et al., 2014).

**2.9Preparation of cementation solution:** cementation solution contained mainly two components; (1) 24hrs old Bacterial culture which was cultivated in nutrient broth with 0.1M $CaCl_2$ and (2) calcium chloride & urea solution contained 82.5 g/L of (0.75M) $CaCl_2$ and 60.0 g/L of (1M) urea respectively. Equal amount of solutions of urea and calcium chloride were taken and mixed. These two components were premixed at the time of injection into the sand. (T. Alhour, 2013).

**2.9.1 Study of sand consolidation**: For the consolidation process 100 ml plastic cup which was contained filter paper in the bottom and drilled from the back. This cup was put inside another plastic cup which is considered as collector cup in which cementation solution was collected from upper plastic cup. 80g of sand was dried packed in drilled plastic cup and up-flushed with water. Cementation (0.75M $CaCl_2$ and1M Urea) solution and bacterial suspension were premixed immediately before injection and poured into cup containing sand. The solution was poured on the sand daily until the process finished after 7 days from the start of the process. After 10-15 days cups were looked for the crust formation cups which indicate consolidation of sand particles. (T. Alhour, 2013).

**3. RESULTS AND DISCUSSION**

**3.1Isolation and screening of urease producing organisms:** Total 14 urease producing organisms were isolated from six different samples including 13 bacteria and 1fungi. Out of all isolates only four isolates coded as U1, U2, U3 & U4 were selected for further work on the basis of urease production within 48hrs. All 4 were bacteria. Also two reference organisms *Klebsiella pneumoniae* &*Proteus vulgaris* were used for the study. (Figure.1)

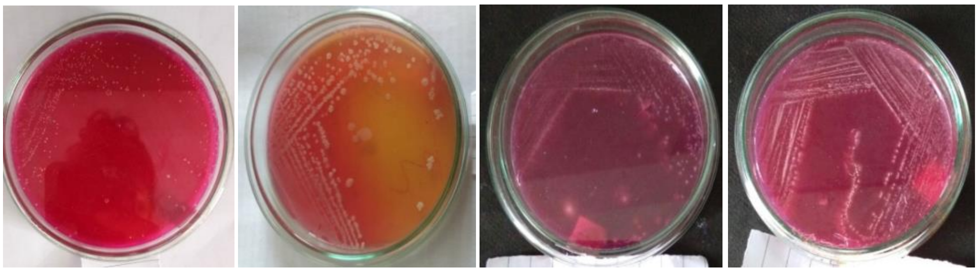


Figure.1 Isolation of urease producers on urea agar plates

**3.1.1Phenotypic characterization:** Among the 13 bacterial isolates 5 were gram-positive rods, 5 were gram-negative rods and 3 were gram-positive cocci.

**3.2 Measurement of Urease activity:** Among the selected four isolates U1 and U3 gave highest urease activity 29 U/ml/min and 28 U/ml/min respectively. And reference organisms *K. pneumoniae* and *P. vulgaris* gave 68 U/ml/min and 88 U/ml/min urease activities respectively. (Figure.2)

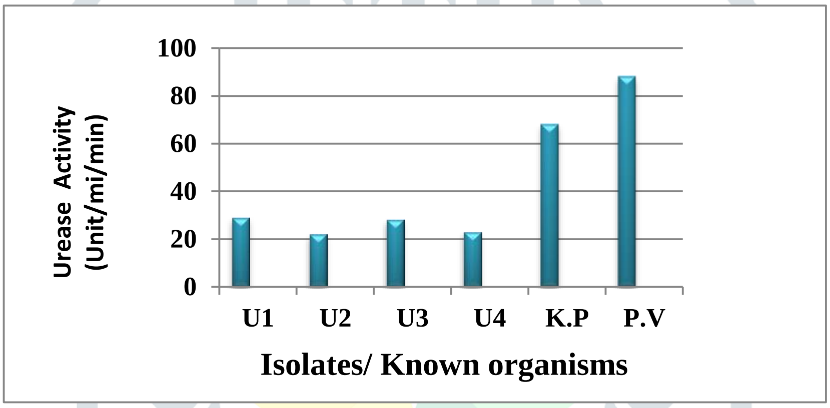



Figure.2Urease activity results

**3.3 Measurement of Urea-tolerance level:** Selected isolated (U1 & U3) and reference organisms showed urea-tolerance capacity up to 2.5M urea concentration as shown in figure.3. 3M and above there was no growth of either isolates or *P. Vulgaris* and *K. pneumoniae*. In one experiment urea tolerance capacity was reported for two bacterial strain of *Proteus* spp. up to 3.5 gm/ml (Varalakshmi et al., 2014).

