Isolation and Identification of Local Bacteria Produced from Soil-Borne Urease

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Abstract—The hydrolysis of urea by the enzyme urease is significant for increasing the irroles in human pathogenicity, biocementation, soil fertilizer, and subsequently in soil improvement. This study devoted to the isolation of urease from urea-rich soil samples collected from seven different locations. Isolation of the various bacterial species was conducted using nutrient agar. The identity of isolated urease was based on morphological characteristics and standard microbiological and biochemical procedures. The urease producing strains of bacteria were obtained using the urease hydrolysis test. The bacterial isolates produced from soil samples collected from different environments and treated by different morphological processes helped in precipitation of large calcium carbonate (CaCO₃) crystal aggregates precipitated within bacterial colonies grown on agar. The different microbial species and functional attributes produced striking differences in the morphology of precipitated crystals. The phylogenetic sequencing of 16S ribosomal RNA genes produced several isolates that are mostly related to the Bacillus group. One strain of promising results was selected and the environmental and nutritional conditions were characterized. The growth curve of the selected strain with an optimized condition was investigated.

Keywords: local bacteria; isolation; calcium carbonate; hydrolysis; microbial analysis; soil improvement.

1. Introduction
Microorganisms including bacteria live in the soil and all over the earth’s surface. Microorganisms play important roles in nutritional chains that are an important part of this biological balance in the life on our planet, and are necessary to complete nutrient and geochemical cycles such as the carbon, nitrogen, sulfur, and phosphorous cycle. Bacteria play an important role in soil without which the soil will not be fertile and organic matter such as leaves and straw will accumulate within a short period [1–3]. Microorganisms are widely spread in the world. Urease (urea amidohydrolase, EC 3.5.1.15) is an enzyme that catalyzes for hydrolysis urea substrate to form carbon dioxide and ammonia [4]. Urease has been well studied from a clinical perspective as it can indicate increased virulence properties in pathogenic bacteria [5–7]. In recent years, the study on the application of using the production of microbial ureases in civil and geotechnical engineering has increased because of the abilities of these microorganisms to induce calcite precipitation in the presence of urea and calcium ions [4]. This process is termed microbial induced calcite precipitation (MICP), an innovative technique that harnesses the activity of bacteria to improve the physical properties of soils as a sustained and environmentally responsible method for soil strengthening and other engineering applications [8, 9].
Microorganisms that can hydrolyze urea are abundant in natural soils, thus, making the process of urea hydrolysis common to soils worldwide [10]. Many soil bacteria can crush urease in the presence of ammonia and other nitrogen compounds. a few bacteria are known to be able to produce urease in the presence of urea [6]. A well-documented application of ureolytic microorganisms has been the improvement of the mechanical properties of soil for construction and environmental purposes, known as biocementation. Biocementation enhances the strength and stiffness properties of soil through microbial activities and products [11]. Various studies have reported the isolation of ureolytic bacteria using enrichment culture from a variety of soil samples, groundwater samples, and cement samples [12–16]. Most Bacillus species can hydrolyze urea using urease enzymes [13]. The previous studies have shown that there is an increasing number of microorganisms being screened from extreme environments with the capability to produce essential enzymes useful for various industrial applications.

2. Materials and Methods

2.1 Soil sample collection and preparation
Ten soil samples were collected from different regions of soil enriched with urea. A total of 10 samples were aseptically collected randomly from various locations. During collection, a clean stick was used to clear the debris away and a 10 cm hole was dug. The soil sample approximately 5 g was collected using a sterile spatula and put in a clean, dry, and sterile polythene bag, then stored in an icebox.

2.2 Enrichment culture of samples
To enrich the samples for urease producing bacteria, 1 g of soil sample was added to 10 mL of nutrient broth (HiMedia, Laboratories Pvt. Ltd) containing 20 g urea (100 mL shaking flasks) and incubated under aerobic batch conditions at 37°C for 120 hrs. under shaking condition at 100 rpm.

