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Diversity of *Vachellia nilotica* rhizosphere bacteria in the savanna of Baluran National Park, Indonesia and their potential for hydrolytic enzyme production

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Abstract. *Ni'matuzahroh, Affandi M, Ramadhan R, Fatimah, Supriyanto A, Salamun, Nurhariyati T, Geraldi A, Junairiah, Setiawan J, Agustiana BND, Priyatama AH, Atmanindya K, Alawiyah DD, Putra EP, Khiftiyah AM, Sari SK, Clement C. 2025. Diversity of* Vachellia nilotica *rhizosphere bacteria in the savanna of Baluran National Park, Indonesia and their potential for hydrolytic enzyme production. Biodiversitas 26: 1754-1767. Vachellia nilotica*, a leguminous plant introduced to Baluran National Park as a firebreak, is now causing environmental problems due to its invasiveness, which hinders the growth of native plants. Despite significant ecological changes, there is no data regarding the impact of this plant invasion on soil microorganisms. This study aimed to assess the bacterial diversity in the rhizosphere of *V. nilotica* and investigate the bacteria's ability to produce hydrolytic enzymes. The bacterial community was identified using Next-Generation Sequencing (NGS), and a conventional isolation technique. A total of 99,520 Operational Taxonomic Units (OTUs) were obtained, encompassing a rich diversity of 4,631 species. These species belonged to four phyla (i) Pseudomonadota; (ii) Actinomycetota; (iii) Bacillota; and (iv) Acidobacteriota. Additionally, 26 isolates were obtained through conventional isolation, and all of them can produce hydrolytic enzymes, including protease, lipase, amylase and cellulase. A total of 9 isolates showed highest proficiency in producing hydrolytic enzymes, of which 8 belonged to the Bacillota phylum and 1 isolate showed similar characteristic with Acidobacteriota phylum. The isolates SAV 2.NA.1.5.6 and SAV 2.NA.2.4.3 exhibited multiple enzyme activities and were classified as Bacillota. The findings indicate that the rhizosphere of *V. nilotica* contains a high diversity of bacteria, including those capable of producing hydrolytic enzymes. This information can serve as a foundation for managing *V. nilotica* and promoting sustainable development by leveraging the potential of rhizosphere bacteria in this plant. To our knowledge, no prior research has been conducted on this topic in the savanna area of BNP.

Keywords: *Vachellia nilotica*, Baluran National Park, biodiversity, hydrolytic enzyme, Next-Generation Sequencing

INTRODUCTION

Baluran National Park (BNP) is one of Indonesia's oldest national parks, and it was established to conserve the distinctive biodiversity and environment of the area. One of the characteristic habitats of BNP is its savanna ecosystem. This national park is often referred to as Little Africa due to its resemblance to the savannas of Africa (Wahono et al. 2023). Savanna regions are primarily characterized by arid vegetation, making them vulnerable to wildfires (Wahono et al. 2023). However, fire plays a crucial role in shaping the savanna ecosystems of BNP by promoting grass regeneration and favoring the natural selection of grass competitors. While fire benefits the savanna, it poses a significant threat to delicate ecosystems like tropical rainforests (Wahono et al. 2023). To prevent the spread of fire from the savanna to the forest, *Vachellia nilotica* was strategically planted along the perimeters of the savanna as a firebreak (Wahono et al. 2023). According to the latest taxonomic classification, *Vachellia nilotica* (L.) P.J.H. Hurter & Mabb., previously known as *Acacia nilotica* (Kyalangalilwa et al. 2013). Since then, the abundance of *V. nilotica* has continued to grow, gradually invading the BNP savanna (Suwono and Pambudi 2018; Zahra et al. 2020).

The fire-resistant characteristic of *Vachellia* allows it to easily dominate the existing vegetation in the BNP savanna (Yousif et al. 2020). Additionally, *V. nilotica* belongs to the legume family and exhibits strong resilience in arid habitats such as savannas (Castro et al. 2017; Taylor and Dhileepan 2019). This adaptation is derived from nodules on *Vachellia* roots that contain rhizobium bacteria, which play a crucial role in nitrogen fixation, enhancing soil fertility in their rhizosphere (Amadou et al. 2020). Furthermore, *V. nilotica* produces allelochemical toxic compounds that inhibit the

growth of nearby plants (Gioria et al. 2023). Other compounds, such as phenols and terpenoids, produced by *Vachellia* also exhibit similar toxicity (Perumal et al. 2023; Tiwari et al. 2023). This ability to adapt gives *Vachellia* a competitive advantage in quickly dominating new habitats. This undoubtedly alter the structure of the ecosystem and create a new environment that supports microorganisms with high biological activity.

One notable biological activity of microbes around *Vachellia* is the rapid decomposition of *V. nilotica* leaf litter. Several reports indicate that there are no visible remnants of litter around *V. nilotica*, likely due to the rapid decomposition activity of microorganisms, including fungi and bacteria (Zhang et al. 2023). Soil microorganisms have the ability to degrade organic compounds, including litter, by secreting enzymes to hydrolyze and oxidize these compounds and performing various processes to alter their structure (Wang et al. 2020a; Dong et al. 2021). Several enzymes, such as cellulase, amylase, lipase, and protease, play roles in degrading plant residues (Chen et al. 2020b). Amylase initiates the process by hydrolyzing starch, while cellulase hydrolyzes cellulose (Wang et al. 2020b; Ilić et al. 2023). Lipase converts biomass into sugar esters (Gattupalli et al. 2024), while protease degrades amino acids and proteins (Lu et al. 2023). The soil around *V. nilotica* can thus be a hotspot for bacteria that produce high levels of hydrolytic enzymes capable of degrading organic compounds.

BNP is currently investigate the biodiversity in the park. These activity align with BNP's mandate to protect, preserve, and sustainably utilize its natural resources. However, the exploration has yet to fully reveal the richness of its microorganisms. The initial stage of microbial bioprospecting involves assessing the microbial diversity within the community. Understanding diversity is essential for mapping various bioprospecting efforts based on published data. Currently, microbial diversity can be easily determined using metataxonomic approaches, making it crucial to analyze the microbial diversity in BNP. The exploration of microorganisms in BNP can begin by studying those associated with the rhizosphere soil of *V. nilotica*. These bacteria have the potential to produce hydrolytic enzymes with various industrial and biotechnological application. Therefore, the purpose of this study was to analyze the biodiversity and bioprospecting potential of hydrolytic enzyme-producing bacteria found in the rhizosphere soil of *V. nilotica*.

MATERIALS AND METHODS

Sampling area

Soil samples were collected from the rhizosphere of *Vachellia nilotica* growing in the savanna area of Baluran National Park, Situbondo, East Java, Indonesia (7º50'31.2"S, 114º26'09.6"E) (Figures 1 and 2). The soil pH at the sample site was ranged between 4.8 to 5.2, soil moisture levels >8%, air humidity over 32%, a temperature of 31°C, and a light intensity of 1408 Cd. An intriguing observation in the sampling area was the absence of litter or grass in the vicinity of *V. nilotica*.

