Research Article

Isolation and Characterization of Urease-Producing Soil Bacteria

Eshetu Mekonnen1, Ameha Kebede2, Asea Nigussie3, Gessese Kebede3, and Mesfin Tafesse3

1Dire Dawa University, College of Natural and Computational Sciences, Department of Biology, Dire Dawa, Ethiopia
2Haramaya University, College of Natural and Computational Sciences, School of Biology and Biotechnology, Haramaya, Ethiopia
3Addis Ababa Science and Technology University, Department of Biotechnology, Addis Ababa, Ethiopia

Correspondence should be addressed to Eshetu Mekonnen; eshetumicro@gmail.com

Received 31 July 2020; Revised 11 March 2021; Accepted 5 July 2021; Published 10 July 2021

Academic Editor: Joseph Falkinham

Copyright © 2021 Eshetu Mekonnen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Urease is an enzyme produced by ureolytic microorganisms which hydrolyzes urea into ammonia and carbon dioxide. Microbial urease has wide applications in biotechnology, agriculture, medicine, construction, and geotechnical engineering. Urease-producing microbes can be isolated from different ecosystems such as soil, oceans, and various geological formations. The aim of this study was to isolate and characterize rapid urease-producing bacteria from Ethiopian soils. Using qualitative urease activity assay, twenty urease-producing bacterias were screened and selected. Among these, three expressed urease at high rates as determined by a conductivity assay. The isolates were further characterized with respect to their biochemical, morphological, molecular, and exoenzyme profile characteristics. The active urease-producing bacterial isolates were found to be nonhalophilic to slightly halophilic neutrophiles and aerobic mesophiles with a range of tolerance towards pH (4.0–10.0), NaCl (0.25—5%), and temperature (20–40°C). According to the API ZYM assays, all three isolates were positive for alkaline phosphatase, leucine aryl amidase, acid phosphatase, and naphthol AS BI phosphohydrolase. The closest described relatives of the selected three isolates (Isolate_3, Isolate_7, and Isolate_11) were Bacillus paramycoides, Citrobacter sedlakii, and Enterobacter bugandensis with 16S rRNA gene sequence identity of 99.0, 99.2, and 98.9%, respectively. From the study, it was concluded that the three strains appear to have a relatively higher potential for urease production and be able to grow under a wider range of growth conditions.

1. Introduction

Urease is an enzyme that catalyzes the hydrolysis of urea by all plants and many algae, fungi, and bacteria [1]. As a consequence, urease activity (urea amidohydrolase: EC 3.5.1.5) is widely distributed in soil [2, 3]. Microbial urease has also been studied in clinical samples as it is related to the virulence of pathogenic microorganisms [4], contributing to urinary stones, pylonephritis, and gastric ulceration [5, 6]. Ureas were immobilized and used as a biosensor in the construction of a flow cell with the incorporation of a urease-modified device for the continuous measurement of urea in flowing systems [7]. They were also used along with urea fertilizer to ease the hydrolysis of ammonium into the soil [8]. However, in the last two decades, the use of microbial urease has switched from clinical relevance to geotechnical engineering and applied biotechnology [9], because of the abilities of microorganisms to induce calcite precipitation, a common natural soil cementing agent, in the presence of urea and calcium ions [10, 11].

Several aerobic bacteria genera (i.e., Proteus, Morganella, Serratia, Pseudomonas, Clostridium, Fusobacterium, Ureaplasma, Providencia, Sarcina, Lactobacillus, Streptococcus, and Enterobacter) are known to produce the enzyme urease and are able to degrade urea in the soil under aerobic conditions [1, 12]. Urease turns the uncharged urea molecule into two charged ions: ammonium (NH4+, positively charged) and carbonate (CO3−2, negatively charged) [4, 12]. As a result, the ammonium (NH4+) released from urea hydrolysis results in local pH rise and commences the precipitation of calcium carbonate [13].
Microbial urease can exist in two possible states in soil. It occurs either intracellularly, associated directly with ureolytic microorganisms, or extracellularly, after being released from cells [14, 15]. Urease-producing bacteria are of particular interest for the production of complex bio-enzymes and are known to produce other soil enzymes [16] that lead to the stabilization of expansive clays [17] through cation exchange and flocculation of the clay minerals [18, 19].

Reference [20] estimated that the microorganisms capable of hydrolyzing urea comprised between 17 and 30% of the aerophilic, microaerophilic, and anaerobic microorganisms isolated from their soil samples. Their ability to produce urease can be exploited to enrich and isolate such bacteria from the environment for future applications [21]. While the occurrence of these bacteria and their characteristics have been explored in some regions and soil types [1, 12, 21, 22] and other novel bacterial strains isolated from Ethiopian sediments and soils [23, 24], this study is the first report on the characterization of ureolytic bacteria from Ethiopian soil. This study aimed to isolate and characterize rapid urease-producing bacteria from Ethiopian soils. Thus, ureolytic bacteria were isolated from soils and were identified based on their urease activity and 16S rRNA gene sequence analysis. Selected rapid urease producer strains were further characterized by biochemical, morphological, molecular, and exoenzyme profile characteristics.

2. Materials and Methods

2.1. Soil Sampling. Soil samples were collected from different types of ecosystems including a urea dumping site, stable soil structures such as termite casts, and rift valley soda lakeshores of Ethiopia. The samples were collected in summer 2017 from Tulu Bolo Fertilizer Factory (pH = 8.15, soil temperature = 28°C, 8.6633*N, 38.2164 °E, and at an elevation of 2193 meters above sea level); shore soil of Lake Abijata (pH = 10.5, soil temperature = 32°C, 7.6167*N, 38.6000 °E, and at an elevation of 1573 meters above sea level); shore soil of lake Chitu (pH = 11.5, soil temperature = 30°C, 7.403599*N, 38.423527 °E, and at an elevation of 1539 meters above sea level); a termite mound in the Wonji area (pH = 7.56, soil temperature = 33°C, 8.450919*N, 39.278972 °E, and at an elevation of 1618.28 meters above sea level); termite mounds near the town of Yabello (pH = 7.9, soil temperature = 31°C, 4.889622*N, 38.084775 °E, and at an elevation of 1,857 meters above sea level); and a termite mound in West Wollega (pH = 6.7, soil temperature = 30°C, 9.487993*N, 35.526785 °E, and at an elevation of 1821 meters above sea level) [25].

