SCIENTIA PLENA
www.scientiaplena.org.br
VOL. 19, NUM. 04 2023
doi: 10.14808/sci.plena.2023.046201

Enzymatic and antimicrobial activity of microorganisms isolated from a cave in the Amazon region

Atividade enzimática e antimicrobiana de microrganismos isolados de uma caverna da região Amazônica

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(Recebido em 11 de novembro de 2022; aceito em 24 de março de 2023)

Cave microorganisms, mainly in iron lithology, are poorly known. These constitute most of the cave's biodiversity and play a key role in maintaining the cave ecosystem. The isolation and characterization of the cave microbiota can lead to identifying new species and obtaining substances of biotechnological interest. This work aimed to isolate and evaluate the enzymatic and antimicrobial activity of microorganisms isolated from the cave soil of Parque Nacional dos Campos Ferruginosos National Park, southeastern Amazon. Soil samples were collected from the inner surface layer of cave GEM-1462 in different zones (aphotic, dysphotic and photic). To characterize the strains, the Gram stain technique and biochemical test were used. The microorganisms' proteolytic, cellulolytic, amylolytic, nitrogen fixation, phosphate solubilization, and actinomycete activities were evaluated. Antimicrobial activity was evaluated by antibiosis assay, disk diffusion test and minimal inhibitory concentration against standard bacterial strains. In total, 10 distinct microbial colonies were isolated and all showed enzymatic activity, highlighting the proteolytic activity. Four microbial strains showed antimicrobial activity against *Bacillus subtilis* and *Klebsiella pneumoniae* strains. This study demonstrated the first discovery of biological activity of iron cave microorganisms, indicating that the GEM-1462 cave is a promising source for prospecting microorganisms with biotechnological potential, mainly of proteolytic enzymes.
Palavras-chave: Carajás, iron lithology, cave microorganisms.

Os microrganismos cavernícolas, principalmente os de litologia ferrífera, são pouco conhecidos. Estes constituem a maior parte da biodiversidade da caverna e desempenham papel fundamental na manutenção do ecossistema cavernícola. O isolamento e a caracterização da microbiota cavernícola podem levar a identificação de novas espécies e a obtenção de substâncias de interesse biotecnológico. O objetivo desse trabalho foi isolar e avaliar as atividades enzimática e antimicrobiana de microrganismos isolados do solo de caverna do Parque Nacional dos Campos Ferruginosos, sudeste amazônico. Foram coletadas amostras de solos da camada superficial interna da caverna GEM-1462 nas diferentes zonas (afótica, disfótica e fótica). Para caracterização das linhagens, utilizou-se a técnica de coloração de Gram e prova bioquímica. Foram avaliadas as atividades proteolíticas, celulolítica, amilolítica, fixação de nitrogênio, solubilização de fosfato e actinomicetos dos microrganismos. A atividade antimicrobiana foi avaliada por meio de ensaio de antibiose, teste de difusão em disco e concentração inibitória mínima contra cepas bacterianas padrão. No total, foram isoladas 10 colônias microbianas distintas e todas apresentaram atividade enzimática, destacando-se a atividade proteolítica. Quatro linhagens microbianas apresentaram atividade antimicrobiana para as cepas *Bacillus subtilis* e *Klebsiella pneumoniae*. Este estudo demonstrou a primeira descoberta de atividade biológica dos microrganismos de caverna ferrífera, indicando que a caverna GEM-1462 é uma promissora fonte para prospecção de microrganismos com potencial biotecnológico, principalmente de enzimas proteolíticas.
Palavras-chave: Carajás, litologia ferrífera, microrganismos cavernícolas.

1. INTRODUCTION

Caves are generally oligotrophic environments with high humidity, constant temperature and low light conditions [1]. Iron ore caves have distinct and unique characteristics when compared to other caves of different lithologies. These are representative and considered in extreme conditions as they are composed of 90% iron oxides and contain very acidic soil, with low fertility rates and temperatures that reach almost 70ºC on the surface [2]. These environments are poorly studied and neglected, especially regarding the cave microbiota [3].

Environments exposed to high concentrations of metals can result in the coexistence of a great diversity of taxa and stimulate survival strategies [4], such as microbial communities that can undergo genotypic and phenotypic changes according to the geochemical variations of the rock that surround [5], adapting to extreme conditions [6].

The current challenge surrounding the study of biological molecules produced by extremophile microorganisms is that their potential applications may not be well known. The most explored studies of cave microorganisms are mainly the production of enzymes, which can function as biocatalysts in various industrial applications [7], and antimicrobial activity [8], due to competition for resources and habitat, especially in the deeper areas of the cave [9].

The Carajás region is located in southeastern Pará, and has the largest number of natural underground cavities known in Brazil, with more than 2,800 recorded cavities and hundreds still to be inventoried in iron lithology with biospeleogenesis, minerals and unique speleothems [10]. The Parque Nacional dos Campos Ferruginosos, study area, has a high number of endemic species adapted to extreme conditions, such as soil poor in nutrients [11], high concentrations of heavy metals (e.g. iron, aluminum and manganese) [12], high relative humidity (~90%) [13], high temperatures (between 34 ºC and 38 ºC), and strong seasonality [2], with annual rainfall ranging from 2,000 to 2,400 mm [14].

The development of studies on the microbiota and the interaction with abiotic and biotic factors that constitute the cave environment of Carajás is essential to generate knowledge about the ecological processes and the biotechnological potential of microorganisms from iron caves, which due to the geological context can present themselves as an important microbial reservoir with specific biological functions. Thus, the aim of this work was to isolate and evaluate the enzymatic and antimicrobial activity of microorganisms isolated from the cave soil of Parque Nacional dos Campos Ferruginosos National Park.

2. MATERIAL AND METHODS

2.1 Localization and cave description

The study was carried out in the Parque Nacional dos Campos Ferruginosos National Park, municipality of Canaã dos Carajás-PA, located in the Southeast region of the Amazon, Brazil, on the Serra da Bocaina plateau. Of these, cave GEM-1462 (SB-0051) was selected (Figure 1), registered in the National Register of Speleological Information and available in the database of the National Center for Research and Conservation of Caves, at coordinates UTM 622283 E / 9301726 N, elevation 606 m, spindle 22 M and DATUM SAD-69, area of 421 m^2 and volume of 1035 m^3.

