

Bioprospecting cultivable microorganisms from the Pantanal reveals taxonomic and functional enzymatic diversity

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Abstract

The Pantanal is one of the world's largest tropical wetlands, yet its cultivable microbial diversity remains underexplored for biotechnological applications. We bioprospected cultivable bacteria, filamentous fungi, and yeasts from environmental samples collected across 10 Pantanal sites and screened them for seven enzymatic activities. A total of 122 isolates were recovered, comprising 73 bacteria, 46 filamentous fungi, and 3 yeasts, representing 51 taxonomic groups and 37 enzymatic profiles. Protease activity was the most frequent among bacteria (71.2%), all filamentous fungi were cellulase-positive, and chitinase activity was detected in only five bacterial isolates. Taxonomic diversity among enzyme-positive isolates varied according to activity and sample origin, with Shannon–Wiener diversity index values ranging from 0 to 2.86. Significant positive associations were observed between protease–lipase, laccase–amylase, and protease–amylase, whereas no origin–activity association remained significant after multiple-testing correction. Phylogenetic analyses identified three particularly divergent or taxonomically unresolved bacterial lineages: P_1.1_002 within *Rothia*, P_13.1_002 within *Pseudomonas*, and P_10.3_001 within the order Enterobacterales. These findings reveal broad taxonomic and functional diversity among cultivable Pantanal microorganisms and identify lineages of interest for further taxonomic and biotechnological investigation.

Introduction

The Pantanal, one of the world's largest continental wetlands, is characterized by high biological diversity and habitat heterogeneity [1]. Located in central South America, the biome comprises a complex mosaic of aquatic and terrestrial environments, including rivers, lakes, seasonally flooded grasslands, savannas, and different forest formations, which support a remarkable diversity of organisms [1, 2]. The wide range of environmental conditions and vegetation physiognomies supports diverse plant and animal assemblages [3]. This environmental heterogeneity is closely linked to the hydrological processes that structure the Pantanal landscape [4].

Among these processes, seasonal hydrological dynamics play a central role in Pantanal ecosystem functioning and are characterized by alternating flood and dry periods [4, 5]. Across these cycles, the expansion and contraction of flooded areas alter connectivity among rivers, lakes, channels, and the floodplain, generating different hydrological conditions across the landscape [6, 7]. Owing to features such as the low topographic gradient, the complexity of the drainage system, and variation in flood duration and intensity, environments across the region are exposed to distinct hydrological regimes [8, 9]. The interaction of these features creates a landscape composed of environments subject to contrasting hydrological regimes and environmental conditions [6].

This diversity is particularly evident in Nhecolândia, a distinctive Pantanal subregion characterized by thousands of shallow lakes with contrasting hydrological and physicochemical properties [10]. Freshwater, oligosaline, and saline-alkaline lakes occur within a landscape associated with seasonally flooded drainage channels and sandy ridges [11, 12]. This combination of conditions increases the

diversity of ecological niches and may favor microorganisms with distinct physiological and metabolic traits [13].

Among these microorganisms, cultivable bacteria and fungi represent promising sources of enzymes with biotechnological potential, as their extracellular activities are involved in the utilization and transformation of different environmental substrates [14, 15]. Previous studies have provided an initial exploration of the enzymatic potential of microorganisms isolated from the Pantanal, supporting its potential as a still underexplored source of microbial resources of biotechnological interest [16–18]. Despite these advances, studies integrating different groups of cultivable microorganisms in a simultaneous and comprehensive screening of multiple enzymatic activities remain relatively scarce in the Pantanal, and this biotechnological potential therefore remains only partially characterized [17–19].

To address this knowledge gap, we bioprospected cultivable microorganisms from the Pantanal, focusing on their biotechnological potential. Bacterial, filamentous fungal, and yeast isolates were taxonomically identified through partial sequencing of the 16S rRNA gene, the internal transcribed spacer (ITS) region and additional taxonomic marker genes, as appropriate for each microbial group. A total of 122 isolates were qualitatively screened for seven enzymatic activities (amylolytic, cellulolytic, proteolytic, lipolytic, chitinolytic, ureolytic, and laccase activities) to identify microorganisms with diverse enzymatic profiles.

Results

Overview of sampling and taxonomic composition of cultivable isolates

Culture-based isolation yielded a total of 122 microbial isolates distributed across seven sample-origin categories (Supplementary Table S1). Bacteria represented 59.8% of the collection ($n = 73$), followed by filamentous fungi (37.7%; $n = 46$) and yeasts (2.5%; $n = 3$) (Fig. 2b). Soil and plant/root samples yielded the largest numbers of isolates ($n = 39$ and $n = 33$, respectively), followed by water ($n = 19$), termite/termite mound ($n = 16$), lichen ($n = 7$), feces ($n = 6$), and fungus/wood ($n = 2$). Yeasts were recovered only from plant/root ($n = 2$) and fecal ($n = 1$) samples. Overall, 51 taxonomic groups were identified among the cultivable isolates (Fig. 2a; Supplementary Table S1). *Penicillium* was the most frequently recovered genus ($n = 12$; 9.8%), followed by *Bacillus* ($n = 11$; 9.0%) and *Priestia* ($n = 10$; 8.2%). *Aspergillus* and *Exiguobacterium* were each represented by six isolates (4.9%), whereas *Cladosporium* and *Fusarium* accounted for five isolates each (4.1%).

The relative composition of taxonomic groups differed among sample origins (Fig. 2a). In water samples, *Penicillium* and *Cladosporium* each accounted for 15.8% of the isolates. In soil, *Bacillus* and *Priestia* represented 12.8% and 10.3%, respectively. In plant/root samples, *Exiguobacterium* accounted for 12.1% of the isolates, while *Priestia* and *Fusarium* each represented 9.1%. In termite/termite mound samples, *Bacillus* represented 37.5% and *Penicillium* 25.0% of the isolates. *Acinetobacter* accounted for

28.6% of lichen-derived isolates, whereas *Escherichia/Shigella* represented 33.3% of fecal isolates. The fungus/wood category comprised two isolates, assigned to *Pseudomonas* and *Trichoderma* (50.0% each). Taxonomic groups pooled as “Other taxa” accounted for 36.8% of water isolates, 48.7% of soil isolates, 45.5% of plant/root isolates, 18.8% of termite/termite mound isolates, 42.9% of lichen isolates, and 16.7% of fecal isolates.

Based on the predefined selection criteria, five bacterial isolates were subjected to 16S rRNA gene phylogenetic analysis: P_1.1_002, P_5.4_002, P_7.2_005, P_10.3_001, and P_13.1_002. Isolate P_5.4_002 showed 98.33% 16S rRNA gene sequence identity to its closest type-strain match, *Ectopseudomonas composti*, and was recovered as its sister lineage in the maximum-likelihood tree, although this relationship received low bootstrap support (50; Supplementary Fig. S1). Isolate P_7.2_005 showed 98.63% sequence identity to *Arthrobacter deserti* and clustered with *A. mobilis* and *A. deserti* in a strongly supported clade (bootstrap = 99); however, its direct sister relationship with *A. mobilis* received low support (bootstrap = 56), and its species-level placement remained unresolved (Supplementary Fig. S1). Among the remaining isolates, P_1.1_002, assigned to *Rothia* sp., showed 97.44% 16S rRNA gene sequence identity to *Rothia aerolata* and formed a well-supported sister relationship with this species (bootstrap = 97; Fig. 3a). Isolate P_13.1_002 was placed within a strongly supported *Pseudomonas* clade (bootstrap = 99; Fig. 3b), although its species-level position remained unresolved; its closest type-strain match was *Pseudomonas panipatensis*, with 98.22% sequence identity. In contrast, P_10.3_001 showed < 98% 16S rRNA gene sequence identity to its closest type-strain matches, which belonged to different genera within Enterobacterales. Consistent with the BLASTn results, the phylogenetic analysis did not support a robust affiliation of P_10.3_001 with any of the represented genera (Fig. 3c).

Enzymatic screening of cultivable isolates

The isolates were screened for seven enzymatic activities using agar-based assays: urease, protease, laccase, lipase, amylase, cellulase, and chitinase (Fig. 4a). Positive reactions for multiple activities were detected in both bacterial and filamentous fungal isolates, whereas fewer positive reactions were observed in yeasts. Under the assay conditions, chitinase activity was detected exclusively among bacterial isolates, with five of the 73 bacterial isolates showing a positive reaction (6.8%). No detectable chitinase activity was observed in filamentous fungi or yeasts. Therefore, this activity was analyzed separately, whereas the remaining six activities were included in comparative analyses across the microbial groups shown in Fig. 4b,c.