The soil samples consisted of homogenized composite samples taken from multiple sample units as described in [26]. The soil samples were collected from the upper 10 cm of the topsoil, sampling was done using a sterile spatula, and the samples were kept in sterile polyethylene bags [27]. The samples were immediately stored in an ice-box at 4°C and transported to the laboratory at Addis Ababa Science and Technology University.

2.2. Enrichment and Screening of Ureolytic Bacterial Isolates. To enrich urease-producing bacteria from soil samples, 1 g of each soil sample was inoculated into 100 mL of urea broth medium (Sigma-Aldrich) consisting of 1.00 mg/L peptone, 1.000 mg/L dextrose, 5.00 mg/L sodium chloride, 1.2 mg/L disodium phosphate, 0.8 mg/L monopotassium phosphate, 0.012 mg/L of phenol red, and 6% (w/v) urea (HiMedia, sterile filtered 0.45 μm, added after autoclaving) (in 250 mL shake flasks) and incubated under aerobic batch conditions at 30°C for 120 h under shaking condition at 130 rpm [21]. For subsequent enrichment, 20% (v/v) of the culture samples were intermittently transferred (up to four times) into a fresh medium [28]. For bacterial isolation, an aliquot of 1 mL was serially diluted and from the last enrichment, 0.1 mL of the sample was inoculated onto urea agar plates and then spread using a sterilized L-shaped spreader until the fluid was evenly distributed [21]. The plates were then incubated under aerobic conditions at 30°C for 24 h. Colonies showing urea hydrolyzing potential were purified by subsequent culturing and plating until single bacterial colonies were obtained. Urease production was tested through visual observation of color changes. Thus, isolates with positive ureolytic potential turned the urea agar medium from pale yellow to a pink-red color [29]. From a total of 153 collected colonies, 20 potential urease-producing isolates were selected for further studies.

2.3. Quantitative Urease Activity Analysis. For direct assays of urease activity, 1.0 mL of a 24 h old culture was inoculated into bottles containing 9.0 mL of 1.11 M urea solution and monitored for 5 min at 25 ± 2°C. The respective conductivity values were measured and recorded by immersing the probe of the conductivity meter (EC800 Laboratory Benchtop Conductivity Meter, APERA) into the bacterial-urea solution [30]. At the end of the assay, a graph was plotted using conductivity values (ms/cm) against time (min). The rate of conductivity change (ms/cm/min) was acquired from the slope of the plotted graph, which was then multiplied by the dilution factor. This was taken as the ratio of the stock bacteria culture to the sampling bacteria culture before inoculation into the urea solution. The specific urease activity (mM urea hydrolysed/min/OD) was derived by dividing the urease activity (mM urea hydrolysed/min) by the bacterial biomass OD600 [31]. The OD was measured using a spectrophotometer (GENESYS™ 20, Thermo Fisher Scientific) at a wavelength of 600 nm:

\[
\text{specific urease activity} (\text{mM urea hydrolysed} \text{ per min} \times \text{OD}^{-1}) = \frac{\text{urease activity} (\text{mM urea hydrolysed} \text{ per min} \times \text{OD}^{-1})}{\text{biomass (OD600)}}
\]

2.4. Colony and Cell Morphology. Morphological characterization such as colony and cell morphology; Gram, India ink, and malachite green stain reaction; and motility tests were performed by standard methods [32]. Microscopic observations were performed under a light microscope.
2.5. 16S RNA Gene Amplification. Genomic DNA of each bacterial isolate was extracted using the freeze and thaw protocol and used as a template in a PCR using the primers 8f (5′-AGAGTTTGATCTGGCTCAG-3′) and 1492r (5′-GGTACCTTGGTACGACTT-3′) as previously described in [34]. Colonies of overnight grown isolates were picked using a sterilized pipette tip, mixed with 10 μL of PCR grade water in a sterile PCR tube, and placed in a thermocycler with freeze-thaw cycles consisting of three stages with 96°C for 15 min, 90 sec, and 60 sec followed by 15°C for 90 sec at each stage. One microliter of the lysed cells was transferred into 20 μL of PCR master mix. The master mix consists of 16.2 μL PCR grade water, 2 μL of 10x PCR buffer (Life Technologies), 0.4 μL of 10 mM DNTP mix (Life Technologies), 0.4 μL of 20 mg/mL BSA, 0.8 μL of 25 mM MgCl₂, 0.08 μL of 50 μM of each primer 8f, 1492r, and dream taq-Polymerase (Life Technologies). DNA amplification was performed using a Thermocycler (Verti Cycler, Applied Biosystems).

2.6. Nucleotide Sequencing and Analysis. Sequencing was done using the Illumina sequencing facility and the raw DNA chromatogram sequences were viewed and edited using the BioEdit Programme [35] and stored in FASTA format. The forward and reverse sequencing products were assembled using MEGA X after removing poor-quality sequences from the 3′ and 5′ sequence ends. The sequences were blasted against existing sequences in the National Centre for Biotechnological Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) nucleotide collection database program to search for the closest best match sequence [36].

2.7. Optimization of Growth Conditions. The one-factor-at-a-time (OVAT) technique was employed to study the effects of culture conditions affecting bacterial growth such as incubation temperature (4–45°C), initial medium pH (4–45°C), and NaCl concentrations (0–20%) in triplicate under oxic conditions. The bacterial cultures were grown in a urea broth base medium (24.0g/L, HiMedia Laboratories Pvt. Ltd.) and supplemented with filtered urea (5% w/v, Thermo Fisher Scientific) using a 0.45 μm sterile syringe filter [3].

2.7.1. Testing for Optimum pH. To test the range and optimum pH, a medium was prepared at different pH (pH = 3–10) at 0.5 pH intervals. The pH within a desired range 10 mM of the following buffers was maintained. MES buffer (2-(N-morpholino)ethanesulfonic acid) was used for the pH range from 3.0 to 6.7; HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for the pH range from 6.8 to 8.2, HEPPS buffer (3-[4-(2-Hydroxyethyl)piperazin-1-yl] propane-1-sulfonic acid) for the range from 7.3 to 8.7; and CHES buffer (N-Cyclohexyl-2-aminoethanesulfonic acid) for the pH range from 8.6 to 10 [37].