Figure 2. The process of soil sampling surrounding of *V. nilotica* roots

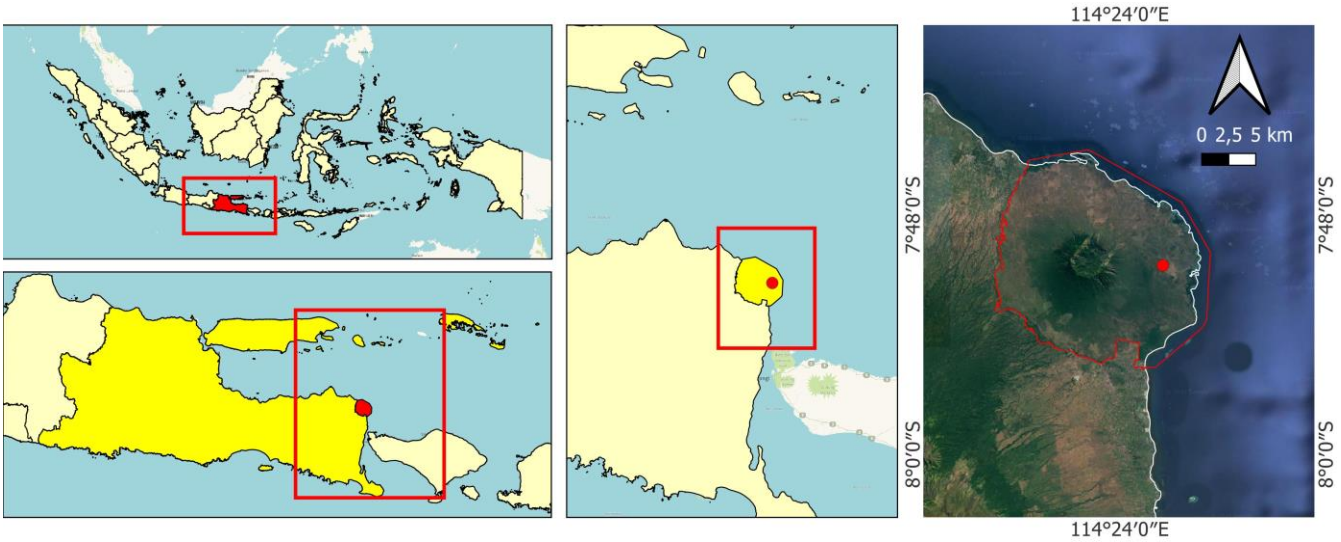

Figure 1. Sampling location point of *Vachellia nilotica* in Baluran National Park, Situbondo, East Java, Indonesia

Sample collection

Soil samples were collected from a depth of 0-10 cm using a soil drill and shovel. The collected soil samples were placed in plastic bags and delivered to the laboratory. The samples were stored at 19°C for long 14 days until analysis.

DNA extraction

A total of 50 g of soil was sent to PT Genetika Science (Indonesia) for analysis. Genomic DNA was extracted from the soil samples using the Zymo BIOMICS DNA Miniprep Kit (Zymo Research, D4300). The quantity and purity of extracted DNA were assessed using a Nanodrop 2000. The DNA was amplified using the polymerase chain reaction (PCR) method with primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). The quality of the PCR product was evaluated using agarose gel electrophoresis with 1% TBE agarose. The DNA quantity and concentration were determined using a Qubit fluorometer. DNA samples with a target band of 1,500 base pairs and a minimum concentration of 20 ng in a volume of 10 µL were subsequently sequenced using Oxford Nanopore technology.

Next-generation sequencing (NGS)

Next-Generation Sequencing (NGS) was performed using the Oxford Nanopore Technologies (ONT) method by PT Genetika Science (Indonesia), following the applicable protocols. Prior to sequencing, library preparation was performed using kits from Oxford Nanopore Technology. Sequencing was performed using MinKNOW software version 23.04.5, followed by base calling using Guppy version 6.5.7 (Wick et al. 2019). The quality of the obtained FASTQ files was checked using NanoPlot version 6.5.7 and quality filtering was conducted using Nanofit version 2.8.0 (de Coster et al. 2018; Nygaard et al. 2020). The obtained reads were classified using the centrifuge classifier (Kim et al. 2016). The bacterial index was constructed using the NCBI 16S Refseq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). Analysis and visualization were performed using Pavian, available at the following GitHub repository: https://github.com/fbreitwieser/pavian.

Isolation and characterization of bacteria

Ten g of soil were placed in a saline solution (0.90% NaCl). Subsequently, the mixture was homogenized for 30 minutes using a shaker. The suspension was then diluted up to 10^{-6}. A total of 1 mL of the suspension from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was inoculated onto Nutrient Agar (NA), using the pour plate method and incubated at room temperature for 24 hours. After incubation, bacterial colonies were enumerated and examined macroscopically and microscopically for characterization. Microscopic observation of bacteria was conducted using Gram staining to determine cell shape, Gram type, and the presence of endospores. Endospore characterization was done by flooded isolate fixation with malachite green dye and heated over a boiling water for about 15-20 minutes to facilitate penetration into the endospore.

The dye was then rinsed with water and counterstained with safranin for 1 minute. Bacterial colonies with distinct macroscopic and microscopic characteristics were classified as different isolates and selected for purification. The purified isolates were subsequently subjected to biochemical tests using Microbact™ 12A (Oxoid) kit and Sulfide Indole Motility (SIM) Medium. Microbact™ 12A kit consisted of 12 tests i.e. lysine decarboxylase, ornithine decarboxylase, H2S production, sugar (glucose, mannitol, and xylose) fermentation, β-galactosidase (ONPG), indole production, urea hydrolysis, Voges-Proskaüer (VP) reaction, citrate utilization, and tryptophan deaminase (TDA). SIM media was used to detect motility test by stabbing a well-isolated colony into the medium then incubated at room temperature for about 24 hours. The positive result was indicated by a turbid area extending away from the line of inoculation while the negative result was on the contrary. The pure isolates obtained were assessed for their capacity to produce hydrolytic enzymes, including cellulase, amylase, lipase, and protease.

Bacterial hydrolytic potential assay

The obtained bacterial isolates were inoculated onto Bushnell Haas media (Himedia) in Petri dishes using various carbon sources. Each isolate was cultured on agar media containing 1% (w/v) sodium carboxymethyl cellulose (Na CMC), 1% (w/v) starch, 2% olive oil, 1% Tween 80, 1% Rhodamine B and 5% skimmed milk to assess their potential in producing cellulase, amylase, lipase, and protease enzymes, respectively (Ni'matuzahroh et al. 2020; Rahmah et al. 2020; Ni'matuzahroh et al. 2024).

Bacterial isolates capable of producing enzymes were identified by the presence of clear zones around the colonies on the respective media. When bacteria were placed on the protease test media, a distinct, clear zone appeared around the colony. For cellulase test, agar media was treated with Congo Red and left undisturbed for 15 minutes, a clear zone showed the presence of cellulose. The media were then rinsed with a solution of NaCl (0.1 N) and left undisturbed for 15 minutes. In amylase test agar media was evaporated with iodine, a distinct, clear zone appeared. The presence of a clear zone in the lipase test media was detected using ultraviolet (UV) light (Ni'matuzahroh et al. 2020; Ni'matuzahroh et al. 2024). The bacterial ability to produce cellulase, amylase, and protease enzymes was quantified using the hydrolytic index. The hydrolytic index was calculated using the following formula (Choi et al. 2005):

$$\text{Hydolytic index} = \frac{\text{Total diameter} - \text{Bacterial colony diameter}}{\text{Bacterial colony diameter}}$$

A hydrolytic index value of less than 1 indicates weak enzyme activity, 1 to 2 indicates moderate activity and 2 or more indicates strong activity (Choi et al. 2005). The method for measuring the total diameter and colony diameter is illustrated in Figure 3. The diameter of the clear zone indicating lipase enzyme production could not measure accurately due to unclear boundaries. Therefore, the bacterial ability to produce lipase enzymes was reported descriptively as either 'yes' or 'no.' The most promising

bacterial isolate was selected for further characterization. The selection criteria included its ability to produce all four hydrolytic enzymes (cellulase, amylase, protease, and lipase) and ranking among the top three isolates in terms of cellulase, amylase, and protease production. The most promising bacterial isolates were subjected to further biochemical testing using Microbact™ 12A (Oxoid), following the procedures described by the manufacturer.