2.3 Isolation of urea hydrolyzing bacteria
For bacterial isolation, 1 mL of soil culture was serially diluted (five-fold) and inoculated on urea nutrient agar. A loopful culture from serially diluted enrichment samples was inoculated onto Petri plates containing urea nutrient agar were then spread using a loopful spreader until the fluid was evenly distributed. Then the plates were incubated under aerobic conditions at 37°C for 48 hrs. When isolates of 20 g urea degradation were grown in Petri dishes containing nutrient agar, a subsequent sub-culture was performed until single colonies of bacteria were obtained.

2.4 Screening for ureolytic bacteria
Urea agar base (Oxoid, Thermo Scientific Microbiology Sdn Bhd) was used as a qualitative urease assay and to rapidly test for bacteria producing urease. Preparation of the medium was performed following the manufacturer's instructions. To test the production of urease, heavy inoculation was performed on the surface of the (UAB) medium in the test Petri plates and incubated under aerobic conditions at 37°C for 48 hrs. The urease test was studied through visual observation for color changes. The isolates that changed the medium from yellow to a pink color were selected for further studies based on their ability to rapidly produce urease qualitatively.

3. Identification and Characterization of Isolates
All isolated were further identified using biochemical tests such as Gram stain reaction, motility test, citrate test, catalase test, sugar fermentation test.

3.1 Isolation of genomic DNA
Genomic DNA was isolated using the Wizard Genomic DNA purification Kit (Promega).

3.2 Extraction protocol of DNA
According to the kit's protocol, the following procedure was implemented to extract genomic DNA from isolated bacterial strains:
3.2.1. For gram-positive bacteria

- One mL from an overnight broth culture of isolated bacterial was added to the Eppendorf tube and pelleted by centrifuge at 14000 rpm for 2 min, then remove supernatant.
- re-suspend cell pellet in (50 mm) EDTA to final volume 480 μL.
- a lytic enzyme (120 μL) (lysozomandor or lysostaphin) was added to the tube and mix again by vortex
- after that incubate at 37°C to 1 h., centrifuge for 2 min at 14000 rpm, finally, remove the supernatant.

3.2.2. For gram-negative bacteria

- Re-suspended cell pellet in Nuclei Lysis Solution to a final volume of 600 μL, and incubate at 80°C for 10 min.
- Five μL of RNase solution was added to the mixture, mix with a pipet for 2-5 times, and incubate at 37°C for 1 h.
- After RNase treatment, 200 μL of Protein Precipitation Solution was added to the tube, and mix the mixture by vortex at high speed for 20 s.
- After that incubate the tube on ice for 5 min, and centrifuge at 14000 rpm for 3 min.
- Transfer the supernatant to a clean tube contains 600 μL of isopropanol, mix gently and centrifuge at 14000 rpm for 2 min.
- Absolute ethanol of 600 μL (70%) was added to the tube and mixed again by vortex at high speed for 15 s.
- Drain the tube to absorbent paper and leave it to dray for 10-15 min.
- Consequently, about 50 μL of DNA rehydration solution was added to the Eppendorf tube and incubate at 65°C for 1 h, then the sample was centrifuged at 6000 rpm for 1 min.
- Finally, the extracted DNA was stored at -20°C freezer in the appropriate sample box until PCR amplification.

3.3. Measuring the concentration and purity of DNA

The concentration of DNA and purity were measured by the Nano-drop UV spectrophotometer (Quawell Technology Inc, USA). The elution buffer that used as the blank was dsH₂O, then 1 μL of DNA was measured at two wavelengths (260 and 280 nm) to conclude DNA concentration and purity respectively. The calculated ratio was gained from the operating software and displayed by the Nano-drop spectrophotometer (157 UV/Vis single wavelength detector).