For each pH step, 4.5 mL of medium was added to test tubes (triplicate) and inoculated with 0.5 mL of fresh culture. Samples were incubated at 33°C under oxic condition and OD₆₀₀ was recorded at intervals of 0 h to 24 h. The sterile medium was used as blank. Finally, growth curves were plotted as LogOD versus time for each pH, and the optimum was determined. Optimal growth was defined as ≥75% of the highest growth rate achieved [38].

2.7.2. Testing for Optimum Temperature. To test the range and optima temperature, media was prepared at the optimum pH as indicated above. For each temperature, 4.5 mL of medium was added to test tubes (triplicate) and inoculated with 0.5 mL of fresh culture. The tubes were incubated under the oxic condition at temperatures between 5 and 45°C at intervals of 5°C. OD₆₀₀ was recorded at intervals of 1 h for 24 h. The uninoculated medium was used as blank. Finally, the growth curve was plotted as time versus log OD for each temperature and the optimum was determined [39].

2.7.3. Testing for Optimum Salinity. Media were prepared with the optimum pH and for each NaCl concentration to be tested (0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 g/L final concentration (w/v)); then, 4.5 mL of medium was added to test tubes (triplicate) and inoculated with 0.5 mL of fresh culture. Tubes were incubated and recorded as indicated above.

2.8. Exoenzyme Analysis. Indole formation, aesculin degradation, urease activity, and further exoenzyme activities were determined by using the API ZYM and API20NE test systems (bioMerieux) following the instructions of the manufacturer. API ZYM is a semiquantitative micromethod designed for the research of enzymatic activities [40]. It allows the systematic and rapid study of 19 enzymatic reactions using very small sample quantities [41]. After inoculation, the reaction mixture was incubated for 4–4.5 h at 35°C (optimum temperature) [42]; then, the data was recorded and interpreted.

2.9. Phylogenetic Analysis. A phylogenetic tree based on 16S rRNA gene sequences was reconstructed using MEGA version 10.0 [43]. Prior to phylogenetic analysis, primer sequences at both ends were removed and the gaps were adjusted to improve the alignment. Nucleotide sequence alignments were inspected visually to identify positions of uncertain alignments to be corrected or omitted for further analysis [44]. Multiple sequence alignments were obtained using the Clustal-W alignment tool from the MEGA-X software with distance options according to the Kimura two-parameter model and clustering with the maximum likelihood statistical method [44]. Bootstrap analysis based on 1000 replications was used to estimate the confidence level of the tree topologies [43].
3. Results

3.1. Isolation, Urease Activity, Phylogenetic Analysis, and Morphological Features of the Isolated Bacteria

3.1.1. Isolation and 16S RNA Gene Similarity. Numerous active urease-producing bacterial cultures were enriched and a total of 153 ureolytic pure bacterial colonies were collected after a consecutive restreaking [45]. Twenty strains with high urease activity were identified based on the rapid development of the pink color of the urea agar plates within 24 h of incubation [12] and selected for further investigation. The selected 20 isolates were subjected to partial 16S rRNA gene sequencing [46] at the Leibniz-Institute DSMZ—German Collection of Microorganisms and Cell Cultures. The BLAST results of the sequences searched against the GenBank database using the BLASTN program [47] are summarized in Table 1.

The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔTm [48]. A 16S rRNA gene sequence similarity of 98.6% [49] was generally used as a threshold value for species definition in prokaryotes taxonomy. Accordingly, in this study, the sequence analysis showed that 3 (15%), 3 (15%), and 14 (70%) of the isolates belong to the genera Bacillus, Citrobacter, and Enterobacter, respectively (Table 1).

3.1.2. Specific Urease Activity Testing. The specific urease activity of each bacterial isolate was measured and the analysis is presented in Figure 1. Based on the quantitative analysis, Isolate_3, Isolate_7, and Isolate_11 showed clearly higher specific urease activity values of 3.88, 3.18, and 3.05 mM urea hydrolysed/min/OD, respectively (p < 0.001), and were selected for further analysis. Only these three isolates with higher specific urease activity were selected for analysis due to the limited budget and time during the study time.

3.1.3. Phylogenetic Analysis of the Selected Isolates. The phylogeny of the isolates was analyzed using the maximum likelihood method and the Kimura 2-parameter model and included bootstrap analysis based on 1000 replications [43] to estimate the confidence level of the tree topology. The analysis revealed that Isolate_3 was affiliated with the genus Bacillus (Figure 2). The highest 16S rRNA gene sequence identity for Isolate_3 (MW723439) was 98.9% and was determined for Bacillus paramycoideus MCCC 1A04098T. The analysis placed Isolate_7 (MW722959) in the vicinity of Citrobacter sedlakii I-75T and within the same group (99.2% 16S rRNA sequence identity). The phylogenetic analysis also showed that Isolate_7 was more closely related to Isolate_11 than Isolate_3. Isolate_11 (MW722969) was placed in the neighborhood of Enterobacter bugandensis 247BMC T which had a 16S rRNA gene sequence identity of 99.0% to this type of strain.

3.1.4. Cellular and Colony Features of the Selected Isolates. Microscopic examination of Isolate_11 showed that cells stained Gram-negative and are single cocci while rod-shaped with an average length of 0.6–1.8 μm (Figure 3(c)). The cells were motile when they were observed in a wet mount with phase-contrast microscopy and by using semisolid agar stabs (agar, 2 mg/L) [50]. Capsules and endospores were not observed after staining with India ink and malachite green, respectively. After incubation at 35°C for 18 h on a nutrient agar medium, the colonies had an average size of 2 mm in diameter and were whitish, smooth, shiny, circular, and convex with entire margins. Colony and microscopic features of Isolate_11 were similar to the recently described strain of Enterobacter bugandensis EB-247T [51]. Cells of Isolate_7 stained Gram-negative and were coccobacilli to roads with an average length of 1.6 μm, Gram-negative, non-spor forming, and noncapsulated and occur as single cells or in short chains (Figure 3(b)). After incubation at 35°C for 18 h on nutrient agar plates, colonies were whitish to gray, convex, and circular with an average size of 2.5 mm. These features were similar to the pathogenic Citrobacter sedlakii isolated from infant brain samples and grown on sheep blood agar plates [52].