Data analysis

The data obtained from Next-Generation Sequencing (NGS) were utilized for a descriptive analysis of bacterial diversity. The hydrolytic enzyme production potential of the bacteria was analyzed to evaluate their prospects as enzyme producers. Additionally, the characteristics of the bacterial isolates were correlated with the results from the metataxonomic analysis.

RESULTS AND DISCUSSION

The NGS analysis conducted on soil samples from the rhizosphere of *Vachellia nilotica*, in the savanna of Baluran, a total of 99,520 reads were retrieved, with an average read length of 1.598.5 ± 43.5 base pairs. The number of reads corresponds to the quantity of Operational Taxonomic Units (OTUs) present in the sample, with nearly uniform read lengths. The OTUs were subsequently classified into their respective taxonomic categories. The rhizosphere soil of *V. nilotica* in the savanna of Baluran National Park (BNP) was predominantly dominated by four bacterial phyla (i) Pseudomonadota; (ii) Actinomycetota; (iii) Bacillota; and (iv) Acidobacteriota, as indicated by the OTU values. In addition to these four phyla, 41 other phyla were identified, although they had a lesser number of OTUs (Figure 4).

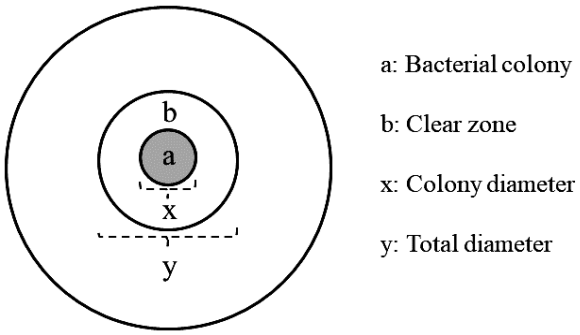

Figure 3. Schematic illustration showing the measurement of the colony diameter and the total diameter of the colony

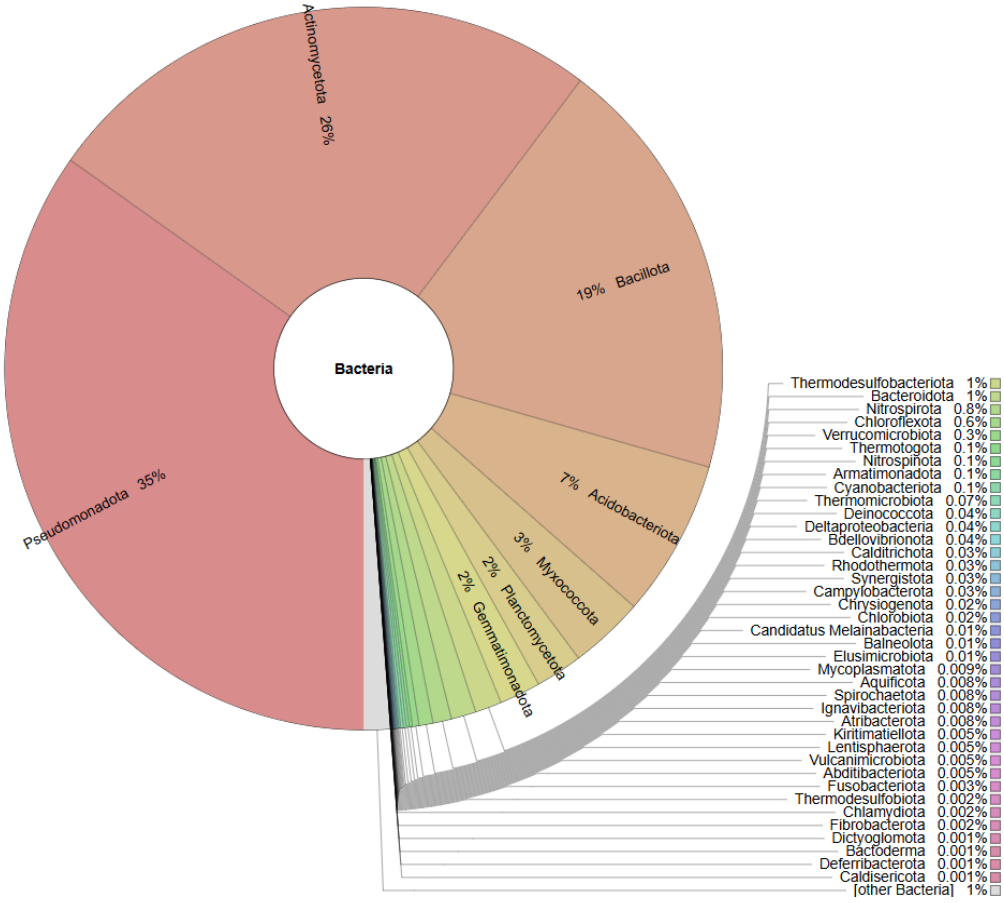

Figure 4. Bacterial phyla constituting the rhizosphere soil bacterial community of *Vachellia nilotica* in the savanna of Baluran National Park, Indonesia

These phyla comprise 4,631 species, 1,844 genera, and 547 families. The 10 families, 10 genera, and 10 species with the highest cumulative number of OTUs are presented in Figure 5. The top 10 genera consisted of *Luteitalea*, *Vicinamibacter*, *Gaiella*, *Rhabdothermincola*, *Rubrobacter*, *Solirubrobacter*, *Bacillus*, *Lysinibacillus*, and *Pradoshia*. Whereas top 10 species were *Luteitalea pratensis*, *Vicinamibacter silvestris*, *Gaiella occulta*, *Rubrobacter marinus*, *Rhabdothermincola sediminis*, *Rubrobacter spartanus*, *Solirubrobacter ginsenosidimutans*, *Lysinibacillus odyssey*, and *Pradoshia eiseniae*. Interestingly, despite Pseudomonadota having the highest OTU values, the species representing this phylum in the rhizosphere soil of *V. nilotica* were highly diverse, resulting in a limited number of dominant species. *Arboricoccus pini* was the sole species that dominated the Pseudomonadota phylum. In the Actinomycetota phylum, five dominant bacterial species were identified (i) *Gaiella occulta*; (ii) *Rhabdothermincola sediminis*; (iii) *Rubrobacter marinus*; (iv) *Rubrobacter spartans*; and (v) *Solirubrobacter ginsenosidimutans*. *Lysinibacillus odysseyi* and *Pradoshia eiseniae* were the dominant species in the Bacillota phylum. Two bacterial species, *Vicinamibacter silvestris* and *Luteitalea pratensis*, were identified in the Acidobacteriota phylum, with *V. silvestris* exhibiting the highest level of dominance.

The bacterial community was highly diverse, as evidenced by the substantial number of OTUs that can be classified into a wide variety of bacterial groups. This study demonstrated the biodiversity of bacteria in the rhizosphere of *V. nilotica*, not only through the number of bacterial groups detected via sequencing but also by the alpha diversity values represented by the Shannon and Simpson indices. In the present study, the Shannon and Simpson index was 6.627 and 0.995, respectively.