The frequency of positive reactions varied according to microbial group and enzymatic activity (Fig. 4b). Among bacterial isolates, protease was the most frequently detected activity (71.2%), followed by urease (47.9%), amylase (46.6%), lipase (34.2%), laccase (16.4%), and cellulase (13.7%). Among filamentous fungi, all isolates were positive for cellulase activity (100.0%), whereas urease and amylase activities were detected in 69.6% and 67.4% of isolates, respectively. Protease activity was detected in 50.0% of filamentous fungal isolates, while lipase and laccase activities were detected in 30.4% and 17.4%, respectively. In yeast isolates, only one showed urease activity (33.3%), and no positive reaction was

detected for the remaining activities. The number of positive activities per isolate ranged from 0 to 5 in bacteria and from 1 to 6 in filamentous fungi (Fig. 4c). The bacterial isolates positive for five activities were *Aeromonas* sp. P_5.1_001, *Priestia megaterium* P_8.2_002, and *Glutamicibacter endophyticus* P_8.2_003. Within the filamentous fungi, *Aspergillus* section *Terrei* P_8.1_007 and *Fusarium* sp. P_15.1_012 were positive for six of the seven activities evaluated. Bacterial isolates had a median of two positive activities per isolate, whereas filamentous fungi had a median of three. Among yeasts, the median number of positive activities was zero.

At the level of recurrent taxa, several groups showed consistent patterns of multiple activities (Supplementary Table S1). All *Bacillus* isolates were positive for protease (11/11), with urease detected in 9/11 and cellulase in 7/11. All *Priestia* isolates were positive for protease and amylase (10/10), and 8/10 were positive for laccase. All *Penicillium* isolates were positive for amylase and cellulase (12/12), whereas 7/12 were positive for protease. All *Aspergillus* isolates were positive for lipase, amylase, and cellulase (6/6). Likewise, all *Cladosporium* isolates were positive for protease and cellulase (5/5), all *Fusarium* isolates were positive for urease and cellulase (5/5), and all *Exiguobacterium* isolates were positive for protease and amylase (6/6).

The Shannon–Wiener diversity of final taxonomic assignments among enzyme-positive isolates varied across enzymatic activities and sample origins (Fig. 5), with H' values ranging from 0 to 2.86. The highest values were observed for protease-positive isolates from soil ($H' = 2.86$) and urease-positive isolates from plant/root samples ($H' = 2.73$), followed by protease in water and plant/root samples ($H' = 2.56$ for both), amylase in soil ($H' = 2.55$) and plant/root samples ($H' = 2.48$), and cellulase and urease in soil ($H' = 2.43$ for both). Chitinase-positive isolates were detected only in water ($H' = 0.69$) and plant/root samples ($H' = 1.10$), whereas laccase H' values ranged from 0 to 1.73, with the highest value observed in soil. In fungus/wood samples, urease showed $H' = 0.69$, while amylase and cellulase each showed $H' = 0$.

Analysis of associations among enzymatic activities identified three significant positive associations after Benjamini–Hochberg correction: protease–lipase (34 isolates; $\phi = 0.362$, $q = 0.0011$), laccase–amylase (17 isolates; $\phi = 0.282$, $q = 0.0217$), and protease–amylase (48 isolates; $\phi = 0.271$, $q = 0.0217$). When stratified by microbial group, bacteria showed significant associations between laccase–amylase ($\phi = 0.475$, $q = 0.0006$) and protease–amylase ($\phi = 0.351$, $q = 0.0413$), with all 12 laccase-positive bacterial isolates also testing positive for amylase. Among filamentous fungi, only the protease–lipase association remained significant ($\phi = 0.567$, $q = 0.0048$). No association between sample origin and enzymatic activity remained significant after correction for multiple comparisons, although the largest effect estimates were observed for cellulase among isolates from termites/termite mounds (13/16; 81.3%; $\phi = 0.276$, $q = 0.138$) and protease among water-derived isolates (17/19; 89.5%; $\phi = 0.247$, $q = 0.215$).

Analysis of each isolate across the seven enzymatic assays identified 37 distinct binary enzymatic profiles in the overall collection (Fig. 6; Supplementary Table S1). Bacterial isolates displayed 26 distinct

profiles, whereas 16 profiles were observed among filamentous fungi and two among yeasts. Only five profiles were shared by bacteria and filamentous fungi, whereas 19 occurred exclusively in bacteria and 11 exclusively in filamentous fungi. The distribution of positive activities per isolate also differed between the two major microbial groups. Among bacteria, 35 of 73 isolates (47.9%) were positive for at least three of the seven activities, 17 (23.3%) for at least four, and three (4.1%) for five activities. Among filamentous fungi, 33 of 46 isolates (71.7%) were positive for at least three activities, 18 (39.1%) for at least four, and 11 (23.9%) for five or more activities. Laccase-like activity occurred only in multienzymatic profiles: all 12 laccase-positive bacteria and all eight laccase-positive filamentous fungi displayed at least three additional activities. All five chitinase-positive bacteria were also positive for protease; four were additionally positive for urease and lipase, and two for amylase.

Discussion

The diversity of cultivable microorganisms recovered in this study reflects the diversity of the Pantanal microenvironments sampled and reinforces the potential of this biome as a source of microorganisms of biotechnological interest. Several identified taxa showed ecological correspondence with their environments of origin, including saline lake sites, plant- and lichen-associated substrates, termite-related materials, and aquatic environments.

At the saline lake sites, microorganisms previously described from environments characterized by salinity, alkalinity, or low water availability were recovered. *Luteimonas deserti*, isolated here from dry soil at saline lake 01, was originally described from desert soil and can grow across a broad pH range and at NaCl concentrations of up to 8% (w/v) [20]. Similarly, *Glutamicibacter endophyticus*, recovered from vegetated soil at Salina 2, has previously been isolated from halophytic plants and their rhizospheric soils in environments characterized by high salinity and saline–alkaline conditions [21, 22]. Other representatives recovered from the saline lake sites, including *Paracoccus aestuarii*, *Rosellomorea marisflavi*, *Aspergillus*, *Cladosporium*, and *Penicillium*, have also been reported from coastal or hypersaline environments, including tidal-flat sediments and waters, as well as solar salterns [23–25]. This correspondence is consistent with the possibility that the environmental conditions of the saline lake sites influenced the cultivable fraction recovered. However, their occurrence in these environments does not by itself allow them to be classified as halophilic or haloalkaliphilic, because the salt tolerance of the isolates obtained in this study was not experimentally evaluated.

The influence of microhabitat was also evident in biologically structured substrates. Lichens and bromeliads yielded microorganisms previously associated with these hosts, including representatives of *Pantoea*, *Acinetobacter*, and *Meyerozyma* [26–28]. Similarly, *Bacillus safensis*, recovered from termites in this study, has previously been isolated from the gut of these insects [29]. The occurrence of *Trichoderma* in termite-related materials and decomposing wood is consistent with its frequent occurrence in lignocellulose-rich environments, as its repertoire of enzymes involved in plant polysaccharide degradation is well documented [30, 31]. Comparable patterns were observed in aquatic environments and macrophyte root-associated samples. *Exiguobacterium indicum* has been reported as

a rhizobacterium of an aquatic macrophyte, whereas *Flectobacillus roseus* was originally described from freshwater environments, including a fish pond and a spring [32, 33]. These findings indicate that the cultivable diversity recovered does not simply reflect the broad environmental distribution of microorganisms but also includes taxa whose known ecology is compatible with specific characteristics of the sampled microhabitats.