4. PCR Amplification

4.1. Oligonucleotides primer pairs

The primer of the bacillus gene used in this study was purchased from Alpha DNA (Canada) in lyophilized, shown in Table 1, the bacillus sequence of isolated bacterial strains. The first twenty nucleotides represent the forward primer and the complementary primer represents the last seventeen nucleotides. Primarily, the stock solution was prepared by dissolving the lyophilized powder in nuclease-free water buffer according to the manufacturer's protocol to give a total concentration of 100 pmol μL⁻¹ for each one. Then the working solution was prepared where 10 μL from Stock solution was added to 90 μL of nuclease-free water to give a total concentration of 10 pmol μL⁻¹ for each reverse/forward primer.

| Primer name | Primer sequence | Reference |
|-------------|-----------------|-----------|
| 27F         | 5’-AGAGTTTGATCTTGCGTCAG-3 | Designed in this study |
| 142R        | 5’-TACCGTACCTTTGTTACGACTT-3 |                 |
4.2. Amplification kit
The PCR amplification mixture which used to amplified bacillus gene includes GoTaq® Green Master Mix, 2X (25 μL), 2.5 μL of 25 ng DNA template, 5 μL (1 mM) of each forwarded and reversed primers and then 22.5 μL of nuclease-free water was added to complete the amplification mixture to 50 μL (Table 2).

| Component                  | Volume | Final concentration |
|---------------------------|--------|---------------------|
| GoTaq® Green Master Mix, 2X | 10 μl  | 1X                  |
| Forward primer            | 1 μl   | 1µM                 |
| Reverse primer            | 1 μl   | 1µM                 |
| DNA template              | 3 μl   | 30 ng               |
| Nuclease free water       | 5 μl   |                     |
| Final volume              | 20 μl  |                     |

4.3. Agarose gel electrophoresis
Extracted DNA and PCR products were tested by (1% [w/v]) agarose gel electrophoresis with a 1X TAE buffer. Firstly, 1 g of agarose was dissolved in 100 mL TAE working buffer (1X) in a flask, then the mixture was heated on a hot plate for 5-10 min until the agarose was completely softened, the solution was left to be warm for 5 min, and 1 μL (10 mg/mL) of ethidium bromide was added. After that, the solution was poured in a gel tray with a good comb and left to solidify at 50-60°C for 20-25 min. For loading samples, DNA samples were mixed with 1X DNA loading dye, then 5 μL of PCR product and DNA samples along with DNA ladder was loaded into the gel wells. The running buffer is 1X TAE, and the gel was run at 100v/mAmp for 1 h and it visualized by Ultraviolet light (UV) device (158), see results in Table 3.

| Optimization          | Temperature, °C | m:s   | Cycle number |
|-----------------------|-----------------|-------|--------------|
| Initial denaturation  | 95              | 05:00 | 1            |
| Denaturation          | 95              | 00:30 |              |
| Annealing             | 60              | 00:45 | 30           |
| Extension             | 72              | 01:00 |              |
| Final extension       | 72              | 07:00 |              |
| Hold                  | 10              | 10:00 | 1            |

4.4. Sequencing
PCR product was sent to the Macrogen Company in Korea for sequencing. Unpurified PCR product at a volume of 50 μL, and forward primers at a concentration of (10 pmol μL⁻¹) were sent to the company for purification to remove all the unwanted elements, then Sanger sequencing was done by using AB13730XL, automated DNA sequences. The result analyzed by the BLAST website on NCBI, Mega, and Bioedite.

5. Measurement of Urease Activity
Urease activity was measured immediately after incubating an overnight bacterial culture. urease activity was determined by [17] In measuring the activity of the enzyme on the conversion of one micromol of urea to ammonia. The reagent used in this study are:

5.1. HEPES (N-2Hydroxy Ethyl Piperazine-N-2-Ethan)
HEPES solution was prepared by dissolving (50 mM of sulfuric acid, (1 mM) of EDTA (Ethylene diamine tetraacetic acid), (1 mM) of 2-mercaptoethanol) in 100 mL of distilled water and the initial pH was adjusted to 7.5 using 0.1 M of NaOH [18]. This buffer is used to dilute the ammonium chloride
storage solution of the standard curve as well as to dilute the samples to estimate the effectiveness of urease.