The results of conventional method showed a diverse collection of bacteria, exhibiting a wide range of colony traits and microscopic cell characteristics. A total of 26 isolates were successfully isolated from the rhizospheric soil of *V. nilotica*. The macroscopic and microscopic characteristics of all bacterial isolates are presented in Table 1.

The bacterial diversity was primarily dominated by rod-shaped, Gram-positive bacteria with oval-shaped endospores located in the central, subterminal, and terminal regions of bacterial cells. The observed macroscopic characteristics of bacterial colonies included circular and irregular shapes, with colors ranging from white and gray to yellowish-white. The colony margins varied, exhibiting curled, entire, and undulate forms, with elevations ranging from flat to elevated (Figure 6). The prevalent colony texture was smooth and dry, with optical characteristics including opaque, translucent, and transparent. One bacterial isolate, coded SAV 2. NA. 1.3.2, exhibited Gram-negative, rod-shaped microscopic characteristics. The colonies were circular, cream-colored, with raised entire margins, dry texture, glistening appearance, and a translucent optical preference. Microscopic characteristics of several bacterial cells isolated from *V. nilotica* rhizosphere soil are presented in Figure 7.

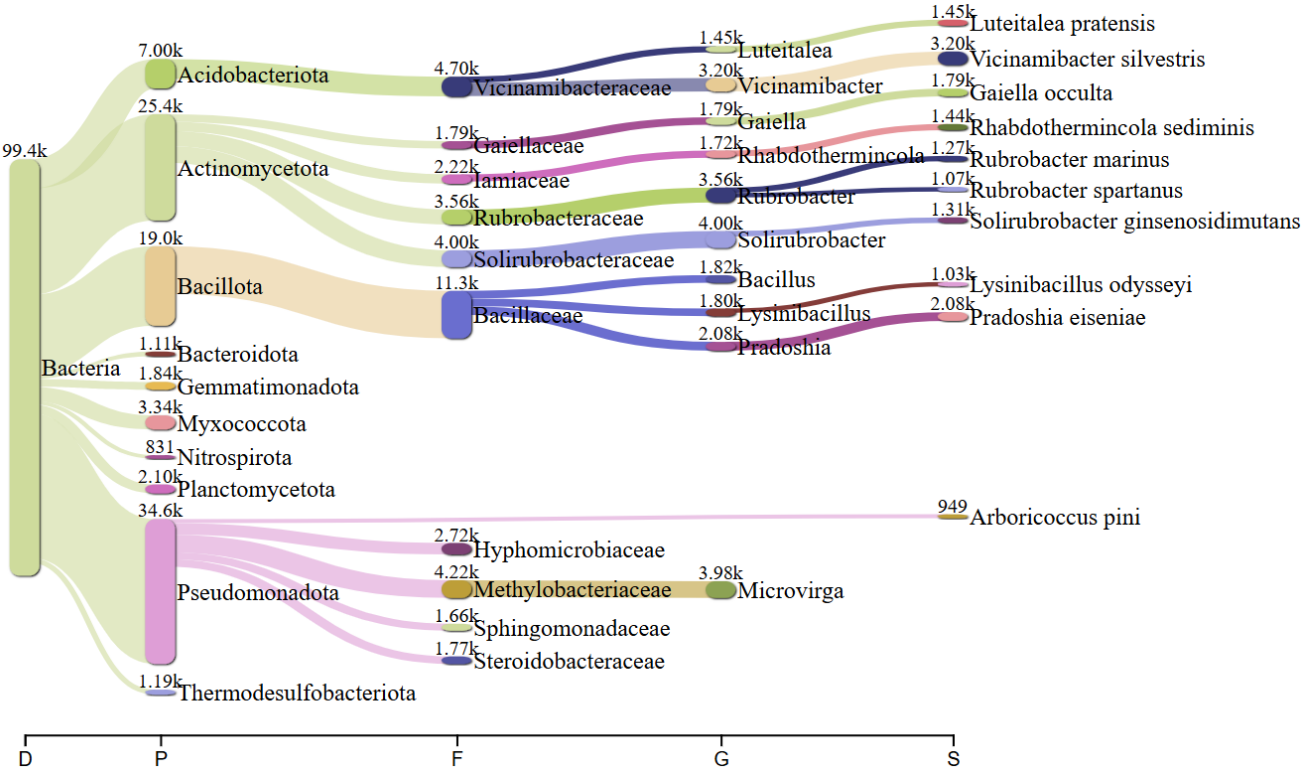

Figure 5. Top ten bacterial species, genera, families, and phyla in the bacterial communities of the rhizosphere soil of *Vachellia nilotica* in the savanna of Baluran National Park. The numbers depicted in the graphic represent the number of OTUs in thousands

Table 1. Diversity of bacteria from the rhizosphere soil of *Vachellia nilotica* based on their macroscopic and microscopic characteristics

Isolates	Macroscopic characteristics							Microscopic characteristics		Endospore	Motility
	Color	Form	Margin	Elevation	Appearance	Optical preference	Texture	Type of Gram	Form		
SAV 2. NA. 1.3.2	Cream	Circular	Entire	Elevated	Glistening	Translucent	Dry	Negative	Bacil	-	-
SAV 2. NA. 1.3.3	White	Irregular	Curled	Flat	Dull	Translucent	Dry	Positive	Bacil	Oval, Central	+
SAV 2. NA. 1.3.6	Cream	Circular	Entire	Elevated	Glistening	Translucent	Dry	Positive	Bacil	Oval, Terminal	-
SAV 2. NA.1.3.7	White-Milk	Irregular	Curled	Elevated	Dull	Opaque	Dry	Positive	Bacil	Oval, Central, and Subterminal	+
SAV 2. NA. 1.3.8	Cream	Circular	Entire	Elevated	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Central	-
SAV 2. NA. 1.4.1	White Cream	Circular	Entire	Growth into medium	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Terminal	+
SAV 2. NA. 1.4.2	White Bone	Irregular	Entire	Elevated	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Central	+
SAV 2. NA. 1.4.3	White-Grey	Irregular	Entire	Flat	Dull	Translucent	Smooth	Positive	Bacil	Oval Terminal	-
SAV 2. NA. 1.4.4	White-Grey	Irregular	Curled	Flat	Dull	Translucent	Smooth	Positive	Bacil	Round, Subterminal	-
SAV 2. NA. 1.4.6	White Cream	Circular	Entire	Elevated	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Subterminal	-
SAV 2. NA. 1.5.1	White-Yellowish	Circular	Entire	Elevated	Dull	Opaque	Smooth	Positive	Bacil	Oval, Terminal	-
SAV 2. NA. 1.5.2	White-Grey	Circular	Entire	Elevated	Glistening	Transparent	Smooth	Positive	Bacil	Oval, Terminal	-
SAV 2. NA. 1.5.3	White-Yellowish	Circular	Entire	Elevated	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Terminal	-
SAV 2. NA. 1.5.4	White-Grey	Circular	Curled	Elevated	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Terminal	+
SAV 2. NA. 1.5.5	White-Yellowish	Circular	Entire	Elevated	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Subterminal	-
SAV 2. NA. 1.5.6	White	Circular	Entire	Elevated	Dull	Opaque	Smooth	Positive	Bacil	Round, Terminal	-
SAV 2. NA. 1.5.7	White-Yellowish	Circular	Entire	Flat	Dull	Opaque	Smooth	Positive	Bacil	Oval, Central	-
SAV 2. NA. 2.3.1	White Milk	Circular	Entire	Flat	Dull	Translucent	Smooth	Positive	Bacil	Oval, Subterminal	+
SAV 2. NA. 2.3.2	White Milk	Circular	Curled	Elevated	Dull	Translucent	Vivid	Positive	Bacil	Oval, Subterminal	+
SAV 2. NA. 2.3.3	White-Grey	Circular	Curled	Flat	Dull	Translucent	Smooth	Positive	Bacil	Oval, Subterminal	+
SAV 2. NA. 2.3.4	Cream	Circular	Entire	Elevated	Glistening	Opaque	Smooth	Positive	Bacil	Oval, Central	+
SAV 2. NA. 2.3.5	White-Grey	Circular	Entire	Flat	Dull	Translucent	Dry	Positive	Bacil	Oval Central	+
SAV 2. NA. 2.4.1	White	Circular	Entire	Flat	Dull	Transparent	Smooth	Positive	Bacil	Oval, Subterminal	+
SAV 2. NA. 2.4.2	White	Irregular	Undulate	Flat	Dull	Opaque	Smooth	Positive	Bacil	Oval, Central	+
SAV 2. NA. 2.4.3	White	Circular	Entire	Flat	Glistening	Translucent	Dry	Positive	Bacil	Oval, Central	+
SAV 2. NA. 2.5.1	White-Grey	Circular	Entire	Flat	Glistening	Transparent	Smooth	Positive	Bacil	Oval, Central	+