From a functional perspective, enzymatic profiles did not show an equally direct relationship with environmental categories. Some trends were biologically plausible, such as the higher occurrence of cellulolytic activity among isolates from termites and termite mounds, because lignocellulose degradation by termites depends on a complex interaction between host enzymes and the associated microbiota [34, 35]. Likewise, the high occurrence of proteolytic activity among aquatic isolates is consistent with the role of extracellular peptidases in the transformation and utilization of proteinaceous organic matter in freshwater ecosystems [36]. However, these trends did not remain statistically significant after correction for multiple comparisons, indicating that sample origin alone does not explain the observed enzymatic repertoire. A similar lack of direct correspondence between environmental origin and enzymatic profile has also been observed in cultivable microorganisms from brackish, saline, and hypersaline lakes, where a broad diversity of hydrolytic activities was detected without significant differences in enzymatic profiles according to sample origin or sample type [37]. Thus, the functional diversity observed here likely reflects a combination of taxonomic composition, strain-level variation, and isolate-specific physiological characteristics.

Regarding the bacterial isolates selected for phylogenetic analysis, the combined BLAST and phylogenetic results revealed different degrees of taxonomic resolution among the isolates examined. Notably, P_5.4_002 and P_7.2_005 also showed 16S rRNA gene sequence identities below the proposed species-level threshold; however, their phylogenetic relationships were not sufficiently resolved to support a clear taxonomic interpretation based on 16S rRNA gene analysis alone. In contrast, P_1.1_002 showed a clear affiliation with *Rothia* but remained sufficiently divergent from its closest type strain to warrant further investigation as a potentially distinct lineage within the genus. Likewise, P_13.1_002 was consistently placed within *Pseudomonas*, while its unresolved relationship with the closest described species supports its consideration as a possible novel species-level lineage. P_10.3_001 showed an even more distinctive pattern, as both sequence similarity and phylogenetic placement failed to associate it robustly with any of the closely related Enterobacterales genera examined, suggesting a potentially distinct taxonomic lineage. Although additional analyses, particularly genomic characterization, are required for formal taxonomic confirmation, the combined BLAST and phylogenetic evidence obtained in this study supports the possibility that P_1.1_002, P_13.1_002, and P_10.3_001 represent distinct bacterial lineages.

From a bioprospecting perspective, the frequent occurrence of multienzymatic profiles was one of the most relevant findings. Microorganisms capable of expressing different hydrolytic or oxidative activities may be advantageous for applications involving complex matrices, and environmental isolates combining amylase, protease, lipase, and cellulase activities have been considered promising for waste

processing and bioremediation of complex organic materials [38–40]. In the present study, this multienzymatic potential was further supported by significant associations among some activities, particularly protease–lipase and protease–amylase. The different association patterns observed between bacteria and filamentous fungi also suggest that these combinations reflect distinct functional repertoires within the collection rather than a uniform pattern across microbial groups.

Complementarily, Shannon–Wiener diversity showed that these enzymatic capacities differed in their taxonomic breadth: protease, urease, amylase, and cellulase were represented across a wider range of taxonomic assignments, whereas chitinase and laccase were concentrated in fewer groups. Although some of the highest H' values occurred in soil, water, and plant/root samples, no association between sample origin and individual enzymatic activities remained significant after correction for multiple comparisons, indicating that the broader taxonomic representation observed in these origins should not be interpreted as evidence of origin-specific functional enrichment. Together, these results indicate that, among the cultivable isolates examined in this study, some enzymatic functions were both widely distributed taxonomically and frequently combined within individual isolates, while others were more restricted. These associations should be interpreted as evidence of multiple functional capacities within the collection rather than as evidence of co-regulation or molecular dependence among the enzymes.

Taxonomic assignments revealed isolates with distinctive enzymatic phenotypes. Among these, *Exiguobacterium indicum* has previously been associated with the production of several hydrolytic enzymes, including proteases, amylases, and lipases, highlighting its considerable enzymatic versatility [38, 41, 42]. However, experimental evidence of chitinolytic activity in *E. indicum* remains limited. This finding is particularly interesting because chitinase was one of the least frequent activities in the collection. The *Flectobacillus roseus* isolate exhibited proteolytic and lipolytic activities. In the original description of the species, however, the detection of activities related to proteolysis and lipolysis varied depending on the assay and substrate used, with negative results reported for protease activity, skim-milk hydrolysis, lipase activity on corn oil, and Tween hydrolysis, but positive reactions for trypsin, α -chymotrypsin, and esterase-lipase [32]. *Glutamicibacter endophyticus* displayed cellulase, amylase, and protease activities, which have previously been described for the species [21, 22], together with laccase-like activity, which appears to be less well documented. The isolate assigned to *Pantoea stewartii* also showed a phenotype distinct from previous reports, with ureolytic activity [43]. These differences may reflect strain-level variability, cultivation conditions, or the limited discriminatory power of the 16S rRNA gene among closely related bacterial species.

The diversity of profiles observed among the isolates highlights the value of strain-level bioprospecting, as microorganisms belonging to the same species may display distinct enzymatic repertoires. This intraspecific variability broadens the potential for discovering phenotypes of interest and reinforces the importance of evaluating individual isolates when selecting promising candidates for biotechnological applications. In this context, the qualitative solid-medium screening used in this study enabled the identification of isolates with different enzymatic profiles and the selection of candidates of particular interest for subsequent enzyme characterization and production studies. Moreover, considering that

microbial enzyme production and activity can be influenced by salinity, isolates originating from distinct environments, such as saline lakes, represent particularly interesting candidates for future investigations of enzyme production under different NaCl concentrations and of their halotolerant potential [37].

An important limitation of this study is that culture-dependent approaches capture only a fraction of the environmental microbial diversity. The isolation media and incubation conditions used here may have favored microorganisms adapted to the selected cultivation conditions, while excluding taxa requiring specific nutrients, physicochemical conditions, or interspecies interactions. Therefore, the taxonomic composition reported here should be interpreted as the cultivable fraction recovered under the experimental conditions rather than as a representation of the total microbial community. Because sampling was conducted during a single campaign under predominantly dry conditions, the recovered cultivable diversity may also reflect seasonal and interannual effects. Sampling across contrasting hydrological periods would be valuable for determining the temporal stability of the observed patterns.

Overall, our results demonstrate that distinct Pantanal microhabitats harbor a diverse cultivable microbial fraction with substantial functional heterogeneity. The recovery of 122 isolates representing 51 taxonomic groups and 37 enzymatic profiles highlights the value of culture-dependent bioprospecting for exploring the functional diversity of microorganisms from this biome. Several isolates displayed multienzymatic phenotypes, while selected strains showed poorly documented enzymatic activities or relatively low sequence similarity to their closest reference taxa, supporting their prioritization for future taxonomic, biochemical, and biotechnological characterization. In addition, several isolates recovered in this study represent promising candidates for further functional characterization and evaluation in enzyme-based biotechnological applications and bioremediation processes.

Methods

Study area and sampling

Environmental samples were collected on 5 and 6 November 2025 at 10 sampling sites in the Pantanal region, Mato Grosso do Sul, Brazil (Fig. 1; Table 1). Sampling was conducted in early November to capture predominantly dry-season conditions, when reduced precipitation and water availability generate contrasting physicochemical conditions across Pantanal microhabitats. This period was selected to capture microorganisms potentially adapted to relatively dry and, at some sites, saline or alkaline conditions. During the sampling campaign, maximum temperatures ranged from 33 to 37°C and relative humidity ranged from 15 to 30% during the hottest hours of the day. Accumulated precipitation in the Pantanal region in early November was substantially below the historical average for the period. Maps were generated in R (version 4.6.1; R Foundation for Statistical Computing, Vienna, Austria; <https://www.R-project.org/>) using the *geobr* package (version 1.9.1) to obtain the geographic base layers and the *ggspatial* package (version 1.1.10) to display and annotate the sampling locations.

Table 1
Sampling points, geographic coordinates, and collected materials in the Pantanal, Mato Grosso do Sul, Brazil.