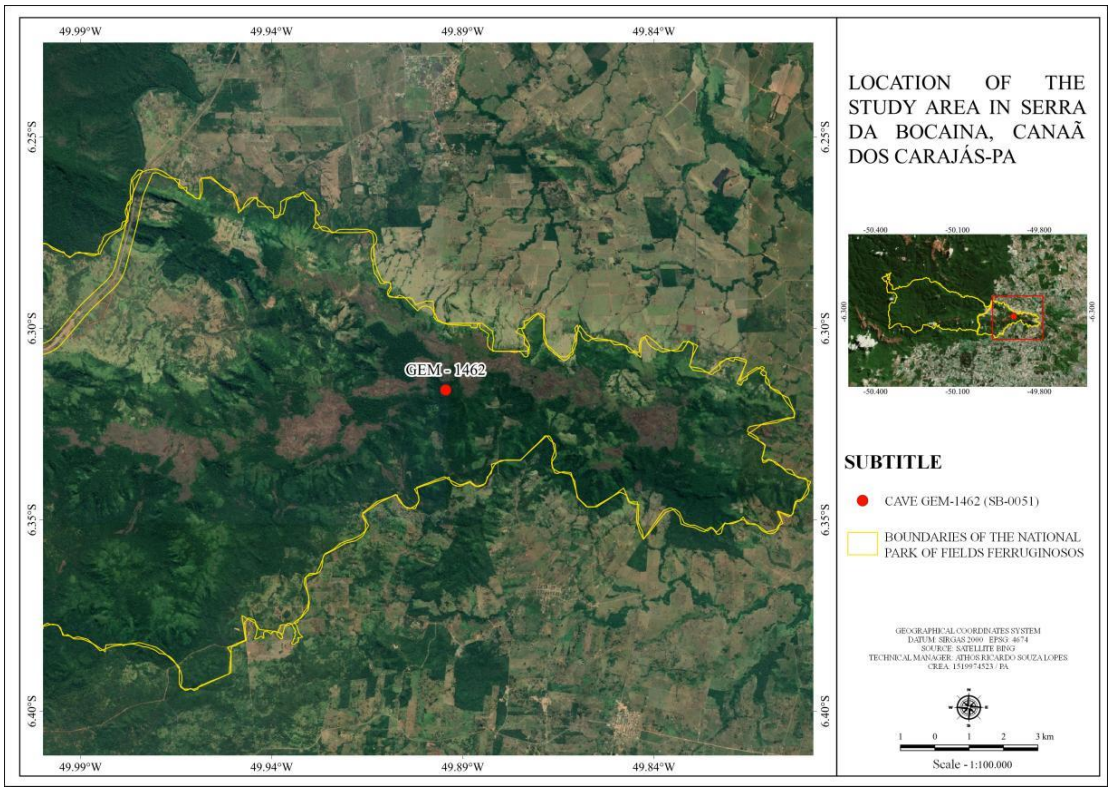

Figura 1: Location map of cave GEM-1462 (SB-0051) in Parque Nacional dos Campos Ferruginosos National Park, Pará.

2.2 Sampling description

The collection was carried out in the dry season, after the permission of the System of Authorization and Information on Biodiversity (SISBIO, nº 69596-1) was released. Between 100 to 600 g of soil was collected in the superficial layer (0 to 10 cm deep) from two random points, delimited in quadrants of 0.25 cm², from each zone, totaling 6 sampling points. The cave was classified into three zones (Figure 2): 30% of the cave area corresponds to the photic zone (GSF: entrance, with light incidence), 50% dysphotic zone (GSD: low luminosity) and 20% of the aphotic zone (GSA: lack of light), according to Trajano and Bichuette (2006) [15].

The samples were aseptically transferred to sterile plastic bags, identified and transportadas em caixa térmica a 4 ºC to the Multipurpose Biology Laboratory at Universidade Federal do Sul e Sudeste do Pará (Unifesspa), located in the municipality of Marabá, Pará, Brazil.

Figure 2: Soil collection in cave GEM-1462: A) photic zone; B) dysphotic zone; and C) aphotic zone.

2.3 Isolation, cultivation and conservation of cave microorganisms

Soil samples were aseptically sieved, homogenized and added (10 g) in Erlenmeyers containing 90 mL of sodium chloride solution (0.9%), in a stage called pre-cultivation. The

Erlenmeyers were incubated in an orbital shaker (150 rpm) at 30ºC (temperature close to cave GEM-1462 temperature), overnight. After the incubation period, the stock solutions were diluted (10^{-1} to 10^{-5}) and 100 µL of the resulting solutions were inoculated on the surface of the plate count agar (PCA) medium, with the aid of the Drigalski spatula. This is a non-selective medium for the cultivation of aerobic bacteria, as the enzymes and antimicrobials producers. The plates were incubated at 30°C, for a period of 24 to 72 h, and then the count of colony-forming units per gram of sample (CFU g^{-1}) was performed. Morphologically distinct colonies were purified on PCA medium and characterized using the Gram stain technique. The microbial strains were preserved in Hogness medium at –20ºC in Multipurpose Biology Laboratory at Unifesspa.

2.4 Determination of enzymatic activity

We analyzed microorganisms' amylolytic, cellulolytic and proteolytic activities (Table 1). These enzymes play an essential role in the carbon cycle. The specific culture medium for the growth of phosphate solubilization (PS), nitrogen-fixing (NF) and actinomycetes microorganisms were also used (Table 1).

Tabela 1: Composition of the culture media used to evaluate enzymatic activity of cave microorganisms.

Enzymatic activity	Culture media (g L^{-1})	References
Actinomycete	Soluble starch, 10 g; hydrolyzed casein, 0.3 g; KNO_3, 2 g; NaCl, 2 g; K_2HPO_4, 2 g; $MgSO_4.7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4$, 0.01 g; agar, 15 g; pH 7.0.	[16]
Amylolytic	Soluble starch, 10 g; yeast extract, 1 mL; $MgSO_4.7H_2O$, 0.1 g; KH_2PO_4, 1.5 g; NH_4NO_3, 0,1 g; NaCl (solution 0.85%), 50 mL; soil extract, 950 mL; agar, 15 g; pH 7.0	[17]
Cellulolytic	Carboxymethyl cellulose, 5 g; NH_4NO_3, 1 g; NaCl (0.85% solution), 50 mL; soil extract, 950 mL; agar, 15 g; pH 7.0.	[18]
Proteolytic	Hydrolyzed casein, 10 g; yeast extract, 1 ml; K_2HPO_4, 1.5 g; $MgSO_4.7H_2O$, 0.5 g; NaCl (0.85% solution), 50 mL; 950 mL of soil extract; agar, 15 g; pH 7.0.	[18]
Phosphate solubilizer	Solution A: dextrose, 10 g; yeast extract, 5 ml; KNO_3, 0.1 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; NaCl, 0.1 g; agar, 15 g. Solution B: $CaCl_2$, 10 g; distilled water, 100 mL. Solution C: K_2HPO_4, 5 g; distilled water, 50 mL. Micronutrient solution: $MoO_4Na.2H_2O$, 0.2 g; $MnSO_4.2H_2O$, 0.24 g; H_3BO_3, 0.28g; $CuSO_4.5H_2O$, 0.008 g; $ZnSO_4.7H_2O$, 0.024 g; distilled water, 200 mL. Solution of FeEDTA-NaEDTA, 6.07 g; FeSO4.7H2O, 6.17 g; pH 7.0.	[19]
Nitrogen fixation	KH_2PO_4, 0.4 g; K_2HPO_4, 0.1 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $FeCl_3$, 0.01 g; $MoO_4Na.2H_2O$, 0.002 g; sodium malate, 5 g; bromothymol blue (0.5%), 5 mL; agar, 15 g; pH 6.8	[20]