Notes: (-): no endospore or negative result; (+): positive result

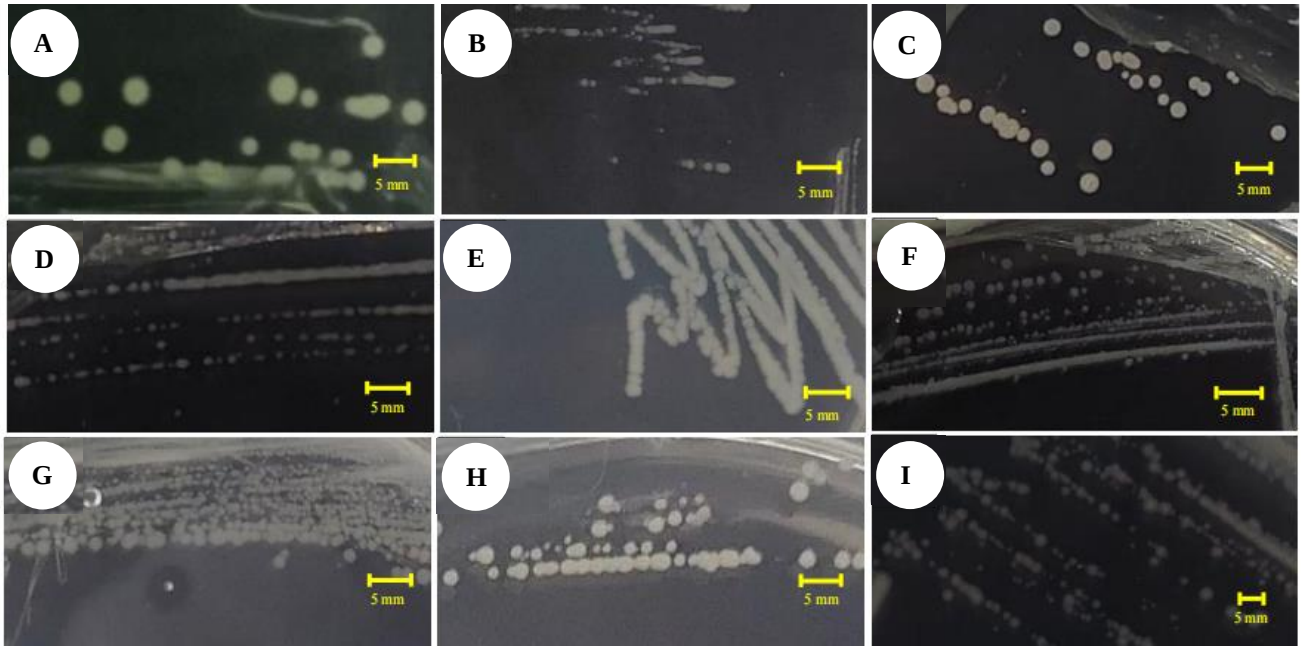

Figure 6. The macroscopic characteristics of bacterial colonies from the rhizosphere soil of *Vachellia nilotica*. A. SAV 2. NA. 1.5.1; B. SAV 2. NA. 1.5.6; C. SAV 2. NA. 1.5.2; D. SAV 2. NA. 1.3.2; E. SAV 2. NA. 1.4.4; F. SAV 2. NA. 1.5.4; G. SAV 2. NA. 2.3.1; H. SAV 2. NA. 2.3.4; I. SAV 2. NA. 2.4.3