Sampling point	Sample ID	Collected material	Sample origin	Latitude (°S)	Longitude (°W)
P1	P1.1	Water	Water	19.53786	56.02791
P5	P5.1	Water from Salina 1	Water	19.88176	56.10139
P5	P5.2	Soil 1 from Salina 1 (dry soil)	Soil	19.61131	56.69122
P5	P5.3	Soil 2 from Salina 1 (intermediate-moisture soil)	Soil	19.13208	56.6789
P5	P5.4	Soil 3 from Salina 1 (wettest soil)	Soil	19.17988	56.23628
P7	P7.1	Bromeliad	Plant/Root	19.1602	56.71687
P7	P7.2	Lichen	Lichen	19.54885	56.03405
P7	P7.3	Soil 4 (sandy soil)	Soil	19.98572	56.81905
P8	P8.1	Soil 5 from Salina 2 (soil without vegetation)	Soil	19.54512	56.02181
P8	P8.2	Soil 6 from Salina 2 (soil with vegetation)	Soil	19.54523	56.02228
P9	P9.1	Termite specimen	Termite/Mound	19.55575	56.01959
P9	P9.2	Termite mound	Termite/Mound	19.55575	56.01959
P10	P10.1	Water	Water	19.58069	56.00647
P10	P10.2	Aquatic macrophyte root in soil	Plant/Root	19.58069	56.00647
P10	P10.3	Aquatic macrophyte root in water	Plant/Root	19.58069	56.00647
P11	P11.1	Cow feces	Feces	19.5812	56.00567
P13	P13.1	Soil 8 from the dry Vermelhinho riverbed, with vegetation	Soil	19.59626	55.99172
P14	P14.1	Fungus associated with a decomposing tree trunk	Fungi/Wood	19.58242	55.99168
P15	P15.1	Root/water suspension	Plant/Root	19.59045	56.00357

Samples included water, soils with different characteristics, swabs collected from plant and root surfaces, lichen, termites and termite-mound material, bovine feces, and fungal material associated with decomposing wood. Several sampling sites included more than one material type, enabling the recovery of microorganisms from different environmental substrates within the study area. Geographic coordinates and detailed descriptions of the materials collected at each sampling site are provided in Table 1. Microorganisms associated with plant and root surfaces were sampled in the field using sterile swabs. Only the swabs were transported to the laboratory for microbial isolation. Soil samples were collected from approximately 0–10 cm depth, and surface-water samples were transferred to sterile polypropylene containers. Biological substrates were collected manually using sterile instruments. Samples were transported in insulated containers at approximately 4°C until laboratory processing.

Microbial isolation

Microorganisms were isolated by serial dilution of environmental material in sterile 0.85% (w/v) NaCl solution. For filamentous fungi and yeast, 0.1-mL aliquots of 10^{-1} to 10^{-4} dilutions were plated on potato dextrose agar (PDA) supplemented with kanamycin ($50 \mu\text{g mL}^{-1}$). For bacteria, 10^{-4} to 10^{-6} dilutions were plated on Luria–Bertani (LB) agar supplemented with nystatin (0.1%, v/v; $100,000 \text{ IU mL}^{-1}$). Plates were incubated at 28°C for up to 7 days, and morphologically distinct colonies were subcultured to obtain pure cultures.

Molecular identification and taxonomic assignment

DNA extraction and Sanger sequencing were performed by GoGenetic (Brazil). Genomic DNA was extracted from bacterial colonies or fungal mycelia using the Bacterial DNA Extraction Kit (Loccus) or the Extracta Kit DNA and RNA Viral Fast (Loccus), as appropriate. PCRs were performed using GoTaq® G2 DNA Polymerase (Promega) according to the manufacturer's instructions. The bacterial 16S rRNA gene was amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') [44, 45], whereas the internal transcribed spacer (ITS) region of filamentous fungi and yeasts was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [46]. For selected bacterial isolates requiring greater taxonomic resolution, *rpoB* and/or *gyrB* were also analyzed when available (Supplementary Table S1). The *rpoB* gene was amplified using primers *rpoB*-F (5'-ATCGAAACGCCTGAAGGTCCAAACAT-3') and *rpoB*-R (5'-ACACCCTTGTTACCGTGACGACC-3'), as described by Mohkam et al. [47], whereas *gyrB* was amplified using the degenerate primers UP-1 (5'-GAAGTCATCATGACCGTTCTGCAYGCNNGGNGNAARTTYGA-3') and UP-2r (5'-AGCAGGGTACGGATGTGCGAGCCRTCACRTCNCGTCAT-3'), described by Yamamoto and Harayama [48].

PCR products were verified by electrophoresis on 1% agarose gels, purified using Exonuclease I and FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific), and sequenced using BigDye™ Terminator v3.1 on a 3500 Genetic Analyzer (Thermo Fisher Scientific). Electropherograms were processed using Sequencing Analysis v5.4, low-quality regions were trimmed using a Phred quality

threshold of > 25, and forward and reverse reads were assembled into consensus sequences using CAP3 [49]. Consensus sequences were compared with reference nucleotide sequences using BLASTn. Bacterial 16S rRNA, *rpoB*, and *gyrB* sequences were compared against the NCBI nucleotide database, whereas ITS sequences from filamentous fungi and yeasts were compared against the NCBI and UNITE databases [50–52].

Bacterial isolates were selected for phylogenetic analysis considering three complementary criteria: (i) 16S rRNA gene sequence identity < 98.65% to the closest type strain, a threshold proposed for preliminary species-level differentiation [53]; (ii) ambiguous or inconclusive BLAST-based taxonomic assignments; and (iii) availability of 16S rRNA gene sequences > 1,000 bp, providing broader sequence coverage for phylogenetic inference. Closely related type-strain sequences were retrieved from NCBI, aligned using MUSCLE [54], and analyzed in MEGA 12 [55]. The best-fitting nucleotide substitution model was selected individually for each dataset according to the lowest Bayesian Information Criterion (BIC), and maximum-likelihood trees were reconstructed using partial deletion with a 95% site-coverage cutoff. Branch support was assessed using the adaptive bootstrap procedure, and trees were rooted with a related taxon outside the focal group as an outgroup.

Enzymatic screening assays

Inoculum preparation

Bacterial and yeast isolates were cultured in 20 mL of LB broth at 28°C with orbital shaking at 200 rpm for approximately 18–24 h. Cultures were inoculated onto the respective solid screening media. Filamentous fungi were screened according to the sporulation capacity of each isolate. For sporulating strains, 10 µL of a spore suspension was spot-inoculated onto the screening media. For non-sporulating isolates, mycelial plugs obtained from cultures preserved by the Castellani method were transferred directly to the center of the plates. All assays were performed at 28°C for 7 days under controlled conditions. Each assay was performed in biological triplicate. Positive activity was assessed qualitatively based on the formation of substrate-degradation halos, precipitates, or diagnostic color changes, depending on the assay.

Urease activity

Ureolytic activity was screened on mineral urea agar adapted [56]. The basal medium contained (g L⁻¹): peptone (1.5), beef extract (0.9), NaCl (0.6), CaCl₂ (2.8), agar (15), and phenol red (0.012). After autoclaving the mineral base (121°C, 15 min), 2% (v/v) urea sterilized by filtration through a 0.22-µm membrane was aseptically added. Enzymatic activity was qualitatively determined by a color change in the indicator resulting from an increase in medium pH.

Protease activity

Proteolytic activity was screened on a selective medium adapted [57]. The basal medium contained (g L⁻¹): casein (0.5), yeast extract (0.25), dextrose (0.1), and agar (15). After autoclaving (121°C, 15 min), the medium was aseptically supplemented with 1% (v/v) sterile skim milk. Proteolytic activity was qualitatively identified by the formation of translucent hydrolysis halos around colonies, indicating casein degradation.

Lipase activity

Lipolytic activity was screened on a solid medium adapted [58], containing (g L⁻¹): peptone (10), NaCl (5), CaCl₂·2H₂O (0.1), and agar (15). After autoclaving (121°C, 15 min) and cooling to 50°C, the medium was aseptically supplemented with 1% (v/v) sterile Tween 80. Enzymatic activity was qualitatively identified by the formation of opaque precipitation halos around colonies, resulting from complexation of calcium salts with fatty acids released by substrate hydrolysis.

Laccase activity

Laccase screening used different indicator media for bacteria and fungi. The bacterial medium, adapted [59], contained (g L⁻¹): glucose, 1; yeast extract, 5; K₂HPO₄, 1; MgSO₄·7H₂O, 0.2; NaCl, 2; CaCl₂, 0.1; CuSO₄·5H₂O, 0.01; and agar, 15. The pH was adjusted to 7.0, and guaiacol was added at 10 mM as a chromogenic substrate. For fungal screening, a medium adapted [60] contained (g L⁻¹): PDA, 39; peptone, 1; NH₄Cl, 4; K₂HPO₄, 1; FeSO₄, 0.01; NaCl, 0.1; CaCl₂, 0.1; MgSO₄·7H₂O, 0.5; and CuSO₄, 0.03, supplemented with 0.2 g L⁻¹ ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)]. Development of a brown coloration around bacterial growth on guaiacol medium or a blue-green/purple coloration around fungal growth on ABTS medium was considered a positive reaction.