Cells of Isolate_3 were long rods, with an average length of 1.5–4.5 μm and formed highly refractile endospores (Figure 3(a)). Consistent with all other characterized members of the genus Bacillus, the cells of Isolate_3 stained Gram-positive [53]. Staining with India ink demonstrated the presence of capsules. Colonies of Isolate_3 were whitish, rough, circular, and nontranslucent and had a rough surface and entire margins, with 1.5–3.5 mm in diameter after incubation at 35°C for 24 h on nutrient agar plates.

3.2. Optimum and Range of Growth for pH, Temperature, and Salinity. The pH tolerance analysis showed that Isolate_11 was able to grow in a wider range of pH (pH 4.0–10.0). The optimum growth defined as ≥75% of the highest growth rate achieved [39] was recorded at pH = 5.5–8.0. The highest rate of growth (100%) was recorded at pH = 7.0 (p < 0.05) and the lowest rate was at pH = 10.0. Isolate_7 was able to grow between pH = 5.5 and pH = 9.5 and optimum growth was recorded between pH = 6.5 and pH = 8.0. The highest rate of growth (100%) was observed at pH = 8.0 and it was unable to grow at lower pH = 4.0 and higher pH = 10.0. Growth was observed for Isolate_3 between pH of 6.5 and pH of 9.5; optimal growth (≥75%) was recorded between pH of 7.0 and pH of 8.0 (p < 0.05); and the highest rate of growth (100%) was recorded at pH = 7.4. Isolate_3 was unable to grow or showed limited growth between pH = 2.8 and pH = 5.0 (Figure 4).

The analysis of the OD values after 24 h of incubation at different temperatures showed that Isolate_11 could grow between 20°C and 40°C with both optimum growth (≥75% of the highest growth rate) and maximum growth rate (100%) at 35°C (p < 0.05). It was unable to grow at a temperature range of 4–15°C. Similarly, Isolate_7 was able to grow between 20°C and 40°C (optimum at 25°C–40°C), with the highest rate of growth (100%) at 30°C and 35°C (p < 0.05). It showed no or very slow growth rate between
4°C–25°C and 45°C. Isolate_3 was able to grow between 25°C and 40°C with optimum growth between 35°C and 40°C. It showed (very) limited or no growth between 4°C–20°C and 45°C (Figure 5).

The study of NaCl concentration tolerance also revealed that Isolate_11 was able to grow in NaCl concentration range of 0.0–5.0%, where it grew best (optimum growth) between 0.25 and 3.0% (w/v) in 48 h of incubation; the highest rate of growth (100%) was recorded at 0.25%. During the study, it was observed that Isolate_11 was unable to grow at NaCl concentrations ≥10%. Optimal growth was observed between 0.0 and 2.0% (w/v) NaCl for Isolate_7 and between 0.0 and 0.5% NaCl for Isolate_3 after 24 h of incubation. The highest rate of growth was recorded at 0.25% NaCl for both strains and they showed zero growth at NaCl concentrations of ≥10% (Figure 6).

Table 1: 16S rRNA genes sequence similarity with the closest strains of the isolates.

| Code          | Closest strain                        | %    | Origin     |
|---------------|---------------------------------------|------|------------|
| Isolate_1     | Bacillus paramycoides MCCC 1A04098T   | 99.6 | Tulubolo   |
| Isolate_2     | Enterobacter tabaci YIM Hb-3T         | 97.1 | Lake Chitu |
| Isolate_3     | Bacillus paramycoides MCCC 1A04098T   | 98.9 | West Wellega |
| Isolate_4     | Enterobacter tabaci YIM Hb-3T         | 97.9 | Lake Chitu |
| Isolate_5     | Enterobacter asburiae JCM 6051T       | 99.0 | Wonji      |
| Isolate_6     | Enterobacter tabaci YIM Hb-3T         | 98.9 | Lake Chitu |
| Isolate_7     | Citrobacter sedlakii I-25T            | 99.8 | Wonji      |
| Isolate_8     | Citrobacter sedlakii I-25T            | 99.6 | Lake Chitu |
| Isolate_9     | Enterobacter tabaci YIM Hb-3T         | 98.9 | Lake Chitu |
| Isolate_10    | Enterobacter hormaechei subsp. hormaechei 10–17T | 98.0 | Yabello |
| Isolate_11    | Enterobacter bugandensis 247BMC       | 99.2 | Wonji      |
| Isolate_12    | Enterobacter hormaechei subsp. hormaechei 10–17T | 99.9 | Lake Abijata |
| Isolate_13    | Enterobacter tabaci YIM Hb-3T         | 98.2 | Lake Abijata |
| Isolate_14    | Bacillus wiedmannii FSLW8-0169T       | 98.7 | Tulubolo   |
| Isolate_15    | Enterobacter tabaci YIM Hb-3T         | 98.9 | West Wellega |
| Isolate_16    | Enterobacter tabaci YIM Hb-3T         | 99.0 | Yabello    |
| Isolate_17    | Enterobacter asburiae JCM 6051T       | 97.2 | Yabello    |
| Isolate_18    | Citrobacter sedlakii I-25T            | 99.2 | Tulubolo   |
| Isolate_19    | Enterobacter tabaci YIM Hb-3T         | 98.2 | Tulubolo   |
| Isolate_20    | Enterobacter tabaci YIM Hb-3T         | 98.8 | Yabello    |

% indicates similarity.