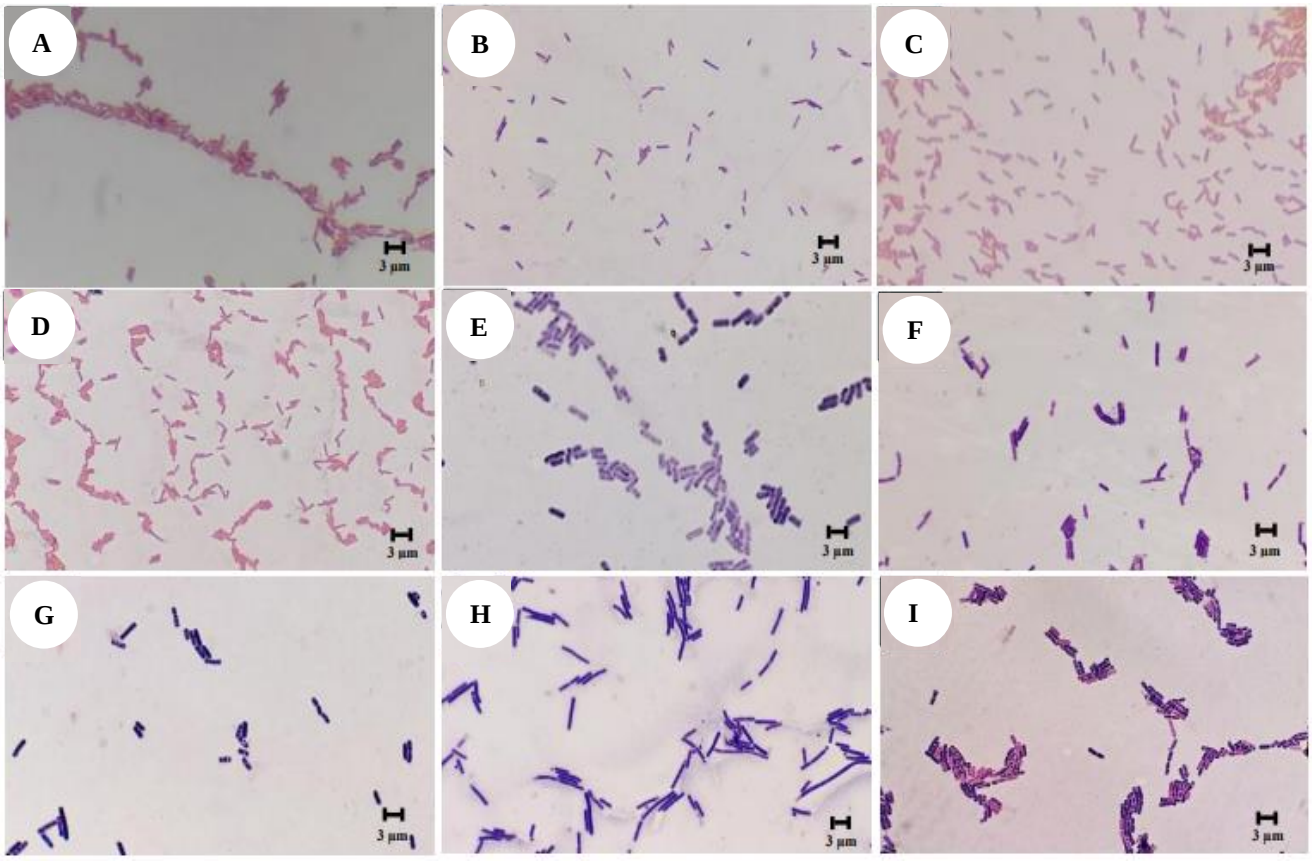

Figure 7. Microscopic characteristics of bacterial cells isolated from *Vachellia nilotica* rhizosphere soil. A. SAV 2. NA. 1.5.1; B. SAV 2. NA. 1.5.6; C. SAV 2. NA. 1.5.2; D. SAV 2. NA. 1.3.2; E. SAV 2. NA. 1.4.4; F. SAV 2. NA. 1.5.4; G. SAV 2. NA. 2.3.1; H. SAV 2. NA. 2.3.4; I. SAV 2. NA. 2.4.3

The bacterial isolates acquired were also assessed for their capacity to produce hydrolytic enzymes, serving as a starting point for exploring bacteria from the rhizosphere soil of *V. nilotica* for potential applications. The presence of hydrolytic enzymes in bacteria was indicated by the formation of a clear zone surrounding bacterial colonies when cultivated on screening media for cellulase, protease, amylase and lipase, also fluorescent isolate when observed using UV light for lipase (Figure 8). The diameter of the clear zone surrounding the bacterial colony can be compared to the diameter of the bacterial colony itself to assess the bacteria's ability to produce hydrolytic enzymes. Table 2 displays the hydrolytic index of all bacterial isolates obtained from the rhizosphere of *V. nilotica*.

Each bacterial isolate exhibited varying hydrolytic abilities. Two isolates, namely SAV 2. NA. 1.5.6 and SAV 2.NA. 2.4.3, demonstrated the ability to hydrolyze all tested substrates (multi-enzyme activity). It was observed that 11 isolates exhibited cellulase hydrolytic activity. Additionally, all isolates demonstrated protease hydrolytic activity, while 13 isolates exhibited amylase hydrolytic activity. These isolates demonstrated prominent clear zones during the enzyme activity assays, indicating low, medium, and strong hydrolytic activity.

The results revealed that the top three isolates capable of hydrolyzing cellulase enzyme were SAV 2. NA. 1.5.1; SAV 2. NA. 1.5.6; and SAV 2. NA. 1.5.2 and classified in low index category. Meanwhile, the isolates that were able to hydrolyze protease enzyme were SAV 2. NA. 1.3.2; SAV 2. NA. 1.4.4; and SAV 2. NA. 1.5.6 which showed strong hydrolytic index category. The isolates having the ability to produce amylase were SAV 2. NA. 1.5.4, SAV 2. NA. 2.3.1, and SAV 2. NA. 2.3.4, which classified in medium hydrolytic index category. The isolates capable of hydrolyzing the lipase enzyme were identified by a noticeable alteration in the color of media. The selected isolates were subsequently subjected to biochemical tests, as presented in Table 3.

The biochemical test results for the nine isolates revealed significant physiological diversity. The ability to metabolize carbohydrates varied among isolates, with SAV 2. NA. 1.4.4, SAV 2. NA. 1.5.2, SAV 2. NA. 1.5.4, SAV 2. NA. 2.3.1, SAV 2.NA. 2.3.4, and SAV 2. NA. 2.4.3 testing positive for glucose metabolism. Additionally, SAV 2. NA. 1.5.1 demonstrated the ability to utilize mannitol and xylose, highlighting its capacity to metabolize alternative carbon sources. Meanwhile, for sucrose or lactose fermentation SAV 2. NA. 1.3.1, SAV 2. NA. 1.5.1, SAV 2. NA. 1.5.2, SAV 2. NA. 1.5.4, SAV 2. 1.5.6, SAV 2. NA. 2.3.4, and SAV 2. NA. 2.3.4 exhibited positive results.

Catalase activity was observed in most isolates, except for SAV 2. NA. 1.4.4 and SAV 2. NA. 1.5.6. However, none of the isolates showed oxidase activity. Furthermore, nitrate reduction was confirmed in SAV 2. NA. 1.5.4, SAV 2. NA. 2.3.1, SAV 2. NA. 2.3.4, and SAV 2. NA. 2.4.3.

TSIA (Triple Sugar Iron Agar) test results demonstrated varying metabolic patterns. For instance, SAV 2. NA. 1.3.2 and SAV 2. NA. 1.5.6 exhibited an A/K (acid/alkaline) reaction whereas SAV 2. NA. 1.5.2, SAV 2. NA. 1.5.4, SAV 2. NA. 2.3.4, and SAV 2. NA. 2.4.3 showed an A/A (acid/acid) reaction. Meanwhile, test for indole, citrate, and urease activity yielded varied results. None of the isolates produced indole or utilized citrate, while urease activity was only detected in SAV 2. NA. 1.5.4.

Table 2. The potential of soil bacteria from *Vachellia nilotica* rhizosphere to produce hydrolytic enzymes based on the hydrolytic index

Isolates	Hydrolytic index			
	Cellulase	Protease	Amylase	Lipase
SAV 2. NA. 1.3.2	0.00	2.46	0.00	No
SAV 2. NA. 1.3.3	0.00	1.38	0.00	No
SAV 2. NA. 1.3.6	0.19	0.14	0.00	Yes
SAV 2. NA. 1.3.7	0.00	1.17	0.00	No
SAV 2. NA. 1.3.8	0.00	1.17	0.03	Yes
SAV 2. NA. 1.4.1	0.00	1.15	0.10	Yes
SAV 2. NA. 1.4.2	0.00	1.26	0.00	Yes
SAV 2. NA. 1.4.3	0.00	1.06	0.00	No
SAV 2. NA. 1.4.4	0.00	2.24	0.00	No
SAV 2. NA. 1.4.6	0.00	1.58	0.00	Yes
SAV 2. NA. 1.5.1	0.92	0.31	0.64	No
SAV 2. NA. 1.5.2	0.67	0.60	0.00	Yes
SAV 2 NA. 1.5.3	0.66	0.00	0.00	No
SAV 2. NA. 1.5.4	0.06	0.73	1.41	No
SAV 2. NA. 1.5.5	0.00	0.63	0.18	Yes
SAV 2. NA. 1.5.6	0.71	2.13	0.56	Yes
SAV 2. NA. 1.5.7	0.00	0.10	0.00	Yes
SAV 2. NA. 2.3.1	0.66	1.62	1.09	No
SAV 2. NA. 2.3.2	0.00	0.12	0.00	No
SAV 2 NA. 2.3.3	0.00	1.36	0.44	No
SAV 2. NA. 2.3.4	0.00	1.82	1.00	No
SAV 2. NA. 2.3.5	0.31	0.80	0.65	No
SAV 2. NA. 2.4.1	0.28	0.55	0.24	No
SAV 2. NA. 2.4.2	0.24	0.75	0.93	No
SAV 2. NA. 2.4.3	0.40	0.77	0.55	Yes
SAV 2. NA. 2.5.1	0.00	0.74	0.87	No