Amylase activity

Amylolytic activity was screened on starch medium adapted [61, 62] containing, per liter: 10.0 g soluble starch (1%, w/v), 0.40 g K₂HPO₄, 0.10 g NH₄Cl, 0.10 g MgSO₄·7H₂O, 0.066 g CaCl₂·2H₂O, 0.5 g yeast extract, and 15.0 g agar; the final pH was adjusted to 6.8. After incubation, each 100-mm plate was flooded with 3 mL of Lugol's iodine solution [63]. A clear zone around microbial growth against the dark iodine-stained background was considered a positive reaction for amylase.

Cellulase activity

Cellulolytic activity was screened on carboxymethylcellulose (CMC) agar adapted [61] containing, per liter: 10.0 g CMC (1%, w/v), 0.40 g K₂HPO₄, 0.10 g NH₄Cl, 0.05 g MgSO₄, 0.05 g CaCl₂, 0.5 g yeast extract, and 15.0 g agar; the final pH was 6.8. After incubation, plates were flooded with 0.1% (w/v) Congo red for 15 min; the dye was removed and plates were washed with 1 M NaCl for 15–20 min. Clear zones around microbial growth were considered positive for extracellular cellulolytic activity [64].

Chitinase activity

Colloidal chitin was prepared by acid treatment based on the classical method [65, 66], with modifications. Commercial chitin (10 g) was dispersed in 160 mL concentrated HCl (37%, w/w) in an ice

bath with intermittent mixing for 60 min. The mixture was precipitated into 2 L of ice-cold distilled water under constant stirring for 20 min. The precipitate was recovered by centrifugation (3,800 rpm, 10 min, 10°C) and repeatedly washed with distilled water until pH 3.4–3.6 was reached. Colloidal chitin was sterilized at 121°C for 20 min and stored at 4°C. The chitinolytic screening medium [67] contained (g L⁻¹): colloidal chitin, 10.0 (1%, w/v); KH₂PO₄, 3.0; K₂HPO₄, 1.0; NH₄Cl, 0.5; NaCl, 0.5; MgSO₄·7H₂O, 0.12; yeast extract, 0.5; and agar, 15.0; the pH was adjusted to 6.8. Formation of a translucent hydrolysis zone around microbial growth was considered positive.

Statistical analysis of enzymatic activities and taxonomic diversity

Taxonomic diversity among enzyme-positive isolates was quantified separately for each enzymatic activity and sample-origin category using the Shannon–Wiener index [68]. For each enzymatic activity *i* and sample-origin category *g*, positive isolates were grouped according to their final taxonomic assignment. The relative frequency of each taxonomic assignment was calculated as:

$$p_{jig} = \frac{n_{jig}}{N_{ig}}$$

1

where p_{jig} is the relative frequency of taxonomic assignment *j*, n_{jig} is the number of positive isolates belonging to taxonomic assignment *j*, and N_{ig} is the total number of isolates positive for enzymatic activity *i* within sample-origin category *g*. Taxonomic diversity was then calculated as [69]:

$$H'_{ig} = - \sum_{j=1}^{S_{ig}} p_{jig} \ln(p_{jig})$$

2

where S_{ig} is the number of distinct final taxonomic assignments represented among positive isolates for enzymatic activity *i* within sample-origin category *g*. Natural logarithms were used. Combinations with no positive isolates were not assigned an H' value, whereas $H' = 0$ indicated that all positive isolates belonged to a single taxonomic assignment. Because final taxonomic assignments included different levels of taxonomic resolution, H' was interpreted as the diversity of taxonomic assignments among enzyme-positive isolates rather than as species diversity.

Enzymatic activities were treated as binary variables (positive/negative). Associations among the seven enzymatic activities were evaluated using 2 × 2 contingency tables, the two-sided Fisher's exact test [70], and the phi coefficient (ϕ) as a measure of association strength. Analyses were performed for the complete dataset and separately for bacteria and filamentous fungi. Multiple comparisons were corrected using the Benjamini–Hochberg procedure to control the false discovery rate (FDR) [71], with the correction applied separately to each dataset.

Associations between sample origin and each enzymatic activity were also evaluated using 2×2 contingency tables, comparing each origin category with all remaining origins combined. The Benjamini–Hochberg correction was applied across all origin–activity comparisons, and adjusted q values < 0.05 were considered statistically significant. Statistical analyses were performed in Python using SciPy [72].

Generative AI statement

ChatGPT (OpenAI) was used for English-language refinement and editorial assistance during manuscript preparation. All AI-assisted content was critically reviewed and verified by the authors, who take full responsibility for the scientific content, analyses, interpretations, and conclusions presented in this manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Author Contribution

Conceptualization, K.S.G., J.C.M.G., and D.S.S.; methodology, M.L.L.M., G.M.M., and K.S.G.; investigation, M.L.L.M., G.M.M., and K.S.G.; collection of materials, B.C.S.C., K.G.F.B., D.C.V., D.S.S., J.C.M.G., and K.S.G.; data curation, M.L.L.M., and K.S.G.; writing - original draft preparation, M.L.L.M.; writing - review and editing, K.S.G., D.S.S., J.C.M.G.; supervision, K.S.G., J.C.M.G., L.C., L.A.O and D.S.S.; resources, L.C., L.A.O and K.S.G; All authors have read and agreed to the published version of the manuscript.

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Data Availability

All data supporting the findings of this study are available within the article and its Supplementary Information. Isolate-level metadata, taxonomic assignments, and qualitative enzymatic screening results

are provided in Supplementary Table S1. The microbial isolates obtained were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGen) under access code A0CF9FB. Nucleotide sequences generated in this study have been deposited in GenBank, with the corresponding accession numbers provided in Supplementary Table S1.