3.3. Exoenzyme Profiles of the Selected Strains. In addition to urease activity, the selected isolates showed activities for various exoenzymes (Table 2). Out of 25 tested exoenzymes, Isolate_11 showed activities for 14 exoenzymes; Isolate_7 showed for 13 exoenzymes; and Isolate_3 for 11 exoenzymes. All the three selected strains showed similar preferences towards phosphate-containing compounds (alkaline phosphatase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase), peptidase activities (leucine arylamidase and valine arylamidase), and nitrate reductase activity. They all assimilated N-acetyl-glucosamine, D-maltose, potassium gluconate, and trisodium citrate. Isolate_3 and Isolate_7 showed a similar positive reaction for lipids (esterase and esterase lipase), while Isolate_11 and Isolate_7 showed similar preferences for trypsin and β-galactosidase. Isolate_11 showed unique preferences for

| Specific urease activity | Figure 1: Specific urease activity. |

Isolate_1, Isolate_2, Isolate_3, Isolate_4, Isolate_5, Isolate_6, Isolate_7, Isolate_8, Isolate_9, Isolate_10, Isolate_11, Isolate_12, Isolate_13, Isolate_14, Isolate_15, Isolate_16, Isolate_17, Isolate_18, Isolate_19, Isolate_20, Sporosarcina
Bacillus cereus ATCC 14579^T (NR_074540)
Bacillus albus MCCC 1A02146^T (NR_157729)
Bacillus paramycoides MCCC 1A04098^T (NR_157734)
Bacillus paramycoides (isolate_3)
Bacillus wiedmannii FSL W8-0169^T (NR_152692)
Bacillus Proteolyticus MCCC 1A00365^T (NR_157735)
Citrobacter sedlakii 1-75^T (NR_028686)
Citrobacter sedlakii (isolate_7)
Citrobacter rodentium DO 14784^T (NR_028685)
Escherichia fergusonii ATCC 35469^T (NR_074902.1)
Citrobacter youngae GTC 1314^T (NR_041527)
Enterobacter ludwigii EN-199^T (NR_042349)
Leclerica adecarboxylate NBRC 102595^T (NR_114154)
Enterobacter bugandensis 247BMC^T (NR_148649)
Enterobacter bugandensis (isolate_11)
Enterobacter cancerogenus LMG 2693^T (NR_044977)

**Figure 2:** Molecular phylogenetic analysis by maximum likelihood method based on almost-full-length 16S rRNA gene sequences illustrating the phylogenetic position of Isolate_3, Isolate_7, and Isolate_11 and related taxa. The percentage of trees in which the associated taxa clustered together are shown next to the branches.

(a) 5 μm (b) 1 μm (c) 2 μm

**Figure 3:** (a, b, c) Phase-contrast photomicrographs of strains: Isolate_3, Isolate_7, and Isolate_11.

**Figure 4:** pH growth optimum and range analysis of the strains Isolate_11, Isolate_7, and Isolate_3, respectively.
various sugars (α-glucosidase, β-glucosidase, and N-acetyl-β-glucosaminidase).

4. Discussions

This study was conducted with the aim of isolation and characterization of rapid urease-producing bacteria from Ethiopian soils. In the study, twenty urease-producing bacterial isolates were identified using a qualitative urease activity assay. Among these, three of them (Bacillus parmycoides, Citrobacter sedlakii, and Enterobacter bugandensis) expressed urease at high rates (3.88, 3.18, and 3.05 of mM urea hydrolysed min⁻¹ OD⁻¹) (p < 0.05) as determined by a conductivity assay. Literature showed that urease was studied from several bacterial strains such as Bacillus [54], Citrobacter, Enterobacter, Pseudomonas, Serratia, and
the genus isolatedescribed here probably represents a new member of
fore, it is significant to note that Isolate_3 showed unique
lated strain of
differentiate our strain from the previously identified re-
producers. ffK_hese are important key characteristics that
waxy colonies after incubation at 32 °C for 48h on LB
μ
nonspore forming, with 1.8–2.2
Citrobacter
Bacillus
urease activity were identified as belonging to the genera

TABLE 2: Biochemical and exoenzyme profiles of the three selected
strains.

| Characteristics                          | Isolate_3 | Isolate_7 | Isolate_11 |
|------------------------------------------|-----------|-----------|------------|
| Control                                  | –         | –         | –          |
| API 20NE                                 | –         | –         | –          |
| Nitrate reductase                        | +         | +         | +          |
| Indole formation                         | –         | –         | –          |
| Arginine dihydrolase                     | –         | W         | +          |
| Urease                                   | +         | +         | +          |
| Protease                                 | –         | –         | +          |
| L-Arabinose                              | –         | –         | +          |
| API ZYM                                  | –         | –         | –          |
| Alkaline phosphatase                     | +         | +         | +          |
| Esterase (C4)                            | W         | W         | –          |
| Esterase lipase (C8)                     | W         | W         | –          |
| Lipase (C14)                             | –         | –         | –          |
| Leucine arylamidase                      | +         | +         | +          |
| Valine arylamidase                       | W         | W         | +          |
| Cystine arylamidase                      | –         | –         | –          |
| Trypsin                                  | W         | W         | W          |
| α-Chymotrypsin                           | +         | –         | –          |
| Acid phosphatase                         | +         | +         | +          |
| Naphthol-AS-Bl-phosphohydrolase          | W         | W         | W          |
| α-Galactosidase                          | –         | –         | –          |
| β-Galactosidase                          | –         | –         | –          |
| β-Glucuronidase                          | –         | –         | –          |
| α-Glucosidase                            | +         | –         | W          |
| β-Glucosidase                            | –         | –         | W          |
| N-Acetyl-β-glucosaminidase               | –         | –         | W          |
| α-Mannosidase                            | –         | –         | –          |
| α-Fucosidase                             | –         | –         | –          |

"+" = positive; "W" = weakly positive; and "−" = negative.

In addition, the morphological and physiological studies of Isolate_11 showed different characters from the previ-
ously characterized nonureolytic E. bugandensis strain EB-
247T [51], as Isolate_11 does not form a capsule but secretes urease and gelatinase enzymes and assimilates D-arabinose
following incubation for 18h at 35°C, while the former was
incubated for 24h at 37°C on MacConkey agar. The most
prominent biochemical feature of Isolate_7 was its ability to
assimilate aesculin ferric citrate, which agrees with the name
of the genus (Citrobacter = citrate utilizing rods) [57].
Furthermore, similar biochemical and morphological fea-
tures were also observed between Isolate_7 and clinical
isolate Citrobacter sedlakii 2596T [52] with respect to urease
activity, arginine hydrolase, and fermentation of arabinose,
mannitol, and maltose. Jacob [57] also reported a similar
positive reaction for urease, arginine, lipase, and β-gluco-
sidase for halophilic Citrobacter strains isolated from the
saline environment. But, the reported strain had filamentous
and rough colonies unlike Isolate_7, which showed convex
and circular colonies with an average size of 2.5 mm.
Brenner et al. [58] reported that Citrobacter sedlakii
ATCC 51118T has similar positive results to Isolate_7 with
respect to urease activity and arginine dihydrolase and is
different in its negative reaction with aesculin substrate
utilization.