Notes: Yes: color change; No: no color change. The values are the results of two repetitions

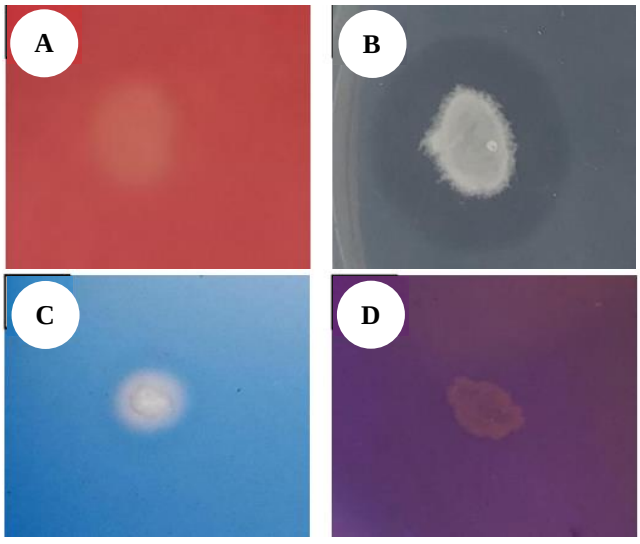

Figure 8. Clear zone surrounding the bacterial colony indicate hydrolytic activities of: A. cellulase; B. protease; C. amylase; D. lipase fluorescent isolate indicate activity of lipase

Table 3. Biochemical test results of potential bacterial isolates from the rhizosphere soil of *Vachellia nilotica*

Biochemical tests	Isolates								
	SAV 2. NA. 1.3.2	**SAV 2. NA. 1.4.4**	**SAV 2. NA. 1.5.1**	**SAV 2. NA. 1.5.2**	**SAV 2. NA. 1.5.4**	**SAV 2. NA. 1.5.6**	**SAV 2. NA. 2.3.1**	**SAV 2. NA. 2.3.4**	**SAV 2. NA. 2.4.3**
Lys	-	-	-	-	(+)	(+)	-	-	-
Orn	-	-	-	-	-	-	-	-	(+)
H2S	-	-	-	-	-	-	-	-	-
Glu	-	(+)	-	(+)	(+)	-	(+)	(+)	(+)
Man	-	-	(+)	-	-	-	-	-	-
Xyl	-	-	(+)	-	-	-	-	-	-
ONPG	-	-	-	-	-	-	-	-	-
Nitrate	-	-	-	-	(+)	-	(+)	(+)	(+)
Indole	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	(+)	-	-	-	-
V.P	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-
TSIA	A/K	K/A	A/K	A/A	A/A	A/K	K/A	A/A	A/A
Sucrose/lactose	(+)	-	(+)	(+)	(+)	(+)	-	(+)	(+)
Catalase	(+)	-	(+)	(+)	(+)	-	(+)	(+)	(+)
Oxidase	-	-	-	-	-	-	-	-	-

Note: A/K: (Yellow/Red or Acidic(A)/Alkaline(K)) indicates sucrose or lactose fermentation; A/A: (Yellow/Yellow or Acidic/Acidic) indicates sucrose and/or lactose fermentation or fermentation of three sugars; K/A: (Red/Yellow or Alkaline(K)/Acid(A)) indicates glucose fermentation only; (-): negative result; (+): positive result)

The results of biochemical tests conducted on the nine best isolates, supported by data on colony morphology and cell morphology, revealed that one isolate, SAV 2. NA. 1.3.2, belonged to the Acidobacteriota phylum, whereas the remaining eight isolates (SAV 2. NA. 1.4.4, SAV 2. NA. 1.5.1, SAV 2. NA. 1.5.2, SAV 2. NA. 1.5.4, SAV 2. NA. 1.5.6, SAV 2. NA. 2.3.1, SAV 2. NA. 2.3.4, and SAV 2. NA. 2.4.3) exhibited characteristics associated with the Bacillota phylum. The diversity in the physical appearance of colonies and the presence of endospores were indicative of the species diversity within the Bacillota phylum.

Discussion

Bacterial biodiversity in the rhizospheric soil of V. nilotica

This study aimed to examine the microbial diversity in the rhizosphere soil of *V. nilotica* and to determine its capacity to produce hydrolytic enzymes. To gain a more comprehensive understanding of the soil conditions in the rhizosphere of *V. nilotica*, two methods were employed: conventional sequencing and nanopore sequencing. The investigation of bacterial diversity in the rhizosphere soil of *V. nilotica* using the nanopore sequencing technique revealed a Shannon index of 6.627 and a Simpson index of 0.995. The Shannon and Simpson indices are metrics used to evaluate the richness of bacterial communities within a particular habitat. The Shannon index measures diversity within a community, with higher values indicating greater diversity. Conversely, the Simpson index ranges from 0 to 1, with higher values indicating lower diversity within the community (Kim et al. 2017).

The alpha diversity of bacteria in the rhizosphere of *V. nilotica* was characterized as low, as indicated by the Simpson index. The low level of bacterial biodiversity, which may be attributed to the release of allelochemicals by *V. nilotica* (Choudhari et al. 2019) that selectively impact the microorganisms in its immediate surroundings. A study conducted by Cheng et al. (2022) suggests that allelochemicals likely have a positive association with specific microbial phyla and a negative association with others. While the Simpson index suggests a lack of bacterial diversity, the Shannon index for bacteria in the rhizosphere of *V. nilotica* in the Baluran National Park (BNP) savanna showed a contrasting result. This finding aligns with the Shannon index derived from various soil samples within the rhizosphere of different *Vachellia* species. The Shannon index in this study is nearly identical to that of non-rhizosphere soil around *Acacia cincinnata* and *Acacia crassicarpa* in Fujian, China (Yuan et al. 2022), but slightly higher than that found in soil in an *Acacia* plantation in So' n Lang commune, K'Bang District, Vietnam, which had values of 6.12 and 6.01 (Sawada et al. 2021).

The microbial community in the rhizosphere soil of *V. nilotica* in the BNP savanna comprised a wide range of bacterial species, resulting in a high level of diversity and multiple phyla. Through nanopore sequencing, four dominant phyla were identified: Pseudomonadota, Actinomycetota, Bacillota, and Acidobacteriota. According to Yuan et al. (2022), these four phyla are among the top 10 dominant phyla in the soil bacterial community around *Acacia* spp. The Pseudomonadota phylum, previously referred to as Proteobacteria, is the predominant phylum in soil ecosystems compared to other bacterial phyla (Liu et al. 2024). Approximately 20-52% of the entire bacterial population in soil microbiota belongs to the Acidobacteriota phylum. Microorganisms belonging to this phylum are notoriously challenging to grow using traditional methods. Therefore, researchers investigate their potential and ecological significance using metagenomics techniques (Coluccia and Besaury 2023). In this study other bacterial phyla were also examined, including Actinomycetota (formerly Actinobacteria),

Planctomycetota (formerly Planctomycetes), Bacteroidota (formerly Bacteroidetes), and Bacillota (formerly Firmicutes). These phyla are the most prevalent in bacterial communities found in various soil samples (Delgado-Baquerizo et al. 2018). Nevertheless, the predominant bacterial species did not originate from the Pseudomonadota phylum. The absence of a dominant species within this phylum in the soil samples is likely due to the enormous diversity of its members.