5.2. Indophenol assay reagents
The indophenol reagents are:

5.2.1. Phenol nitroprusside (A Reagent). Prepare dissolved (2.5 g) of (NaOH) in 500 mL of distilled water added (4.2 mL) of sodium hypochlorite (6%), shake well, and kept in a dark bottle in the refrigerator for a period not to exceed one month.

5.2.2. Alkaline hypochlorite (B Reagent). Prepare (2.5 g) of sodium hydroxide (NaOH) in 500 mL of distilled water and 4.2 mL of sodium hypochlorite (6%), shake well and keep in a dark bottle in the refrigerator for Do not exceed a month [19].

5.2.3. Urea stored solution (500 mM). Prepared by dissolving 300 mg of urea in 10 mL of distilled water.

6. Procedure of Testing
The procedure of testing is conducted as follows:
- Putting 215 µL of HEPES buffer solution and 25 µL of stored urea solution in test tubes and left in a water bath at (37°C) for three minutes.
- Adding 10 µL of the enzyme solution to the reaction components to become the final volume of the reaction solution 250 µL and the final concentration of urea is 50 mM. The tubes were incubated in a water bath at a temperature of 37°C for 15 min (reaction time by which urea was hydrolysis in the presence of urease).
- Adding 5 mL of reagent (A) and 5 mL of reagent (B) to the tubes with rapid shaking, and left in a water bath at 37°C temperature for 20 min for the blue color to appear.
- The light absorbance of the resulting color of the reaction was measured at a wavelength of 625 nm.
- The amount of ammonia released in micromol (µmol) was measured according to the standard curve of the ammonia solution as shown in Fig. 1. The enzymatic activity (unit/mL) was estimated according to the following equation:

\[ \text{Activity (unit/mL)} = \frac{\text{Amount of ammonia (µmol)}}{0.01\times15\times2} \]  

(1)

![Figure 1](image)

**Figure 1.** Variation of ammonia concentration with absorbance at a wavelength of 625 nm.
7. Results and Discussion

7.1. Selection of ureolytic bacteria
Among all six isolated bacteria, six bacterial isolates designated as C11, D11, H11, A11, B12, and D12 were selected based on their quick urease production when tested on urea agar base medium. Urea agar base media is used for the diagnosis of a variety of microorganisms, especially based on urease production [20]. Bacterial isolates that can produce urease turned the UAB medium in the Petri plate from yellow to pink as shown in Fig. 2, while the isolates which were unable to produce urease turned the media yellow. This medium contains urea and a pH indicator, phenol red. When urea is hydrolyzed, ammonia accumulates in the media which increases the pH of the environment [21]. This increase in pH causes the pH indicator to change from pale orange to pink, confirming a positive result for urea hydrolysis. Several studies have reported using urea agar base media as a preferred qualitative urease assay for isolation and differentiation of ureolytic microorganisms [13–16, 21–23].

![Figure 2. Urease producing test UAB medium.](image)

7.2. Preliminary bacterial identification
The isolates C11, D11, H11, A11, B12, and D12 were characterized using standard methods. All six isolates were Gram-positive rod-shaped and smooth surfaces (Table 4). There were notable morphological differences among bacterial isolates. Bacterial community diversity in the soil environment due to its alkaline state, only organisms that can grow in these conditions can survive in such an environment [22]. The dominant species that may occur during the period of fertilization cultivation where bacteria are usually chosen in isolation and cultivation methods [24]. The phenotypic and biochemical properties of the bacteria isolates have a similar resemblance to those Bacillus species reported previously by [22].

| Characteristic      | C11  | D11  | F11  | A12  | B12  | C12  |
|--------------------|------|------|------|------|------|------|
| Gram stain         | +ve rod | +ve rod | +ve rod | +ve rod | +ve rod | +ve rod |
| End spore staining | Spore forming | Spore forming | Spore forming | Spore forming | Spore forming | Spore forming |
| Catalase           | +    | +    | +    | +    | +    | +    |
| Motility           | +    | +    | +    | +    | -    | -    |
| Citrate            | +    | +    | +    | +    | +    | +    |
| Anaerobic growth   | +    | +    | +    | +    | +    | -    |
| NaCl 7%            | +    | +    | +    | +    | +    | +    |
| NaCl 10%           | -    | -    | -    | +    | -    | +    |
7.3. **Bacterial identification using 16S rRNA sequence analysis**