To detect the enzymatic activity, 25 mL of the culture media shown in Table 1 were aseptically transferred to a Petri dish (90 x 15 mm). A fresh culture colony (24 h) of the microorganisms isolated under the surface of the culture media was inoculated. The plate was

incubated at 30°C for up to 5 days, being monitored daily. The enzymatic index (EI) and solubilization index (SI) was calculated by using the following equation: EI or SI = mean halo diameter (MHD) / mean colony diameter (MCD), where MHD is the total diameter of the hydrolysis or solubilization halo (mm) and the MCD is the diameter of bacterial colonies (mm), according to Oliveira et al. (2006) [21].

2.5 Determination of antimicrobial activity

The screening of antimicrobial-producing microorganisms was performed through the antagonism test against strains from the American Type Culture Collection (ATCC): *Bacillus subtilis* (ATCC 23857), *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 700603) and *Staphylococcus aureus* (ATCC 25923). ATCC strains were cultivated in tryptone soybean agar (TSA) medium at 37°C for 18 to 24 h. After the incubation period, the inoculum of the strains was prepared in saline solution (NaCl, 0.85%) using the 0.5 McFarland scale (1.5 x 10^8 bacteria mL^{-1}). According to the pour plate method, one milliliter of the inoculum was transferred to a Petri dish (90 x 15 mm) containing 25 mL of Mueller Hinton agar (AMH). Then, a recent (24 h) culture loop of the microorganisms was inoculated under the surface of the AMH medium. Plates were incubated at 37°C for 24 to 48 hours. After the incubation period, the formation of inhibition zones around the inoculum of the microorganisms was observed, with at least 1 mm of an area without growth of ATCC strains [22], modified.

Cave microorganisms that showed antimicrobial activity in the antagonism test were selected to obtain the extracts and were cultivated in 250 mL Erlenmeyers containing 100 ml of YPD broth (2.5 g yeast extract, 5 g peptone and 1 g dextrose per liter of distilled water). This culture medium presented a composition similar to PCA and was chosen to obtain cell biomass and antibiotic production. The flasks were incubated in an orbital shaker at 28°C, 180 rpm for 3 days. Then, cultures were filtered and centrifuged at 6000 rpm for 20 minutes. The supernatant was aseptically transferred to a screw-capped bottle and stored at 4°C.

The antimicrobial activity of microorganism extracts was tested against ATCC strains using the disk diffusion method in AMH medium, following the National Committee for Clinical Laboratory Standards recommendations [23]. The 6 mm diameter sterile paper disc (MN 618) was impregnated with 50 µl of microbial extract (impregnation and drying process was repeated three times). In the negative control, uninoculated YPD broth extract was applied and in the positive control the antibiotic tetracycline (25 µg $disc^{-1}$). The inoculum of ATCC strains was prepared in saline solution (NaCl, 0.85%), from a 24 h culture in TSA. The suspension was adjusted using the standard 0.5 McFarland scale (1.5 x 10^8 CFU mL^{-1}) and seeded on the surface of the AMH medium, with the aid of a sterile swab. The disc containing the microbial extract was placed on the surface of the AMH medium and the plate was incubated in an oven at 37°C for 24 h. After the incubation period, the diameter of the bacterial growth inhibition halos was read.

The extracts of microorganisms that showed antimicrobial activity in the disk diffusion test were selected for the minimum inhibitory concentration (MIC) determination test [24], with adaptations. The assay was performed in a sterile 96-well cell culture plate containing Mueller-Hinton broth (CMH). The microbial extracts were transferred to the wells, following the serial dilution technique (0.04% to 80%). In the negative control, the uninoculated sterile YPD broth extract was applied and in the positive control tetracycline (25 µg). Subsequently, 5 µL of the standard strain was inoculated into each well, adjusted using the 0.5 McFarland standard scale (1.5 x 10^8 CFU mL^{-1}). The plate was incubated at 37°C, between 20 to 22 h. After the incubation period, 10 µL of the developing solution of 2,3,5-triphenyltetrazolium chloride (TTC) was applied to the wells and incubated in the oven for another 2 hours. The reading of the MIC test was performed by changing the color of the revealing solution, considering the bacterial growth as the presence of a pink or reddish color [25].

2.6 Statistical analysis

All tests were performed in triplicate. To characterize the composition of microorganisms in relation to enzyme index and functional profiles, non-metric multidimensional scaling (nMDS) was used, based on similarity matrices calculated using the Bray-Curtis index. A Similarity Analysis (ANOSIM) was also applied to determine the effect of metabolic profiles in relation to the enzymatic indices of the functional groups.

In the evaluation of enzymatic indices, clustering analysis (Clustering Dendrogram) was performed to estimate the distance between microorganisms and metabolic profiles, using the nMDS and ANOSIM results, which showed significant effects ($p < 0.05$ and $R^2 > 0.60$). For cluster analysis, Ward's method was used [26]. The "Vegan" package was used, for the nMDS, the "metaMDS" function was used together with the "envfit" function, to verify which enzymatic activities influenced the ordering of the nMDS. A dendrogram was performed with the enzymatic activities of greatest influence for the setting up of clusters.

3. RESULTS

Ten microbial strains from cave GEM-1462 were isolated. Of these strains, 9 are bacteria and 1 yeast fungus, 4 were isolated in the photic zone, 3 in the dysphotic zone and 3 in the aphotic zone (Figure 3). The bacteria presented the form of bacilli and cocci, being 8 Gram-positive and 1 Gram-negative (Figure 3).