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Figures

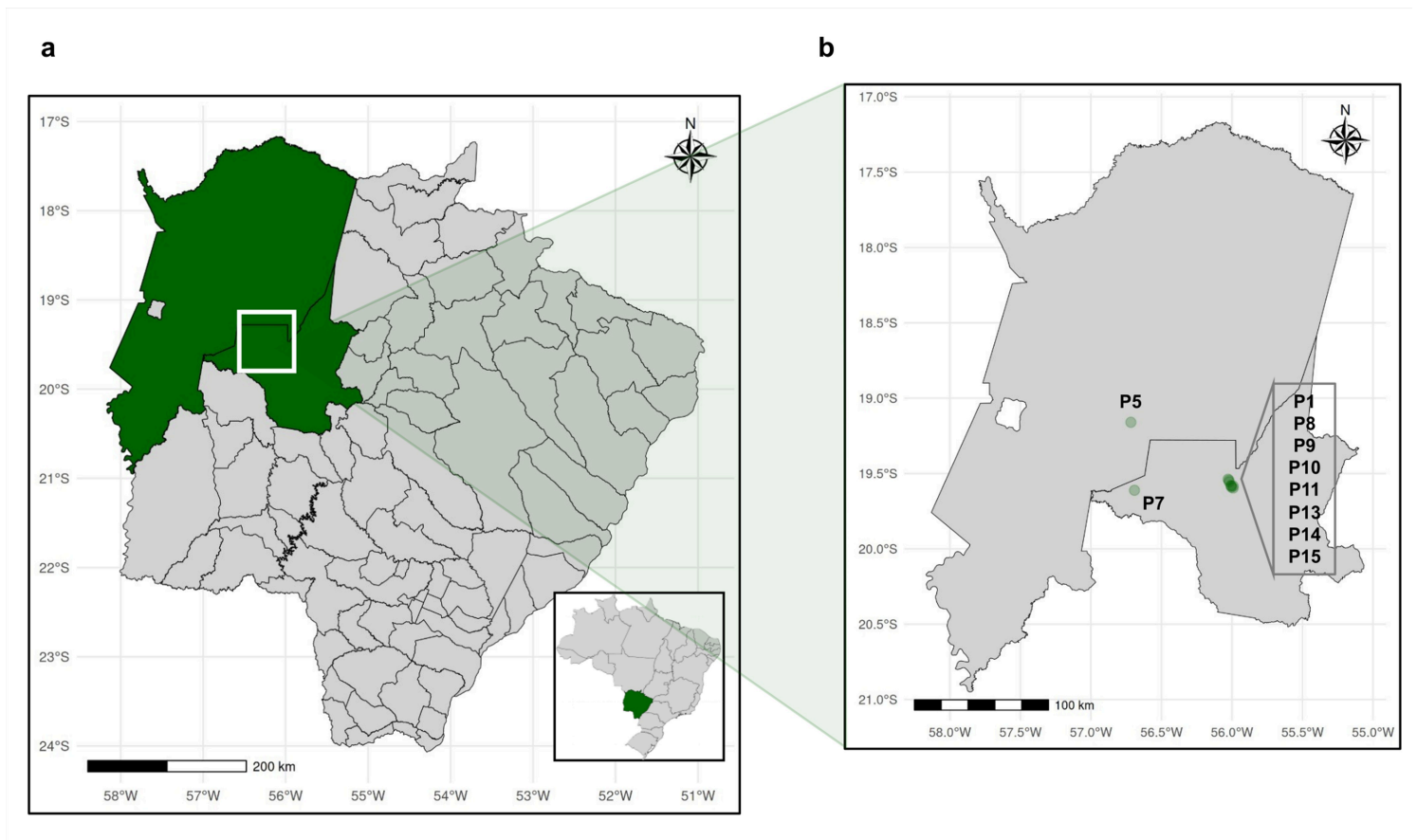


Figure 1

Location of the study area and sampling points in the Pantanal, Mato Grosso do Sul, Brazil. a, Geographic location of the study area in Mato Grosso do Sul, with the sampling region indicated by the dashed box. The inset map shows the location of Mato Grosso do Sul within Brazil. **b,** Detailed view of the study area showing the sampling points (P1, P5, P7, P8, P9, P10, P11, P13, P14, and P15). Geographic coordinates and the materials collected at each sampling point are provided in Table 1.

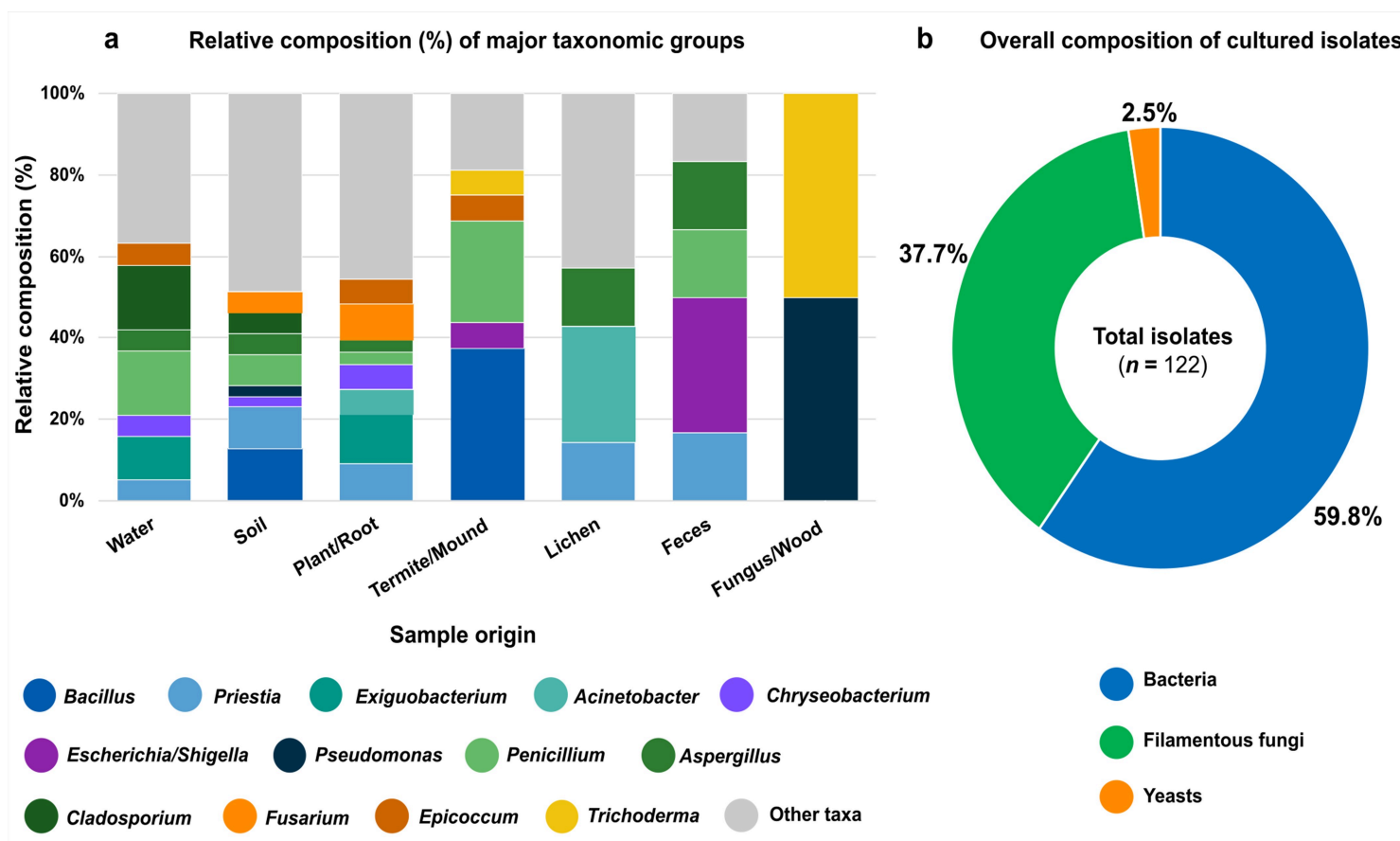


Figure 2

Taxonomic composition of cultivable microbial isolates across sample origins. (a) Relative composition of the major taxonomic groups within each sample origin. Taxonomic groups represented by at least four isolates in the overall collection or accounting for $\geq 25\%$ of the isolates within at least one sample origin are shown individually; the remaining groups were pooled as “Other taxa”. (b) Overall distribution of the 122 cultivable isolates among bacteria ($n = 73$; 59.8%), filamentous fungi ($n = 46$; 37.7%), and yeasts ($n = 3$; 2.5%).