Vicinamibacter silvestris, a species belonging to the Acidobacteriota phylum, was found to be the most prevalent in this investigation. The taxonomic classification of *V. silvestris* species was initially documented in 2016 by Huber et al. (2016). Despite the scarcity of articles elucidating the role and potential of this species, it has been established that this species lacks the ability to break down cellulose, starch, xylan, pectin, lignin, and chitin (Huber et al. 2016). *V. silvestris* was previously discovered in sub-tropical savanna soil in Namibia, but in the present study the species was also present in the savanna of BNP. The next most prevalent bacterial species was *Pradoshia eiseniae*, which belongs to the Bacillota phylum. The *Pradoshia eiseniae* species have been identified and documented as new genera and species. This species is obtainable from the intestinal tract of *Eisenia fetida* worms. It has been established that this species lacks the ability to break down esculin, gelatin, casein, and starch (Saha et al. 2019). The identification of this species in the soil adjacent to the roots of *V. nilotica* represents a new and unique finding for this particular species.

The subsequent dominant species, ranked from third to tenth, include *Gaiella occulta*, which was first isolated from soil and had the potential for bioremediation due to its ability to degrade polycyclic aromatic hydrocarbons (Albuquerque et al. 2011; Zhang et al. 2023). *Rhabdothermincola sediminis* is a newly discovered species isolated from hot spring sediments, with limited information available regarding its potential. However, according to the publication by Delgado-Baquerizo (2018), this species is known to hydrolyze gelatin, cellulose, and starch and to produce various enzymes important in degradation, such as α-glucosidase, α-mannosidase, α-chymotrypsin, α-galactosidase, acidic phosphatase, alkaline phosphatase, β-glucuronidase, and β-fucosidase. Another bacterium, *Solirubrobacter ginsenosidimutans*, has the capability to break down ginsenoside chemicals present in ginseng (An et al. 2011). The bacterium's capacity to break down organic compounds has not been well documented. However, it has been recognized to generate the enzyme β-galactosidase, which is involved in the decomposition of lactose (An et al. 2011). The following prevailing organisms arise from the *Rubrobacter* genus, which includes two distinct species *Rubrobacter marinus* and *Rubrobacter spartanus*. Both species are newly discovered, and there is limited knowledge regarding their potential. *R. marinus* was first obtained from sediments in the South China Sea (Chen et al. 2020a), while *R. spartanus* was discovered in the caldera of Mount Kilauea in Hawaii (Norman et al. 2017). Interestingly, BNP also encompasses the caldera region of Mount Baluran. This finding represents the initial documentation disclosing the presence of a unique bacterial species in the caldera region, which was also detected in BNP. The next species is *Lysinibacillus odysseyi*, which was originally detected on the Mars Odyssey spacecraft (Duc et al. 2004). Nevertheless, there have been other documented instances of *L. odysseyi* isolates being discovered, including its connection with the *Gracilaria canaliculata* Sonder 1871 red alga (Karthick and Mohanraju 2020) and its presence in bird feces (Ravanal et al. 2019). Although the capacity of these bacteria to produce hydrolytic enzymes has not been thoroughly investigated, it has been determined that this species lacks the ability to produce amylase and lipase (Duc et al. 2004). The last dominant species is *Arboricoccus pini*, an endophytic bacterium from *Pinus pinaster* Aiton, which was recently published in 2018 (Proença et al. 2018). This species is unable to degrade a range of organic compounds, such as esculin, xylan, casein, gelatin, chitin, and carboxymethylcellulose, in the context of organic decay.

Conventional methods and screening of bacteria

Most enzymes in soil are produced by microbial metabolism, particularly by bacteria. The enzyme activities are essential for facilitating several processes involved in the decomposition of organic matter and nutrient cycling. Consequently, the presence of enzymes in soil is strongly linked to soil fertility (Zungu et al. 2020; Daunoras et al. 2024). Bacteria found in the rhizosphere soil of *V. nilotica* in Baluran National Park exhibit diverse cellular morphologies, predominantly rod-shaped forms. These rod-shaped bacteria are identified as Gram-positive and closely related to the characteristics of the Firmicutes and Actinobacteriota phyla. Additionally, these bacterial groups exhibit variations in macroscopic colony morphology, which is an essential characteristic in bacterial identification (Franco-Duarte et al. 2019). This finding aligns with the study by Kamutando et al. (2018), which reported the dominance of these two phyla in the rhizosphere soil of *Acacia dealbata* Link.

The prevalence of rod-shaped bacteria in this study is suspected to be a direct response to the drought conditions experienced in the savanna ecosystem of Baluran National Park. Various bacterial species have developed adaptations to thrive under arid and high-temperature conditions, such as spore formation (Maquia et al. 2020). Similarly, a study by Bogati et al. (2023) revealed an increased abundance of Bacillota and Actinomycetota phyla in response to drought-induced stress. Additionally, soil enzymes have been recognized as indicators of microbial activity that are highly sensitive to environmental factors, including drought conditions (Bogati et al. 2022). Moreover, the isolation of bacterial isolates through conventional methods is significantly influenced by the specific type of media used. The Nutrient Agar (NA) medium utilized in this investigation may not adequately meet the nutritional requirements of all bacterial species present in the rhizosphere of *V. nilotica*. Employing a variety of media can enhance the efficacy of culturing soil microorganisms. While metagenomic analysis has effectively identified the existence of numerous bacterial species from various

locations, a substantial proportion of these species remain difficult or even unfeasible to cultivate. Nevertheless, these bacteria are indeed engaged in metabolic activity and play vital roles in the ecosystem. The fact that most of these bacteria cannot be cultured does not imply that they cannot grow in laboratory conditions. Rather, it indicates that our understanding of their optimal growth conditions is incomplete (Chaudhary et al. 2019; Bodor et al. 2020).

All 26 bacterial isolates cultivated through conventional methods were capable of producing hydrolytic enzymes, including protease, lipase, amylase, and cellulase. Among these isolates, 9 showed the highest proficiency in producing hydrolytic enzymes. Interestingly, 8 of these isolates exhibited characteristics associated with the Bacillota phylum, while 1 isolate showed similar characteristics with the Acidobacteriota phylum. Two of the isolates had the ability to synthesize multiple enzymes, both sharing characteristics with the Bacillota phylum. According to many literature sources, soil bacteria capable of producing hydrolytic enzymes are found in the Pseudomonadota phylum (Rathakrishnan and Gopalan 2022), Actinomycetota (Miralles et al. 2021), Bacillota (Caballero et al. 2020), and Acidobacteriota (Coluccia and Besaury 2023).

Interestingly, the presence of rhizospheric microorganisms plays a crucial role in helping *Vachellia* adapt to environmental stress, possibly through Plant Growth-Promoting (PGP) bacteria that regulate stress-tolerance hormones (Elsheikh et al. 2021). Several bacterial species isolated from rhizosphere soil have been found to enhance plant tolerance to drought conditions. A study by Getahun et al. (2020) demonstrated that certain bacteria from the genera *Bacillus*, *Enterococcus*, and *Paenibacillus*, which belong to the phylum Bacillota, can improve the drought tolerance of *Acacia abyssinica.* This capability is achieved through various mechanisms, including the production of ACC deaminase (Camaille et al. 2021), the regulation of cytokinin and gibberellin hormones, and biofilm formation (Chieb and Gachomo 2023).

The association between next-generation sequencing data and the results from conventional enzymatic hydrolysis assays suggests that the dominant phylum plays a significant role in the activity of hydrolytic enzymes in the rhizosphere soil of *V. nilotica*. To our knowledge, no prior research has been conducted on this topic in the savanna area of BNP.

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