Though the selected strains were isolated from moun-
tainous agricultural land termite mound soil (Isolate_11)
with a slightly neutral pH of 6.7 and rift valley grassland
termite mound soil (Isolate_3 and Isolate_7) with slightly
alkaline pH, respectively, they tolerated a broader pH range
as explained above and based on their optimum growth pH
of 5.5–8.5 (p < 0.05), they are categorized as neutrophiles
[59]. They were grown at moderate temperatures between
20°C and 40°C and with an optimum growth temperature in
the range of 30–39°C. Therefore, they are categorized as
mesophilic bacteria [60]. The isolates showed a narrow
tolerance range for temperature corresponding to their
origin from Ethiopian soil.

As shown in Figure 5, all the three selected strains
tolerated NaCl concentrations of up to 5% (w/v), which
exceeds the maximum NaCl tolerance of common soil
bacteria. This increased NaCl tolerance constitutes an im-
portant differential characteristic of the selected species. It
best explains the higher salinity in Ethiopian soils [61],
which could be due to heavy fertilizer application, use of
poor quality irrigation water, and inadequate drainage [64,
65, 66]. Nonhalophiles grow optimally at less than 2% NaCl;
slight halophiles grow optimally at 2–5% NaCl; moderate
halophiles grow optimally at 5–20% NaCl; and extreme
halophiles grow optimally above 20–30% NaCl [62]. This
implies that the rapid urease-producing isolates in this study
could be considered as nonhalophilic to slightly halophilic
bacteria. Further studies of such halophilic bacteria could
help to discover new enzymes to be applied in biocatalytic
processes that are faster, more accurate, specific, and en-
vironmentally friendly [63]. These enzymes could keep high
activity and stability in salty environments and could have
potential application values in agriculture, engineering, and
medicine.

Yersinia [55]. In our study, the identified strains exhibiting
urease activity were identified as belonging to the genera
Bacillus, Citrobacter, and Enterobacter.

Consistent with previous studies done on Bacillus
paramyocoides MCCC 1A04098T, Citrobacter sedlakii 2596T
and Enterobacter bugandensis EB-261T [51, 52, 56], similar
morphological and physiological characteristics were ob-
erved with Isolate_3, Isolate_7, and Isolate_11, respecti-
vely, and later confirmed by 16S rRNA gene sequencing.
However, morphological studies have revealed that Isola-
t_strain was a spore former with conspicuous spore and had a
rough colony and cells having a length of 1.5–4.5 μm after
incubation at 35°C for 18h. This makes Isolate_3 different
from the previously characterized novel strain of
B. paramyocoides MCCC 1A04098T which was reported as
nonsporing, with 1.8–2.2 μm in length and with waxy
colonies after incubation at 32°C for 48h on LB
medium [56]. Besides, unlike the nonureolytic strain of
B. paramyocoides MCCC 1A04098T, Isolate_3 is a urease
producer. These are important key characteristics that
differentiate our strain from the previously identified re-
lated strain of B. paramyocoides MCCC 1A04098T. There-
fore, it is significant to note that Isolate_3 showed unique
morphological and physiological features. On this basis, the
isolate described here probably represents a new member of
the genus Bacillus.
5. Conclusions

The results obtained from this research confirmed the presence of ureolytic bacteria in Ethiopian soil indicating their adaptation from the rift valley to mountainous ecosystems of the country. In the study, new strains of Bacillus, Citrobacter, and Enterobacter were isolated from Ethiopian soil and characterized based on their distinctive physiological and morphological characteristics. From the study, it was shown that the three isolates (Isolate_3, Isolate_7, and Isolate_11) had relatively more rapid rates of urea hydrolysis and were found to be nonhalophilic to slightly halophilic neutrophiles and aerobic mesophiles with a range of tolerance towards pH (4.0–10.0), NaCl (0.25–5%), and temperature (20–40°C). Further studies on the growth profiles of the isolates, calcite precipitation, soil biocementation, and scanning electron microscopy analysis were recommended for future studies.

Data Availability

The data are available upon request to the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding this paper.

Acknowledgments

The authors acknowledge Addis Ababa Science and Technology, Ethiopia, for providing laboratory facilities during soil sample collection, bacterial isolation, and culturing work. They also acknowledge Leibniz-Institute DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, for providing facility during molecular and physiological characterization of the isolates. This work was supported by Ministry of Science and Higher Education (Ethiopia), Ministry of Innovation and Technology (Ethiopia), and DAAD (Grant no. 57381332) 2018.