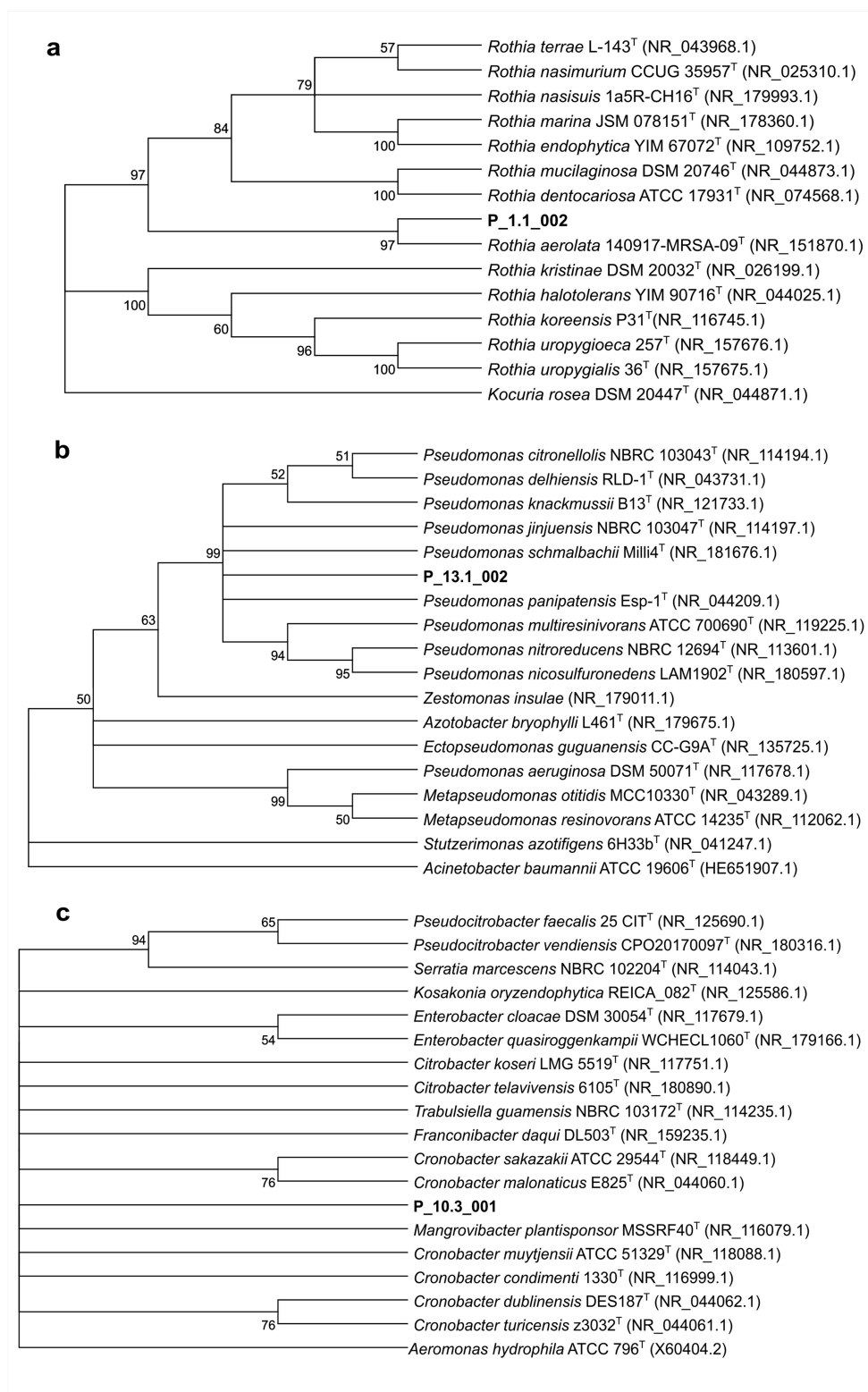


Figure 3

Maximum-likelihood phylogenetic placement of selected bacterial isolates based on 16S rRNA gene sequences. Phylogenetic trees showing the placement of (a) P-1.1-002 within *Rothia*, (b) P-13.1-002 within *Pseudomonas* and related genera, and (c) P-10.3-001 among representative genera of Enterobacterales. Trees were reconstructed using the maximum-likelihood method in MEGA 12, with the best-fitting nucleotide substitution model selected independently for each dataset according to the

lowest Bayesian Information Criterion (BIC). Branch support was assessed using the adaptive bootstrap procedure, and values at the nodes indicate bootstrap support (%). Positions with less than 95% site coverage were excluded using partial deletion. Trees were rooted using *Kocuria rosea* DSM 20447 (a), *Acinetobacter baumannii* ATCC 19606 (b), and *Aeromonas hydrophila* ATCC 796 (c) as outgroups. Isolates obtained in the present study are indicated in bold. Accession numbers of reference sequences are shown in parentheses.

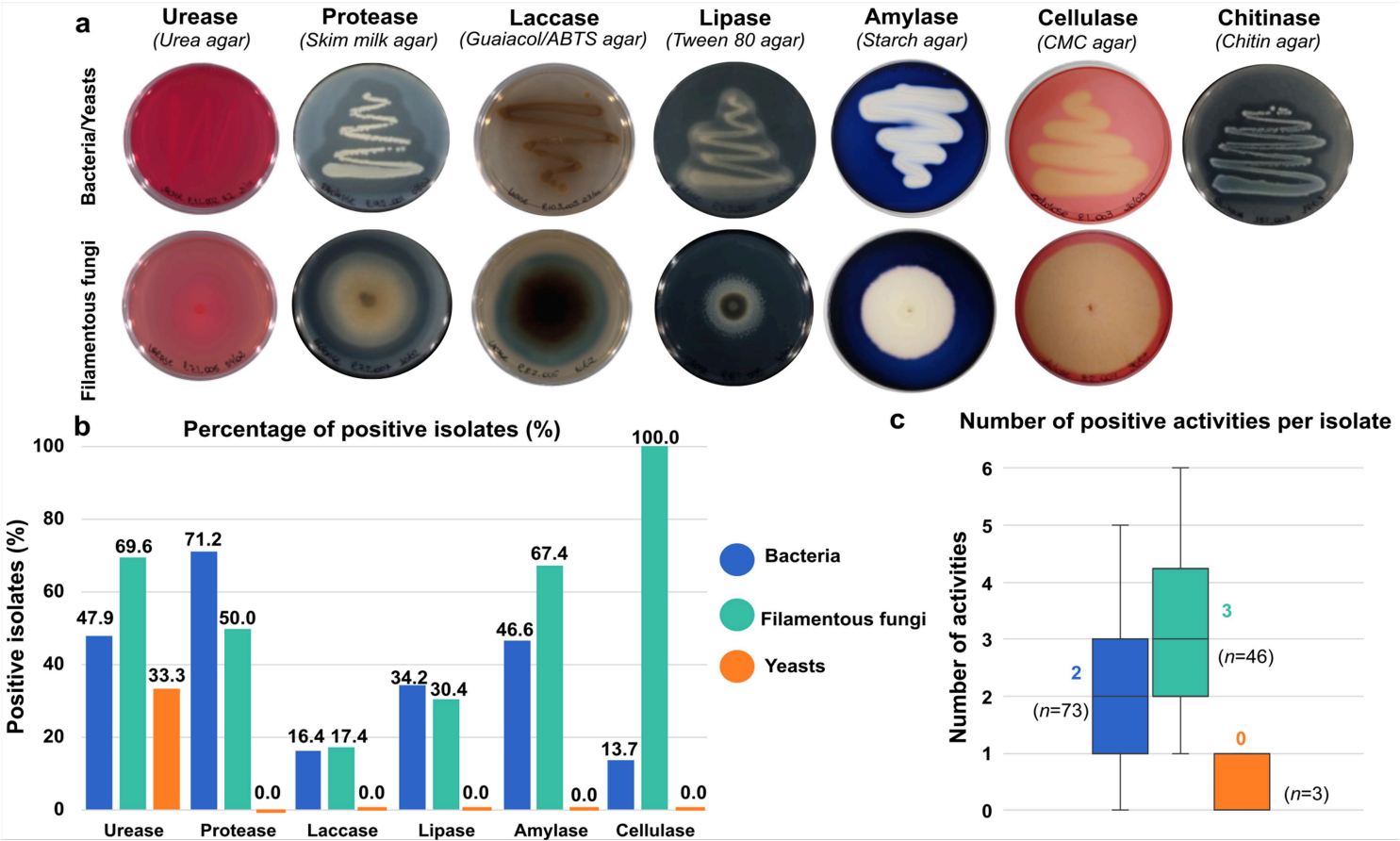


Figure 4

Enzymatic screening of cultivable microbial isolates. **a**, Representative reactions observed in agar-based screening assays for urease (urea agar), protease (skim milk agar), laccase (guaiacol and ABTS agar), lipase (Tween 80 agar), amylase (starch agar), cellulase (CMC agar), and chitinase (chitin agar) activities in bacterial, yeast and filamentous fungal isolates. Under the assay conditions and media tested, detectable chitinase activity was observed only among bacterial isolates. **b**, Percentage of bacterial, filamentous fungal, and yeast isolates showing positive reactions for the six enzymatic activities included in the comparative analysis (urease, protease, laccase, lipase, amylase, and cellulase). **c**, Distribution of the number of positive activities per isolate considering the same six enzymatic assays. Numbers adjacent to the boxes indicate median values; boxes represent the interquartile range and whiskers indicate the distribution beyond the quartiles. Chitinase activity was analyzed separately and was not included in the comparative summaries shown in panels **b** and **c**.