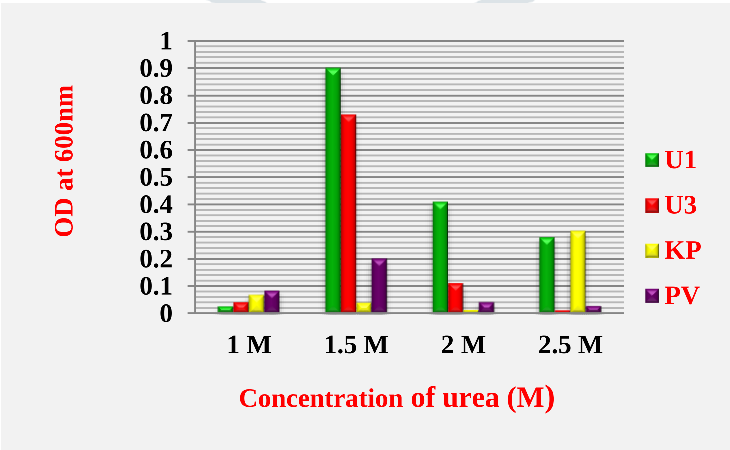



Figure.3Results of urea tolerance capacity

**3.4 Screening of Calcium carbonate precipitation ($CaCO_3$):** Both isolated organisms and reference organisms able to precipitate Calcium carbonated which was observed into agar plates (figure.4) as well as microscopically (figure.5). Such phenomena of crystals generation were also observed by Hammes et al in 2003.

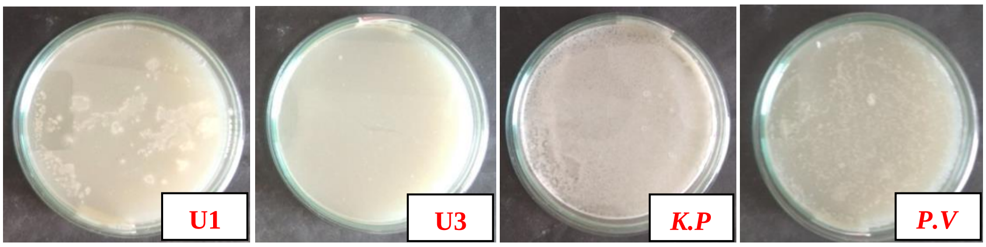



Figure.4 Calcium carbonate precipitation agar plates with precipitates

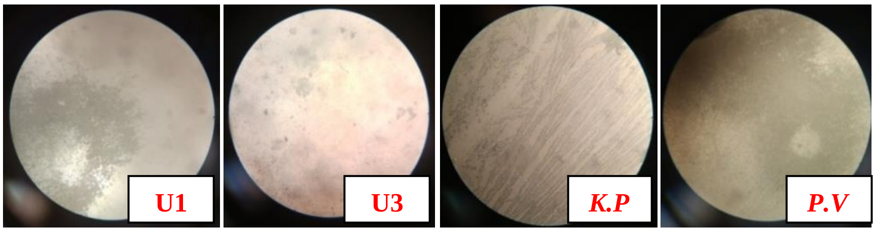



Figure.5Microscopic observation of $CaCO_3$ crystals

**3.5 Determination of water hardness:** As shown in figure.6 the change of color from wine red to blue by reducing EDTA was observed. Laboratory tap water and ground water showed 347.2 mg/L and 487.5 mg/L hardness respectively. It was observed that ground water had higher hardness than laboratory tap water. Hence, ground water was selected for the biocement production.

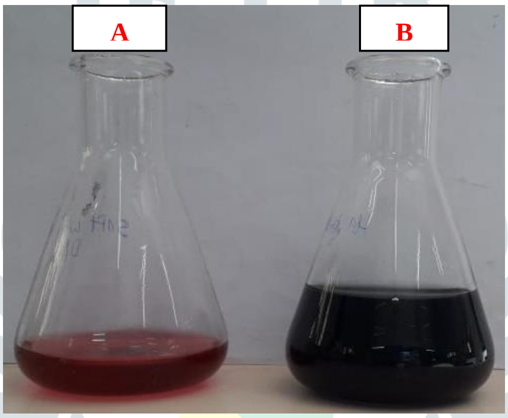
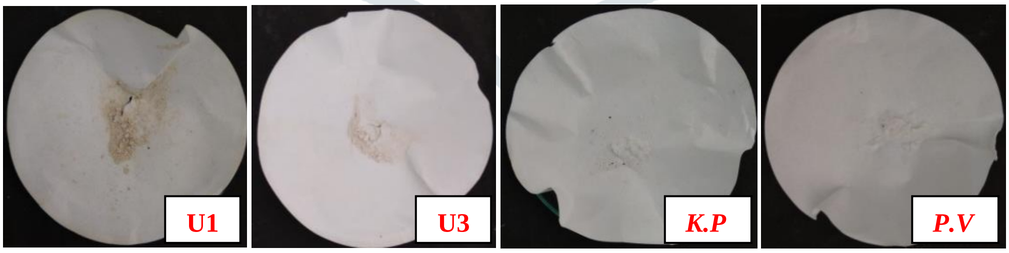