References

[1] L. Kigigha, D. Silas-Olu, and D. Finebone, “Characterization of ureolytic bacteria from nitrogenous waste dumps in port harcourt Nigeria,” Continental Journal of Microbiology, vol. 6, no. 1, pp. 9–13, 2012.
[2] R. Guettes, W. Dott, and A. Eisenbraug, “Determination of urease activity in soils by carbon dioxide release for ecotoxicological evaluation of contaminated soils,” Ecotoxicology, vol. 11, no. 5, pp. 357–364, 2002.
[3] A. Omorogie, N. Senian, P. Ye Li et al., “Ureolytic bacteria isolated from Sarawak limestone caves show high urease enzyme activity comparable to that of Sporosarcina pasteurii (DSM 33),” Malaysian Journal of Microbiology, vol. 12, no. 6, pp. 463–470, 2016.
[4] A. I. Omorogie, G. Khoshdelnezamiha, N. Senian, D. E. L. Ong, and P. M. Nissom, “Experimental optimisation of various cultural conditions on urease activity for isolated Sporosarcina pasteurii strains and evaluation of their bioce-ment potentials,” Ecological Engineering, vol. 109, pp. 65–75, 2017.
[5] C. M. Collins and S. E. F. D’Orazio, “Bacterial ureases: structure, regulation of expression and role in pathogenesis,” Molecular Microbiology, vol. 9, no. 5, pp. 907–913, 1993.
[6] H. L. T. Mobley and R. P. Hausinger, “Microbial ureases: significance, regulation, and molecular characterization,” American Society for Microbiology, vol. 53, no. 1, pp. 85–108, 1999.
[7] D. C. Cullen, R. S. Sethi, and C. R. Lowe, “Multi-analyte miniature conductance biosensor,” Analytica Chimica Acta, vol. 231, no. 1, pp. 33–40, 1990.
[8] P. M. Gilbert, J. Harrison, C. Heil, and S. Seitzinger, “Escalating worldwide use of urea—a global change contributing to coastal eutrophication,” Biogeochemistry, vol. 77, no. 3, pp. 441–463, 2006.
[9] A. Omorogie, D. Ong, and P. Nissom, “Assessing ureolytic bacteria with calcifying abilities isolated from limestone caves for biocalcification,” Letters in Applied Microbiology, vol. 68, no. 2, pp. 173–181, 2019.
[10] V. Achal, A. Mukherjee, and M. S. Reddy, “Original research: biocalcification by Sporosarcina pasteuriiusing corn steep liquor as the nutrient source,” Industrial Biotechnology, vol. 6, no. 3, pp. 170–174, 2010.
[11] J. T. DeJong, K. Soga, S. A. Banwart et al., “Soil engineering in vivo: harnessing natural biogeochemical systems for sustainable, multi-functional engineering solutions,” Journal of The Royal Society Interface, vol. 8, no. 54, pp. 1–15, 2011.
[12] I. R. K. Phang, Y. S. Chan, K. S. Wong, and S. Y. Lau, “Isolation and characterization of urease-producing bacteria from tropical peat,” Biocatalysis and Agricultural Biotechnology, vol. 13, pp. 168–175, 2018.
[13] O. A. Cuzman, S. Rescic, K. Richter, L. Wittig, and P. Tiano, “Sporosarcina pasteurii use in extreme alkaline conditions for recycling solid industrial wastes,” Journal of Biotechnology, vol. 214, pp. 49–56, 2015.
[14] K. N. Paulson and L. T. Kurt, “Locus of urease activity in soil,” Soil Science Society of America, vol. 33, pp. 897–901, 1969.
[15] S. Skujip, “Extracellular enzymes in soil,” Critical Reviews in Microbiology, vol. 4, no. 4, pp. 383–421, 1976.
[16] L. Lindenbaum, Soil Stabilization System, United States Patent Application Publication, Alexandria, VA, USA, 2008.
[17] J. Tingle and R. Santoni, “Stabilization of clay soils with nontraditional additives,” Transportation Research Record, vol. 1819, no. 1, 2003.
[18] D. E. Scholen, “Stabilizer mechanisms in nonstandard stabilizers,” in Proceedings of the Sixth International Conference on Low-Volume Roads, II (TRB), pp. 252–260, National Research Council, May 1995, Washington, DC, USA.
[19] S. J. Tingle, J. K. Newman, S. L. Larson, C. A. Weiss, and J. F. Rushing, “Stabilization mechanisms of nontraditional additives,” Journal of the Transportation Research Board, vol. 1989, no. 2, pp. 59–67, 2007.
[20] A. B. Lloyd and M. J. Sheaffe, “Urease activity in soils,” Plant and Soil, vol. 39, pp. 71–80, 1973.
[21] A. I. Omorogie, N. Senian, P. Ye Li et al., “Screening for urease-producing bacteria from limestone caves of sarawak,” Borneo Journal of Resource Science and Technology, vol. 1989, no. 2, pp. 59–67, 2007.
[23] G. Gebreyohannes, F. Moges, S. Sahile, and N. Raja, "Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia," Asian Pacific Journal of Tropical Biomedicine, vol. 3, no. 6, pp. 426–435, 2013.

[24] M. Kibret, J. F. Guerrero-Garzón, E. Urban et al., "Streptomycetes spp. from Ethiopia producing antimicrobial compounds: characterization via bioassays, genome analyses, and mass spectrometry," Frontiers in Microbiology, vol. 9, p. 1270, 2018.

[25] Eshetu Mekonnen, Ameha Kebede, Tekle Tafesse, and Mesfin Tafesse, "Investigation of carbon substrate utilization patterns of three ureolytic bacteria," Biocatalysis and Agricultural Biotechnology, vol. 22, Article ID 101429, 2019.

[26] V. A. Lancaster and S. Keller-McNulty, "A review of composite sampling methods," Journal of the American Statistical Association, vol. 93, pp. 1216–1230, 1998.

[27] R. Sadati, A. Barghi, and M. Jafarpour, "Isolation and identification of new lipase-producing microbacterium spp. from coastal soils of the Caspian Sea (North of Iran)," World Applied Sciences Journal, vol. 23, no. 1, pp. 65–69, 2013.

[28] T. T. Fida, S. Palmamura, and G. Pandey, "Acidic biodegradation of 2,4-Dinitroanisole by nocardoides sp. strain JS1661," Applied and Environmental Microbiology, vol. 80, no. 24, pp. 7725–7731, 2014.

[29] I. A. Hammad, F. N. Talhkan, and A. E. Zoheir, "Urease activity and induction of calcium carbonate precipitation by Sporosarcina pasteurii NCIMB 8841," Journal of Applied Sciences Research, vol. 9, no. 3, pp. 1525–1533, 2013.

[30] M. P. Harkes, L. A. van Paassen, J. L. Booster, V. S. Whiffin, and M. C. M. van Loosdrecht, "Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement," Ecological Engineering, vol. 36, no. 2, pp. 112–117, 2010.

[31] V. S. Whiffin, Microbial CaCO₃ Precipitation for the Production of Biocement, Murdoch University Western Australia, Perth, Australia, 2004.

[32] R. Moyes, J. Reynolds, and D. Breakwell, "Differential staining of bacteria: gram stain," Current Protocols in microbiologyAppendix, vol. 3, no. 1, 2009.

[33] S. Vieira, M. Luckner, G. Wanner, and J. Overmann, "Luteitalae protiens gen. nov., sp. nov. a new member of subdivision 6 acidobacteria isolated from temperate grassland soil," International Journal of Systematic and Evolutionary Microbiology, vol. 67, pp. 1408–1414, 2017.

[34] K. R. Arora, Soil Mechanics and Foundation Engineering, Springer, Berlin, Germany, 2019.

[35] T. Hall, "BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT," Nucleic Acids Symposium Series, vol. 41, no. 41, pp. 95–98, 1999.