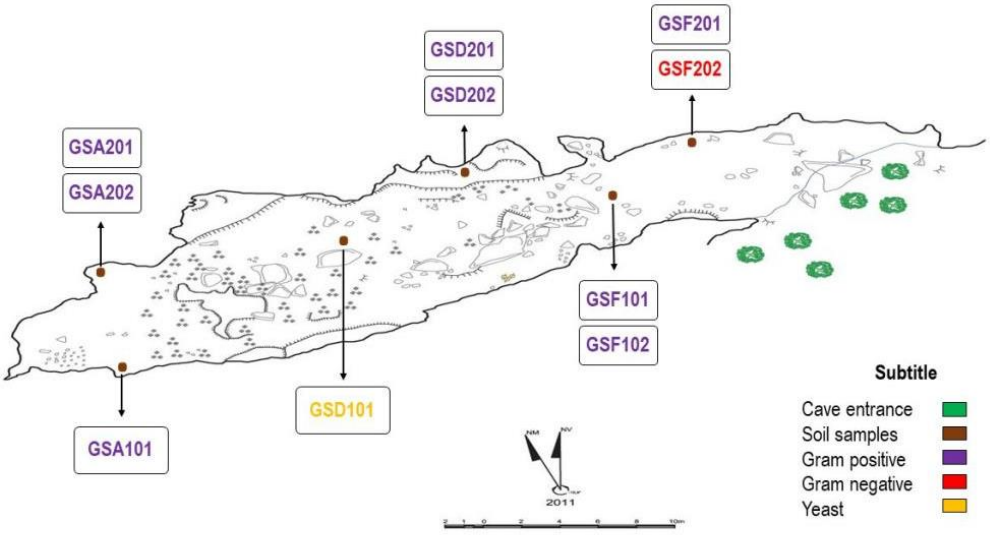

Figure 3: Topographic map of cave GEM-1462, with collection points: GSF (photic zone soil samples); GSD (dysphotic zone soil samples); and GSA (aphotic zone soil samples); and morphotintorial representation of isolated microbial strains.

All microbial strains showed proteolytic activity, 7 cellulolytic, 1 amylolytic, 2 phosphate solubilizers and 3 starch and casein degraders (actinomycetes) (Table 2). Strains isolated from the aphotic zone produced the highest enzymatic indices, followed by the dysphotic and photic zones. The GSF202 strain was the only nitrogen fixative (Table 2).

Table 2: Enzymatic activity (amylolytic, cellulolytic and proteolytic) and phosphate solubilization (PS) of cave microbial strains.

Isolated strains	Proteolytic	Cellulolytic	Amylolytic	Actynomycete	PS
GSA101	20.33	2.30	-	-	-
GSA201	13.50	1.15	-	-	-
GSA202	12.58	-	-	-	-
GSD101	7.83	1.52	0.31	-	-
GSD201	17.83	-	-	0.75	0.59
GSD202	15.25	1.08	-	0.54	-
GSF101	14.92	1.55	-	-	-
GSF102	19.00	1.33	-	0.52	-
GSF201	11.58	1.14	-	-	-
GSF202	12.33	-	-	-	0.59

(-) absence of enzymatic activity; (EI) The enzymatic index values represent the mean of three repetitions of the ratio between the mean diameters of the degradation halo and the colony.

The nMDS analysis of the functional activities of the microbial strains showed that there is a pattern of similarity between the data ($P < 0.05$ and stress 0.04). In Figure 4, we can see that the points sampled in the cave presented cultivable microbial communities with similar enzymatic activities, despite the significant differences between the indices measured by the PERMANOVA test ($P < 0.05$). Proteolytic ($R2 = 0.98$) and cellulolytic ($R2 = 0.65$) activities were predominant in most of the isolated microbial groups.

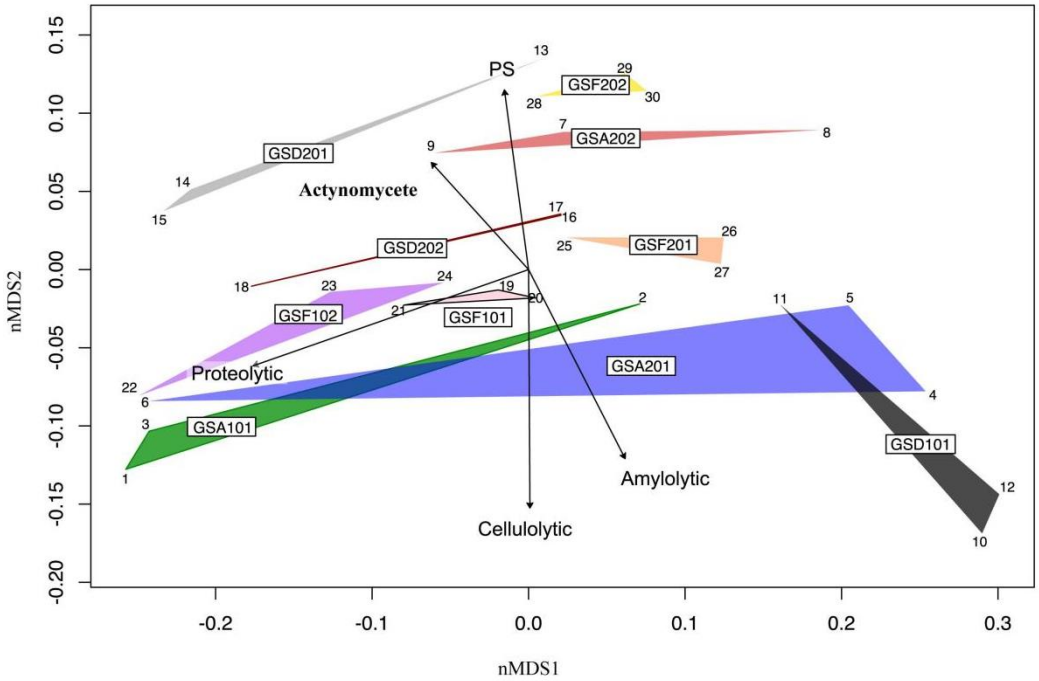

Figure 4: Bray-Curtis dissimilarity in the non-metric multidimensional scaling matrix (nMDS) to assess the functional diversity of cave microorganisms. The colors in the graph refer to the isolated microbial strains: GSF101 (pink); GSF102 (purple); GSF201 (orange); GSF202 (yellow); GSD101 (black); GSD201 (grey); GSD202 (brown); GSA101 (green); GSA201 (blue); and GSA202 (red).