Figure.6 Color change from wine red to blue (A) Before titration (B) After titration

**3.5.1 Biocement production from hard water:** Selected isolates and known bacteria were able to produce biocement in hard water.U1, U3, *K. pneumoniae* and *P. vulgaris* produced 0.88g/L, 0.76g/L, 0.60g/L and 0.72g/L of biocement respectively (figure.7). In similar study Varalakshmi et al.,( 2014) reported 5gm of biocement from 500ml of water sample after incubation of one week.




Figur.7 Biocement production produced by isolates and reference organisms

**3.6 Study of Sand consolidation:** Crust formation was observed due to sand consolidation (figure.8). Among the selected isolates (U1 & U3) and two reference organisms, two organisms U1 and *P. vulgaris* showed positive results to consolidate the sand while 2 others are not. Similar experiment was carried out by T. Alhour in 2013 in which 13 isolates were found to pack the sand tightly. Whiffin et al., (2007) also reported similar results.

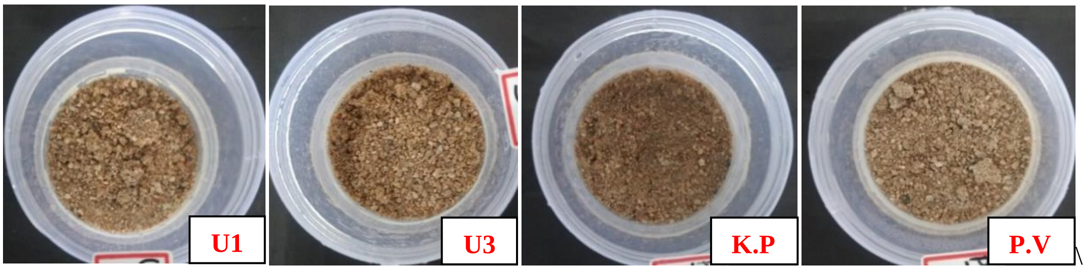


Figure.8 Crust formations through sand consolidation

## 4. CONCLUSION

This study indicates that urease producing microorganisms were able to precipitate calcium carbonate naturally means they are capable to produce biocement and also able to consolidate the sand. This unique property of urease producers make them suitable for applicable in civil engineering as construction material as well as an innovative idea for wall crack filling.

## 5. ACKNOWLEDGEMENT

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## 6. REFERENCES

1) Al- Thawadi, S. M. (2011). Ureolytic bacteria and calcium carbonate formation as a mechanism of strength enhancement of sand. J. Adv. Sci. Eng. Res, 1(1), 98-114.
2) Ariyanti, D., &Handayani, N. A. (2012). Hadiyanto (2012) Feasibility of Using Microalgae for Biocement Production through Biocementation. J Bioprocess Biotechniq, *2*(111), 2.
3) Elmanama, A. A., &Alhour, M. T. (2013). Isolation, characterization and application of calcite producing bacteria from urea rich soils. J Adv Sci Eng Res, 3, 388-399.
4) Hammes, F., Boon, N., Clement, G., de Villiers, J., Siciliano, S. D., &Verstraete, W. (2003). Molecular, biochemical and ecological characterisation of a bio-catalytic calcification reactor. Applied microbiology and biotechnology, 62(2-3), 191-201.
5) Krajewska, B. (2018). Urease-aided calcium carbonate mineralization for engineering applications: A review. Journal of advanced research, *13*, 59-67.
6) Mobley, H. L., &Hausinger, R. P. (1989). Microbial urease: significance, regulation, and molecular characterization. Microbiological reviews, *53*(1), 85-108.
7) Phang, I. R. K., San Chan, Y., Wong, K. S., & Lau, S. Y. (2018). Isolation and characterization of urease-producing bacteria from tropical peat. Biocatalysis and agricultural biotechnology, 13, 168-175.
8) Stabnikov, V., Naeimi, M., Ivanov, V., & Chu, J. (2011). Formation of water-impermeable crust on sand surface using biocement. Cement and Concrete Research, 41(11), 1143-1149
9) Varalakshmi, A. D., & Devi, A. (2014). Isolation and characterization of urease utilizing bacteria to produce biocement. IOSR-JESTFT, 8, 52-57.
10) Whiffin, V. S., van Paassen, L. A., &Harkes, M. P. (2007). Microbial carbonate precipitation as a soil improvement technique. Geomicrobiology Journal, 24(5), 417-423.