[36] A. Naomi, S. Andre, and R. O. Cavalcanti, "Using NCBI BLAST," in Current Protocols, Essential Laboratory Techniques, 14, pp. 11.1.1–11.1.34, Wiley, Hoboken, NJ, USA, 2017.

[37] K. J. Huber, P. K. Wust, M. Rohde, A. Jorg Overmann, and B. U. Foesel, "Aridibacter famidurans gen. nov., sp. nov. and Aridibacter kavangonensis sp. nov., two novel members of subdivision 4 of the Acidobacteria isolated from semiarid savannah soil," International Journal of Systematic and Evolutionary Microbiology, vol. 64, pp. 1866–1875, 2014.

[38] M. Uyttebroek, S. Vermee, P. Wattiaux, A. Ryngaert, and D. Springael, "Characterization of cultures enriched from acidic polycyclic aromatic hydrocarbon-contaminated soil for growth on pyrene at low pH," Applied and Environmental Microbiology, vol. 73, no. 10, pp. 3159–3164, 2007.

[39] J. Pascual, B. U. Foesel, A. Geppert, K. J. Huber, and J. Overmann, "Flaviaesturariibacter luteus sp. nov., isolated from an agricultural floodplain soil, and emended description of the genus flaviaesturariibacter," International Journal of Systematic and Evolutionary Microbiology, vol. 67, no. 6, pp. 1727–1734, 2017.

[40] S. M. Tiqiu, J. H. C. Wan, and N. F. Y. Tam, "Extracellular enzyme profiles during co-composting of poultry manure and yard trimmings," Process Biochemistry, vol. 36, no. 8–9, pp. 813–820, 2001.

[41] M. W. Hubble, A. King, and I. Phillips, "API ZYM: a simple rapid system for the detection of bacterial enzymes," Journal of Clinical Pathology, vol. 30, no. 3, pp. 275–277, 1977.

[42] S. Bascomb and M. Manafi, "Use of enzyme tests in characterization and identification of aerobic and facultatively anaerobic gram-positive cocci," Clinical Microbiology Reviews, vol. 11, no. 2, pp. 318–340, 1998.

[43] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, "Mega 10: molecular evolutionary genetics analysis version 6.0," Molecular Biology and Evolution, vol. 30, no. 12, pp. 2725–2729, 2013.

[44] N. Saitou and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees," Molecular Biology and Evolution, vol. 4, no. 4, pp. 406–425, 1987.

[45] D. S. Katz, "The streak plate protocol," American Society for Microbiology, vol. 4, 1954.

[46] J. M. Janda and S. L. Abbott, "16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls," Journal of Clinical Microbiology, vol. 45, no. 9, pp. 2761–2764, 2007.

[47] J. Ye, S. McGinnis, and T. L. Madden, "BLAST: improvements for better sequence analysis," Nucleic Acids Research, vol. 34, no. Web Server issue, pp. W6–W9, 2006.

[48] L. G. Wayne, D. J. Brenner, R. R. Colwell, P. A. D. Grimont, M. I. Krichevsky, and H. G. Truper, "Report of the ad hoc committee on reconciliation of approaches to bacterial systematics," International Journal of Systematic Bacteriology, vol. 37, no. 4, pp. 463–464, 1987.

[49] E. Stackebrandt and J. Ebers, "Taxonomic parameters revisited: tarnished gold standards," Microbiol Today, vol. 33, no. 4, pp. 152–155, 2006.

[50] R. P. Tittsler and L. A. Sandholzer, "ffK he use of semi-solidagar

[51] J. Pascual, B. U. Foesel, A. Geppert, K. J. Huber, and J. Overmann, "Flaviaesturariibacter luteus sp. nov., isolated from an agricultural floodplain soil, and emended description of the genus flaviaesturariibacter," International Journal of Systematic and Evolutionary Microbiology, vol. 67, no. 6, pp. 1727–1734, 2017.
in *Bio Protection*, pp. 78–85, Lincoln University, Lincoln, UK, 2014.

[56] Y. Liu, J. Du, Q. Lai et al., "Proposal of nine novel species of the *Bacillus cereus* group," *International Journal of Systematic Evolutionary Microbiology*, vol. 67, pp. 2499–2508, 2017.

[57] H. Jacob and F. I. Irshaid, "Biochemical and molecular taxonomy of a mild halophilic strain of *Citrobacter* isolated from hypersaline environment," *Research Journal of Microbiology*, vol. 7, no. 4, pp. 219–226, 2012.

[58] D. J. Brenner, P. A. Grimont, A. G. Steigerwal, E. Ageron, G. R. Fanning, and C. F. Riddle, "Classification of *Citrobacter* by DNA hybridization: designation of *Citrobacter famzerti* sp. nov., *Citrobacter youngae* sp. nov., *Citrobacter braakii* sp. nov., *Citrobacter werkmanii* sp. nov., *Citrobacter sedlakii* sp. nov., and three unnamed *Citrobacter* genomospecies," *International Journal of Systematic Bacteriology*, vol. 43, no. 4, pp. 645–658, 1993.

[59] R. Sánchez Clemente, M. Isabel Igeño, G. Población et al., "Study of pH changes in media during bacterial growth of several environmental strains," *Proceedings*, vol. 2, p. 1297, 2018.

[60] C. Schiraldi and M. Rosa, *Mesophilic Organisms. Encyclopedia of Membranes*, Springer, Berlin, Germany, 2014.

[61] M. Asmamaw, A. Haile, and G. Abera, "Characterization and classification of salt affected soils and irrigation water in Tendaho sugarcane production farm, North-Eastern Rift valley of Ethiopia," *African Journal of Agricultural Research*, vol. 13, no. 9, pp. 403–411, 2018.

[62] I. G. Proca, F. Matei, C. Filofteia, D. ˘ a, and ˘ . Jurcoane, "Salt tolerance of bacterial strains isolated from hypersaline water located in lopatari, Romania," *Scientific Bulletin. Series F. Biotechnologies*, vol. 21, pp. 229–232, 2017.

[63] B. van den Burg, "Extremophiles as a source for novel enzymes," *Current Opinion in Microbiology*, vol. 6, no. 3, pp. 213–218, 2003.