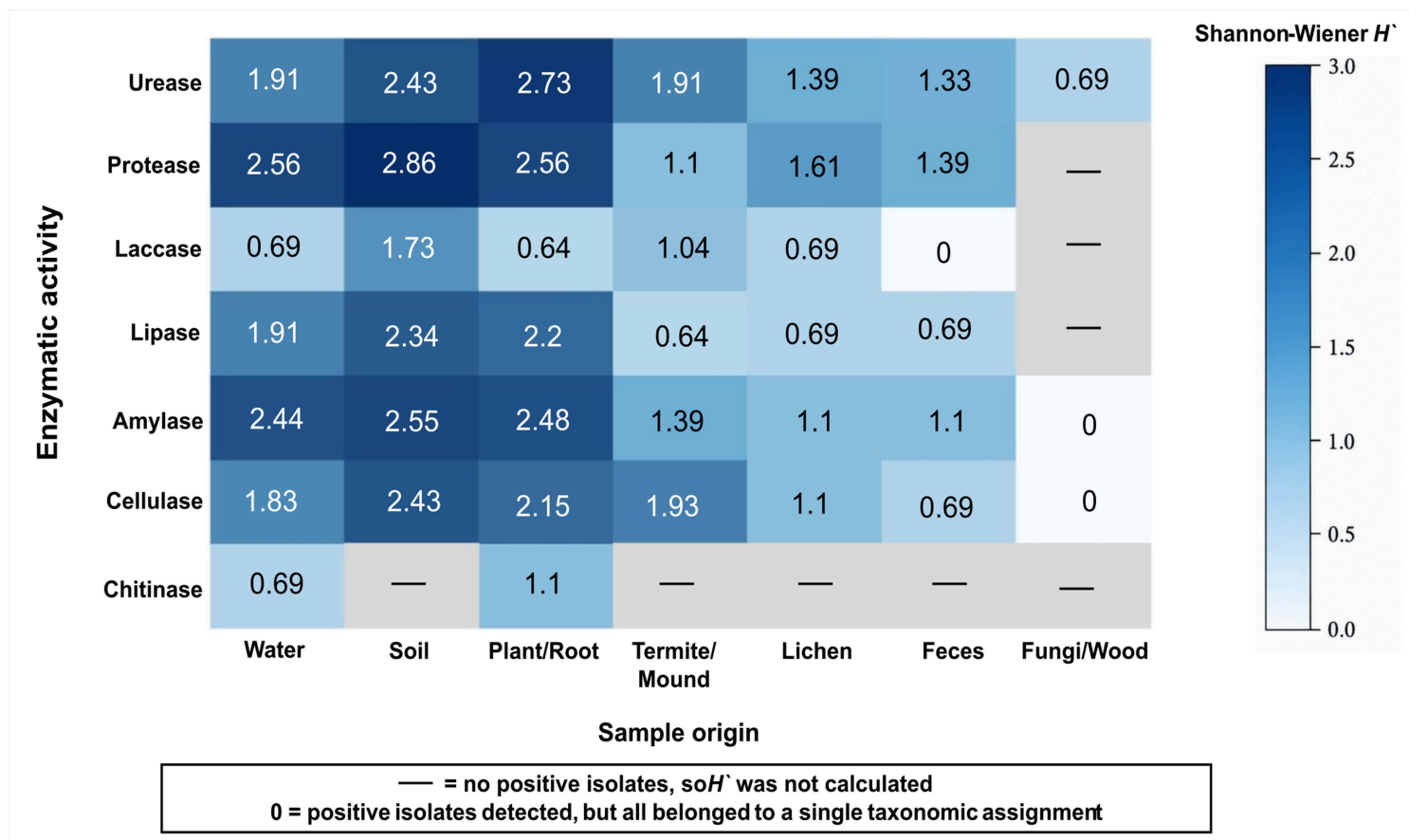


Figure 5

Taxonomic diversity of enzyme-positive isolates across sample origins. Shannon–Wiener diversity (H') was calculated from the distribution of final taxonomic assignments among isolates positive for each enzymatic activity within each sample-origin category. Higher H' values indicate a broader taxonomic distribution of the corresponding enzymatic activity. Color intensity represents H' values ranging from 0 to 3. Cells marked with a dash (—) indicate combinations with no positive isolates, for which H' was not calculated, whereas $H' = 0$ indicates that all positive isolates belonged to a single taxonomic assignment.

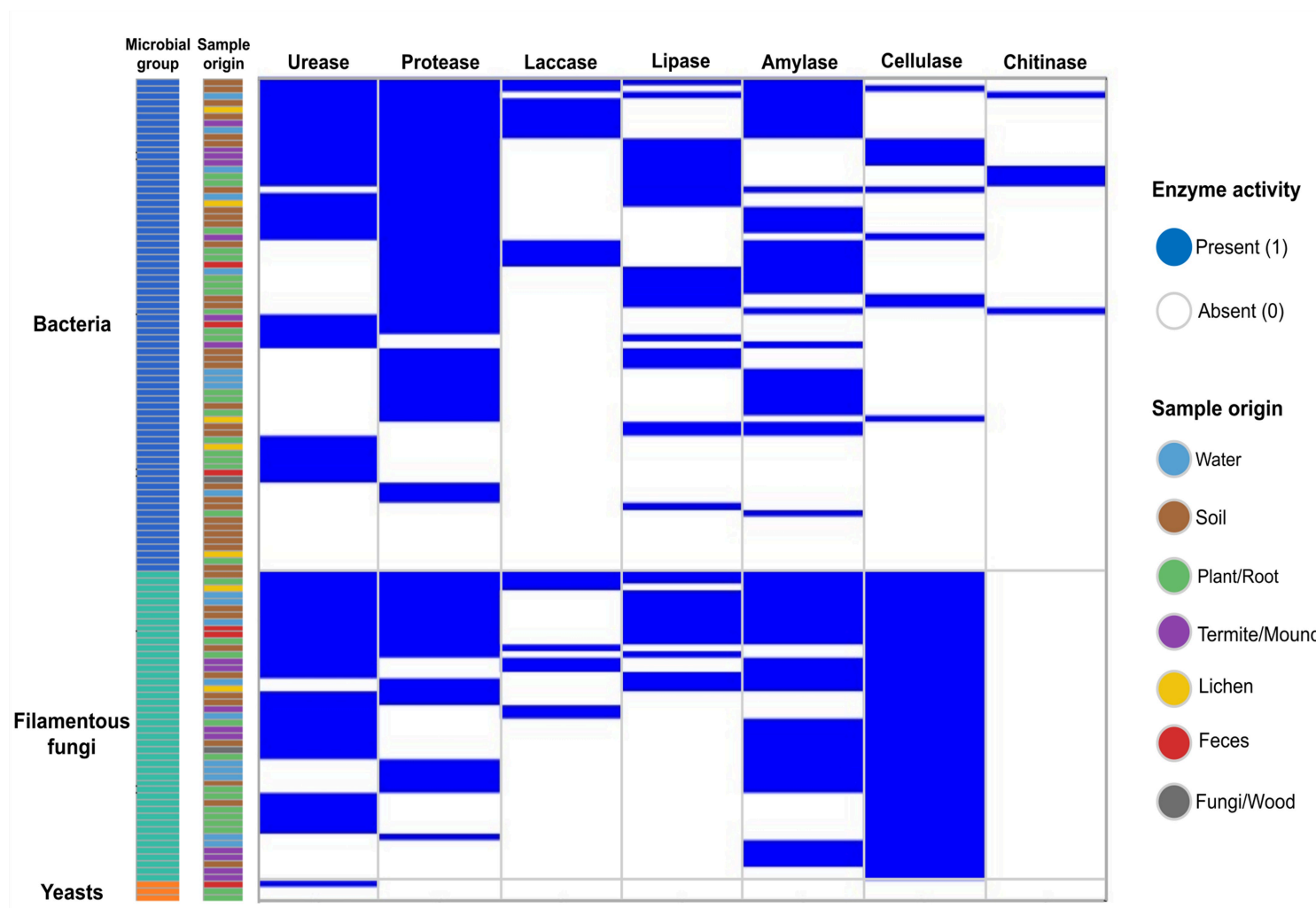


Figure 6

Isolate-level enzymatic profiles of cultivable microbial isolates. Binary heatmap showing the presence (blue; 1) or absence (white; 0) of detectable urease, protease, laccase, lipase, amylase, cellulase, and chitinase activities among the 122 isolates. Rows represent individual isolates grouped as bacteria ($n = 73$), filamentous fungi ($n = 46$), and yeasts ($n = 3$). The annotation bars on the left indicate the microbial group and sample origin of each isolate. Sample origins comprise water, soil, plant/root, termite/mound, lichen, feces, and fungus/wood. Chitinase activity is included in the heatmap for descriptive isolate-level visualization; under the assay conditions and media tested, positive chitinase reactions were detected only in five bacterial isolates and were not detected among filamentous fungi or yeasts.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableS1.xlsx](#)
- [SupplementaryInformation.pdf](#)