The six selected urease producing bacteria isolated from soil were identified and characterized by the sequencing of 16S rRNA. These sequences were BLAST searched against the GenBank database using the BLASTN program. The nucleotide BLAST analysis of the 16S rRNA region showed a reasonable degree of correlation with the physiological characterization especially the morphological classification schemes of species within the genus. The BLAST analysis revealed that C11 had 99% similarity (100% coverage) with *Bacillus sonorensis*, D11 had 99% similarity (100% coverage) with *Bacillus sonorensis*, H11 had 97% similarity (100% coverage) with *Bacillus sonorensis*, A11 had 98% similarity (99% coverage) with *Bacillus licheniformis*, B12 had 92% (99% coverage) with *Bacillus licheniformis*, D12 had 95% similarity (99% coverage) with *Bacillus sonorensis*. Based on sequence data of the 16S of the rRNA region, all bacterial isolates from soil showed a high degree of similarity (91-100%) to their closest species. The relationship between the locally isolated ureolytic bacteria and their closest species are shown in the phylogenetic tree see Fig. 3.

![Phylogenetic tree](image)

**Figure 3.** Phylogenetic tree based on the 16sRNA gene sequence of isolated bacteria in the current study along with sequences available in the GenBank database.

7.4. **Measurement of urease activity**

Among all the bacterial cultures, C11 had the highest urease activity (10.25 mM urea hydrolyzed/min/OD) when compared to other local cultures, while C12 showed the lowest specific urease activity (1.43 mM urea hydrolyzed/min/OD) as shown in Fig. 4. Numerous studies have reported that the usage of reported studies on locally isolated strains of *Bacillus* bacteria had urease activity 3.3 to 8.8 mM urea hydrolyzed/min/OD [25, 26].

![Urease activity chart](image)

**Figure 4.** Comparison of urease activity among the isolated urease producing strains. Two bacterial isolates, C11 and D11 show high urease activity, almost comparable to control strain.
8. Conclusions
The BLAST analysis of the 16S rRNA gene sequence from the locally isolated ureolytic bacteria identified them as Bacillus sonorensis and Bacillus licheniformis. The specific urease activity of the measured bacterial strains indicates that these ureolytic strains serve as a good urea hydrolytic agent, potentially useful for biocementation applications.