The dendrogram of similarity of proteolytic activities generated the formation of two large groups, one formed exclusively by microbial strains with a high enzymatic index and the other mixed, based on nMDS (Figure 5). The isolation zones of the microbial strains did not influence the similarity of the proteolytic activity data.

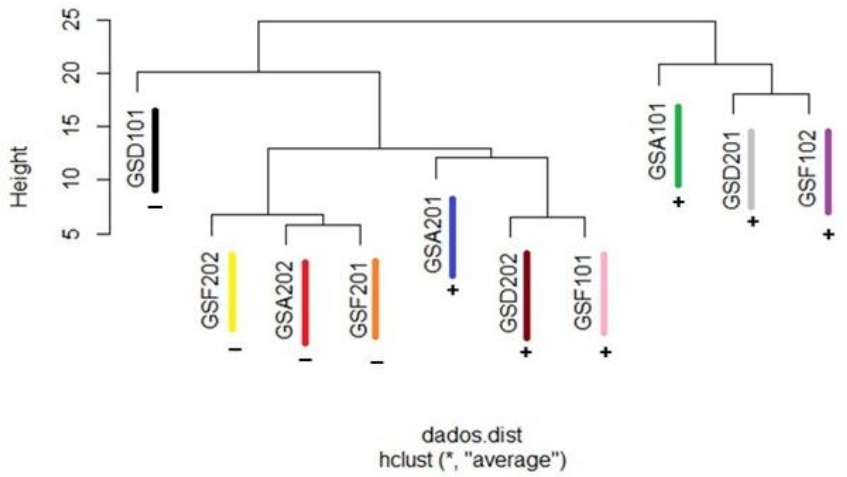

Figure 5: Dendrogram of the hierarchical cluster analysis (Ward) of cave microorganisms based on non-metric multidimensional scaling (NMDS) of proteolytic activity (Enzyme Index: +, high activity; –, low activity). The colors in the graph refer to the isolated microbial strains: GSF101 (pink); GSF102 (purple); GSF201 (orange); GSF202 (yellow); GSD101 (black); GSD201 (grey); GSD202 (brown); GSA101 (green); GSA201 (blue); and GSA202 (red).

Cellulolytic activity also presented two large groups, one with a low index of enzymatic activity and the other high (Figure 6). Within these groups, the similarity between cellulolytic activities was high and the isolation zone of the microbial strains did not influence the similarity of the data.

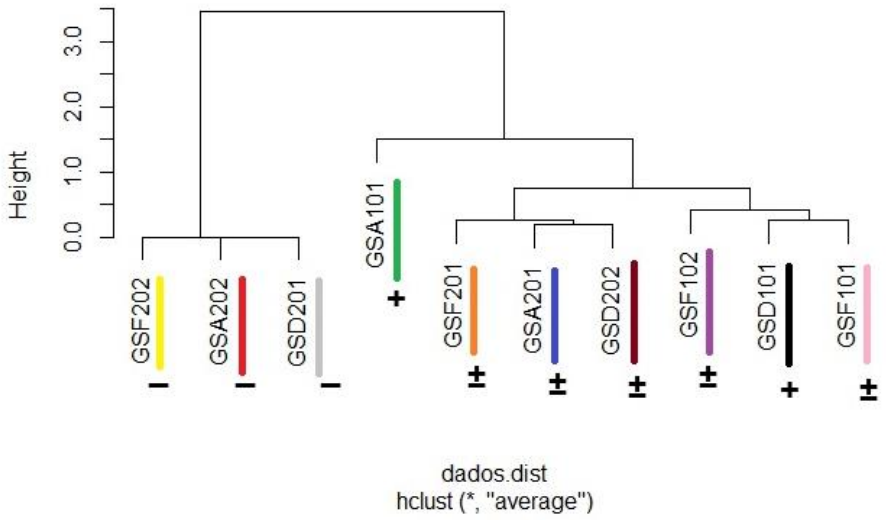

Figure 6: Dendrogram of the hierarchical cluster analysis (Ward) of cave microorganisms based on non-metric multidimensional scaling (NMDS) of cellulolytic activity (Enzyme Index: +, high activity; –, low activity). The colors in the graph refer to the isolated microbial strains: GSF101 (pink); GSF102 (purple); GSF201 (orange); GSF202 (yellow); GSD101 (black); GSD201 (grey); GSD202 (brown); GSA101 (green); GSA201 (blue); and GSA202 (red).

Among the ten microbial strains isolated, only four (GSF102, GSF201, GSF202 and GSD201) showed antimicrobial activity against *B. subtilis* and *K. pneumoniae* strains in the antagonism assay, with different degrees of sensitivity. The four strains presented zones of inhibition for *B. subtilis* and did not differ from each other (Table 3), while for inhibition of *K. pneumoniae* the GSF102 strains, GSF201 and GSD201 presented the highest inhibition values

and did not differ from each other when compared to the GSF202 strain (Table 3). The negative control did not presented a zone of inhibition and the positive was of 20 mm (± 0.6) against the strains tested. The minimum inhibitory concentration of extracts from strains GSF202 and GSD201 was 40% and 80% for *K. pneumoniae*, respectively, and 40% of extract from strain GSF202 for *B. subtilis*.

Tabela 3: The mean inhibition halos of the ATCC B. subtilis and K. pneumoniae strains against the microbial strains isoleted.

Isolated strains	ATCC strains	Disk diffusion (mm)*
GSF102	*B. subtillis*	1.4 ± 0.3
	K. pneumoniae	1.4 ± 0.1
GSF201	*B. subtillis*	1.7 ± 0.2
	K. pneumoniae	1.6 ± 0.3
GSF202	*B. subtillis*	1.8 ± 0.5
	K. pneumoniae	0.9 ± 0.3
GSD201	*B. subtillis*	1.0 ± 0.2
	K. pneumoniae	1.1 ± 0.3

*Inhibition halo, including disc diameter (6 mm); - There was no inhibition.

4. DISCUSSION

4.1 Cave microorganisms

The main microbial group found in this study was that of Gram-positive bacteria. According to literature reports, the dominant phyla identified in caves are Proteobacteria and Actinobacteria, characterized by Gram-negative and Gram-positive bacteria, respectively [27, 28], with proteobacteria being detected mainly through molecular tools, while actinobacteria represent most culture-dependent isolates [29]. However, Lemes et al. (2021) [30] identified that the phylum Acidobacteria (Gram-negative) was the most abundant in the Canga caves analyzed in the Iron Quadrangle, followed by Proteobacteria and Actinobacteria.

The zones of natural underground cavities suffer interference from the penetration and intensity of light. Thus, each zone has specific physicochemical and nutritional conditions [31], which may influence the colonization and diversity of microorganisms, such as a high concentration of iron. In Carajás, the results showed that the cavity zones did not influence the quantity and diversity of microbial strains isolated in each zone. Zhu et al. (2019) [28] suggest that specific niches drive the evolution of the microbial community, which may favor the enrichment of unique microbial populations, despite the general similarity of the microbial communities found in caves. Thus, caves may be sources of new microorganisms with bioactive metabolites with biotechnological potentials, such as enzymes and antibiotics [32].

4.2 Enzymatic activity

Studies have shown that microbial isolates have great potential to produce hydrolytic enzymes. Ntabo et al. (2018) [33], Oliveira et al. (2006) [21] and Ramos et al. (2018) [34] reported that bacterial isolates showed proteolytic, cellulolytic, and aminolytic activities with IE in the range of 1.1 to 6.6, 1.1 to 7.6, and 1.1 to 4.8, respectively. Ramos et al. (2018) [34] also showed that bacterial isolates from rhizospheric soils were phosphate solubilizers, varying the IS between 1.0 and 1.4. For Lealem and Gashe (1994) [35] and Stamford et al. (1998) [36], microorganisms can be considered potential enzyme producers in solid media when the enzyme index is equal or greater than 2.0. The cave microorganisms isolated in this work showed proteolytic, cellulolytic and amylolytic activities, however, the most satisfactory and promising

result was the proteolytic activity. The proteolytic activity may be indicative not only of the biological capacity of soil for the enzymatic conversion of the substrate, which is independent of the extent of microbial activity, but might also have an important role in the ecology of micro-organisms in the ecosystem [37]. These extracellular hydrolytic enzymes are mainly responsible for the biochemical reactions of transformation of organic matter in the soil, acting in the processes of cycling of elements and decomposition [38], through the degradation of insoluble macromolecules into soluble micro-molecules [37], which are absorbed by cells.

Bat guano is considered one of the main energy sources for many species found in caves, contributing to the renewal of organic resources and allowing the development of a varied biota, including microorganisms [39]. In this work, it was possible to observe the presence of guano in the three zones of cave GEM-1462, with the largest amount in the aphotic zone, environment of greater colonization of bats. The distribution of guano between the zones may have occurred due to the process of water percolation in the aphotic zone, such as dripping to the back of the cave, forming reservoirs of organic material (micro-habitat) for microbial growth. The intermittent presence of water stimulates colonization and microbial activity in the guano, favoring the degradation of organic matter (e.g. carbohydrates, lipids and proteins) by hydrolytic enzymes [40] and providing essential elements such as phosphorus and nitrogen in the soil [41], containing about 25% and 10%, respectively [39].

From the results of this research, it is possible to infer that the protein, possibly derived from guano, was the main source of energy for cave microbiota, and that the greater proteolytic activity is not related to the amount of isolated microorganisms, but with the enzymatic indices produced by these strains. Phosphate and nitrogen available in the soil may also have the same origin, and these elements are essential for the vital activities of microorganisms and the maintenance of ecological processes in iron caves.

In addition to guano, plant material, such as litter, roots and seeds from bat food or that were carried into the cavity, as well as guano residues containing structural carbohydrates (e.g. cellulose, hemicellulose and pectin) are important sources of organic matter for cellulolytic microorganisms. Cellulolytic enzymes play an essential role in the degradation of carboxymethyl cellulose, providing compounds for further absorption and processing by the cellular metabolism of other microorganisms [42]. Thus, cellulose and plant biomass residues may be oxidized by amylase-producing microorganisms and actinomycetes, generating easily degraded carbon sources, such as monosaccharides [43].

According to Barton and Jurado (2007) [5], the reduced availability and chemical complexity of some nutrients present in caves limit the growth of many microbial species, and the strategy created by many communities of microorganisms has been to carry out a mutualistic association to overcome this nutritional limitation. For Yin and Keller (2011) [44], there is constant communication between the microbial communities in the cave ecosystem through the quorum sensing, allowing the recognition of the conditions and changes that are being submitted, communicating with each other, developing survival strategies, such as the production of hydrolytic enzymes, to carry out important functions of defense, competition, signaling and ecological interactions.

Microorganisms, such as bacteria and fungi, are considered the best sources for obtaining hydrolytic enzymes, due to their rapid growth and easy handling for the desired yield [45]. Hydrolytic enzymes (ie protease, cellulase and amylase) are the most used in industrial processes, mainly in the areas of biotechnology, food, biofuel, paper, cellulose, feed, dyeing [46], with proteases being the most studied group of hydrolases [21]. Enzymes produced by microorganisms living in extreme environments, such as cave GEM-1462, are considered versatile tools for developing and improving a variety of industrial and biotechnological processes due to their stability in extreme conditions and biodegradability [47].

4.3 Antimicrobian activity

Caves are considered oligotrophic environments, with low light and different mineral formations. These conditions encourage complex interactions (competitive or cooperative) between different microorganisms, enhancing the production of secondary metabolites, such as antibiotics, which can be used to inhibit the growth of other microorganisms in the competition for nutrients [48]. Thus, caves represent strategic environments for prospecting new antimicrobial compounds.

Recent studies have shown the antimicrobial potential of cave microorganisms against pathogenic microbial strains, especially actinomycetes [49, 50], which produce about 2/3 of the antibacterial agents in use [51]. In this work, four isolated microbial strains showed antimicrobial activity, and two strains were characterized as actinobacteria (GSF102 and GSD201).

Isolated cave microorganisms have shown greater growth inhibition of the *B. subtilis* species and reduced or no activity against the *Klebsiella pneumoniae* species [8, 49, 50]. The microbial strains isolated in this work showed antimicrobial activity against both strains, *B. subtilis* and *K. pneumoniae*.

Natural products are the most promising source of antibiotics and cave environments have a high potential for prospecting new microorganisms and antimicrobial agents [32]. The emergence of new pathogenic bacteria associated with the rapid development of resistance to commercially available antibiotics increases the importance of discovering new antimicrobial agents [4, 52].

5. CONCLUSION

Studies on ecological processes and the biotechnological potential of microorganisms from iron caves in the Carajás region are limited or even non-existent. This study presents unprecedented results on enzymatic and antimicrobial activities of microorganisms isolated in iron cave in the region of Carajás.