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References
[1] Kummerer, K. (2004) Resistance in the environment, Journal of Antimicrobial Chemotherapy, 45, 311-320.
[2] Sylvia, D. M., Fuhrmann, J. J., Hartel, P. G., and Zuberer, D. A. (2005) Principles and applications of soil microbiology, No. QR111 S674 2005, Pearson.
[3] Anyadoh-Nwadike, S. O., Ezeanyika, C., and Okorondu, S. I. (2017) Isolation and characteristic of soil-borne urease producing bacteria, European Journal of pharmaceutical and medical Research, 4, 30-38.
[4] Cheng, L. and Cord-Ruwisch, R. (2013) Selective enrichment and production of highly urease active bacteria by non-sterile (open) chemostat culture, Journal of industrial microbiology & biotechnology, 40, 1095-1104.
[5] Collins, C. M. and D'Orazio, S. E. (1993) Bacterial ureases: structure, regulation of expression and role in pathogenesis, Molecular microbiology, 9, 907-913.
[6] Mobley, H. L., Island, M. D., and Hausinger, R. P. (1995.) Molecular biology of microbial ureases, Microbiol. Mol. Biol. Rev., 59, 451-480.
[7] Lee S. G. and Calhoun, D. H. (1997) Urease from a potentially pathogenic coccoid isolate: Purification, characterisation, and comparison to other microbial ureases, Infection and Immunity, 65, 3991-3996.
[8] DeJong, J. T., Soga, K., Banwart, S. A., Whalley, W. R., Ginn, T. R., Nelson, D. C., Mortensen, B. M., Martinez, B. C. and Barkouki, T. (2011) Soil engineering in vivo: harnessing natural biogeochemical systems for sustainable, multi-functional engineering solutions, Journal of the Royal Society Interface, 8,1-15.
[9] Gat, D., Tsesarsky, M., Shamir, D., and Ronen, Z. (2014) Accelerated microbial-induced CaCO3 precipitation in a defined coculture of ureolytic and non-ureolytic bacteria, Biogeosciences, 11, 2561-2569.
[10] Mобиль H. L. and Hausinger, R. P. (1989) Microbial ureases: Significance, regulation, and molecular characterization, Microbiological Reviews, 53, 85-108.
[11] Ivanov, V. and Chu, J. (2008) Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ, Reviews in Environmental Science and Bio/Technology, 7, 139-153.
[12] Rivadeneyra, M. A., Delgado, R., Delgado, G., Moral, A. D., Ferrer, M. R., and Ramos-Cormenzana, A. (1993) Precipitation of carbonates by Bacillus sp. isolated from saline soils, Geomicrobiology Journal, 11, 175-184.
[13] Hammes, F., Boon, N., de Villiers, J., Verstraete, W., and Siciliano, S. D. (2003) Strain-specific ureolytic microbial calcium carbonate precipitation, Appl. Environ. Microbiol., 69, 4901-4909.
[14] Achal V. and Pan, X. (2011) Characterization of urease and carbonic anhydrase producing bacteria and their role in calcite precipitation, Current Microbiology, 62, 894-902.
[15] Burbank, M. B., Weaver, T. J., Williams, B. C., and Crawford, R. L. (2012) Urease activity of ureolytic bacteria isolated from six soils in which calcite was precipitated by indigenous bacteria, Geomicrobiology Journal, 29, 389-395.
[16] Elmanama, A. A. and Alhour, M. T. (2013) Isolation, characterization and application of calcite producing bacteria from urea rich soils, Journal of Advanced Science and Engineering Research, 3, 377-399.
[17] Achakzai, A. K. K., Kayani, S. A., Yaqoob, M., and Nabi, A. (2003) Effect of fertilization and inoculation on lipase and urease activity of mature soybean cv. Williams-82 seeds, Asian Journal of Plant Sciences, 9, 692-695.

[18] Todd, M. J. and Hausinger, R. P. (1991) Reactivity of the essential thiol of Klebsiella aerogenes urease. Effect of pH and ligands on thiol modification, Journal of Biological Chemistry, 266, 10260-10267.

[19] Weatherburn, M. W. (1967) Phenol-hypochlorite reaction for determination of ammonia., Analytical Chemistry, 39, 971-974.

[20] Atlas, R. M. (2010) Handbook of microbiological media, CRC press.

[21] Hammad, I. A., Talkhan, F. N., and Zoheir, A. E. (2013) Urease activity and induction of calcium carbonate precipitation by Sporosarcina pasteurii NCIMB 8841, Journal of Applied Sciences Research, 9, 1525-1533.

[22] Achal, V. (2010) Characterization of two urease-producing and calcifying Bacillus spp. Isolated from cement, Journal of Microbiology and Biotechnology, 20, 1571-1576.

[23] Dhami, N. K. (2013) Biominerlization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites, Journal of Microbiology and Biotechnology, 23, 707-714.

[24] Stocks-Fischer, S., Galinat, J. K., and Bang, S. S. (1999) Microbiological precipitation of CaCO₃, Soil Biology and Biochemistry, 31, 1563-1571.

[25] Stabnikov, V., Jian, C., Ivanov, V. and Li, Y. (2013) Halotolerant, alkaliphilic urease-producing bacteria from different climate zones and their application for biocementation of sand, World Journal of Microbiology and Biotechnology, 29, 1453-1460.

[26] Al-Thawadiand, S. and Cord-Ruwisch, R. (2012) Calcium carbonate crystals formation by ureolytic bacteria isolated from Australian soil and sludge, Journal of Advanced Science and Engineering Research, 2, 12-16.