The prospection of microorganisms that produce enzymes and antimicrobial agents of biotechnological relevance in the cave environment helps to elucidate the molecular mechanisms and ecological interactions that occur in the cave. The analyzes carried out in this work demonstrate that the GEM-1462 cave is a promising source of natural products for research and biotechnological application, mainly of proteolytic enzymes. The knowledge of this cave microbiota will provide subsidies for the conservation and proper use of the speleological heritage, in addition to enabling the development of new resears to discover molecules with industrial application.

6. ACKNOWLEDGMENTS

We are very thankful by collaboration of Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio).

7. REFERENCES

1. Ghosh S, Kuisiene N, Cheeptham N. The cave microbiome as a source for drug discovery: Reality or pipe dream? Bioch Pharmacol. 2017 Jun;134:18-34. doi: 10.1016/j.bcp.2016.11.018
2. Mota NFO, Silva LVC, Martins FD, Viana PL. Vegetação sobre sistemas ferruginosos da serra dos Carajás. In: Carmo FF, Kamino LHY, editors. Geossistemas ferruginosos do Brasil: áreas prioritárias para conservação da diversidade geológica e biológica, patrimônio cultural e serviços ambientais. Belo Horizonte (MG): Instituto Prístino; 2015. p. 289-315.
3. Wu Y, Tan L, Liu W, Wang B, Wang J, Cai Y, et al. Profiling bacterial diversity in a limestone cave of the western Loess Plateau of China. Fronti Microbiol. 2015;6:244. doi: 10.3389/fmicb.2015.00244

4. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, et al. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE. 2012;7(4):e34953. doi: 10.1371/journal.pone.0034953
5. Barton HA, Jurado V. What's up down there? Microbial diversity in caves. Microbe. 2007;2:132-8.
6. Kour D, Rana KL, Kaur T, Singh B, Chauhan VS, Kumar A, et al. Extremophiles for hydrolytic enzymes productions: biodiversity and potential biotechnological applications, bioprocessing for biomolecules production. In: Molina G, Gupta V, Singh B, Gathergood N, editors. Bioprocessing for biomolecules production. Wiley Online Library [online]; 2019. p. 321-72. doi: 10.1002/9781119434436.ch16
7. Hamedi J, Kafshnouchi M, Ranjbaran M. A study on actinobacterial diversity of Hampoeil cave and screening of their biological activities. J Biol Sci. 2019;26(7):1587-95. doi: 10.1016/j.sjbs.2018.10.010
8. Axenov-Gribanov DV, Voytsekhovskaya IV, Tokovenko BT, Protasov ES, Gamaiunov SV, Rebets YV, et al. Actinobacteria Isolated from an underground lake and moonmilk speleothem from the biggest conglomeratic karstic cave in Siberia as sources of novel biologically active compounds. PLoS One. 2016;11(2):e0149216. doi: 10.1371/journal.pone.0149216
9. Maciejewski L, Lepareur F, Viry D, Bensettiti F, Puissauve R, Touroult J. État de conservation des habitats: propositions de définitions et de concepts pour l'évaluation à l'échelle d'un site Natura 2000. Revue d'Ecologie. 2016;71(1):3-20.
10. Albuquerque ARL, Angélica RS, Gonçalves DF, Paz SPA. Phosphate speleothems in caves developed in iron ores and laterites of the Carajás Mineral Province (Brazil) and a new occurrence of spheniscidite. Int J Speleol. 2018;47:53-67. doi: 10.5038/1827-806X.47.1.2135
11. Nunes CS, Bastos MNC, Gil ASB. Flora of the cangas of the Serra dos Carajás, Pará, Brasil: Cyperaceae. Rodriguésia. 2016;67:1329-66. doi: 10.1590/2175-7860201667532
12. Schettini AT, Leite MGP, Messias MCTB, Gauthier A, Li H, Kozovits AR. Exploring Al, Mn and Fe phytoextraction in 27 ferruginous rocky outcrops plant species. Flora. 2018;238:175-82. doi: 10.1016/j.flora.2017.05.004
13. Ladle RJ, Firmino JVL, Malhado ACM, Rodríguez-Durán A. Unexplored diversity and conservation potential of neotropical hot caves. Conserv Biol. 2012 Dec;26(6):978-8. doi: 10.1111/j.1523-1739.2012.01936.x
14. Chaves PP, Ferreira LV. O efeito da sazonalidade da precipitação na florística e na estrutura da regeneração natural dos campos rupestres da Serra Norte de Carajás, Pará, Brasil. Bol Mus Para Emílio Goeldi Cienc Nat. 2016;11:103-16.
15. Trajano E, Bichuette ME. Biologia subterrânea: Introdução. São Paulo (SP): Redespeleo; 2006.
16. Kuster E, Williams ST. Selective media for the isolation of *Streptomycetes*. Nature. 1964;202:928-9. doi: 10.1038/202928a0
17. Pontecorvo G, Roper JA, Chemmons LM, Macdonald KD, Bufton AWJ. The genetics of *Aspergillus nidulans*. Advances in Genetics. 1953;5:141-238. doi: 10.1016/S0065-2660(08)60408-3
18. Wood PJ. Specificity in the interaction of direct dyes with polysaccharides. Carbohydr research. 1980;85:271-287. doi: 10.1016/S0008-6215(00)84676-5
19. Sylvester-Bradley R, Asakawa N, La Torraca S, Magalhães FMM, Oliveira LA, Pereira RM. Levantamento quantitativo de microrganismos solubilizadores de fosfatos na rizosfera de gramíneas e leguminosas forrageiras na Amazônia. Acta Amaz. 1982;12:15-22. doi: 10.1590/1809-43921982121015
20. Döbereiner J, Day JM. Associative symbiosis in tropical grasses, characterization of microorganisms and dinitrogen fixing sites. In: Newton WE, Nyman CJN, editors. Proceedings of the 1st international symposium of nitrogen fixation. Pullman (US): Washington State University Press; 1976. p. 518-38.
21. Oliveira AN, Oliveira LA, Andrade JS, Chagas Júnior AF. Enzimas hidrolíticas extracelulares de isolados de rizóbia nativos da Amazônia Central, Amazonas, Brasil. Food Sci Technol. 2006;26:853-60. doi: 10.1590/S0101-20612006000400022
22. Mohseni M, Norouzi H, Hamedi J, Roohi A. Screening of antibacterial producing actinomycetes from sediments of the Caspian Sea. Int J Mol Cell Med. 2013;2(2):64-71.
23. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests: approved standard. 8. ed. Pennsylvania (US): NCCLS; 2003.
24. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. 6. ed. Pennsylvania (US): NCCLS; 2003.
25. Mohr LC, Capelezzo AP, Rippel T, Ternus RZ, Dalcanton F, Fiori MA, et al. Efeito antimicrobiano de nanopartículas de ZnO e TiO2 frente as bactérias *S. aureus* e *E. coli*. Rev CSBEA. 2017;3(1): 1-10.

26. Angel R, Soares MIM, Ungar ED, Gillor O. Biogeography of soil archaea and bacteria along a steep precipitation gradient. The ISME J. 2010;4:553-63.
27. Bender KE, Glover K, Archey A, Barton HA. The impact of sample processing and media chemistry on the culturable diversity of bacteria isolated from a cave. Int J Speleol. 2020;49:209-20. doi: 10.5038/1827-806X.49.3.2337
28. Zhu H-Z, Zhang Z-F, Zhou N, Jiang C-Y, Wang B-J, Cai L, et al. Diversity, distribution and co-occurrence patterns of bacterial communities in a karst cave system. Front Microbiol. 2019 Aug;10:1-12. doi: 10.3389/fmicb.2019.01726
29. Gherman VD, Boboescu IZ, Pap B, Kondorosi E, Gherman G, Maróti G. An acidophilic bacterial-archaeal-fungal ecosystem linked to formation of ferruginous crusts and stalactites. Geomicrobiol J. 2014;31:407-18. doi: 10.1080/01490451.2013.836580
30. Lemes CGC, Villa MM, Felestrino EB, Perucci LO, Assis RAB, Cordeiro IF, et al. 16S rRNA Gene amplicon sequencing data of the Iron Quadrangle Ferruginous Caves (Brazil) shows the importance of conserving this singular and threatened geosystem. Diversity. 2021;13:1-21. doi: 10.3390/d13100494
31. Culver DC, Pipan T. The biology of caves and other subterranean habitats. Oxford (UK): Oxford University Press; 2019.
32. Riquelme C, Hathaway JJM, Dapkevicius MLE, Miller AZ, Kooser A, Northup DE, et al. Actinobacterial diversity in volcanic caves and associated geomicrobiological interactions. Front Microbiol. 2015;6:1342. doi: 10.3389/fmicb.2015.01342
33. Ntabo RM, Nyamache AK, Lwande W, Kabii J, Nonoh J. Enzymatic activity of endophytic bacterial isolates from selected mangrove plants in Kenya. The Open Microbiol J. 2018;12:354-63. doi: 10.2174/1874285801812010354
34. Ramos, RF, Sobucki L, Rohrig B, Ludwig J, Daroit DJ. Functional diversity of bacteria isolated from rhizosphere and non-rhizosphere soils under maize cultivation. R Ci Agrovet. 2018;17(3):417-27.
35. Lealem F, Gashe BA. Amylase production by a gram-positive bacterium isolated from fermenting tef (Eraglostis tef). J Appl Bacteriol. 1994;77(3):348-52. doi: 10.1111/j.1365-2672.1994.tb03084.x
36. Stamford TLM, Araújo JM, Stamford NP. Atividade enzimática de microrganismos isolados do jacatupé (*Pachyrhizus erosus* L. Urban). Ci Tecnol Aliment. 1998;18(4):382-5.
37. Burns RG, De Forest JL, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, et al. Soil enzymes in a changing environment: current knowledge and future directions. Soil Biol Biochem. 2013;58:216-34. doi: 10.1016/j.soilbio.2012.11.009
38. Arias ME, Gonzalez-Perez, JA, Gonzalez-Vila FJ, Ball AS. Soil health: a new challenge for microbiologists and chemists. Int Microbiol. 2005;8(1):13-21.
39. Sakoui S, Derdak R, Addoum B, Serrano-Delgado A, Soukri A, El Khalfi B. The life hidden inside caves: ecological and economic importance of bat guano. Int J Ecol. 2020;2020:9872532. doi: 10.1155/2020/9872532
40. Chen D, Krol A. Hydrogeology of Heron Island, great barrier reef, Australia. In: Vacher LHL, Quinn TM, editors. Developments in sedimentology. New York (US): Elsevier; 1997. p. 867-84. doi: 10.1016/S0070-4571(04)80052-9
41. Karimou AH, Abdou GF, Guero Y. Physico-chemical characterization of bat guano from the village of Magarawa in Niger. Int J Current Res. 2021;13(1):15953-9. doi: 10.24941/ijcr.40607.01.2021
42. Menezes CR, Barreto AR. Biodegradação de resíduos lignocelulósicos por fungos basidiomicetos: caracterização dos resíduos e estudo do complexo enzimático fúngico. Rev Eletr Gestão, Ed Tecnol Amb. 2015;19:1365-91. doi: 105902/2236117016853
43. Shan Q, Yu Y, Yu J, Zhang J. Soil enzyme activities and their indication for fertility of urban forest soil. Front Environ Sci Eng China. 2008 Jun;2:218-23.
44. Yin W, Keller NP. Transcriptional regulatory elements in fungal secondary metabolism. J Microbiol. 2011 Jun;49(3):329-39. doi: 10.1007/s12275-011-1009-1
45. Gurung N, Ray S, Bose S, Rai V. A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. BioMed Res Int. 2013;2013:329121. doi: 10.1155/2013/329121
46. Manisha SKY. Technological advances and applications of hydrolytic enzymes for valorization of lignocellulosic biomass. Bioresour Technol. 2017 Dec;245(part B):1727-39. doi: 10.1016/j.biortech.2017.05.066
47. Kumar L, Awashi G, Singh B. Extremophiles: a novel source of industrially important enzymes. Biotechnol. 2011;10:121-35.
48. Rajput Y, Biswas J, Rai V. Potentiality test in antimicrobial activity and antibiotic sensitivity of subterranean *Streptomyces* strains isolated from Kotumsar Cave of India. Int J Biol Chem. 2012;6:53-60. doi: 10.3923/ijbc.2012.53.60

49. Belyagoubi L, Belyagoubi-Benhammou N, Jurado V, Dupont J, Lacoste S, Djebbah F, et al. Antimicrobial activities of culturable microorganisms (actinomycetes and fungi) isolated from Chaabe Cave, Algeria. Int J Speleol. 2018:47:189-99. doi: 10.5038/1827-806X.47.2.2148
50. Avguštin JA, Petrič1 P, Pašić L. Screening the cultivable cave microbial mats for the production of antimicrobial compounds and antibiotic resistance. Int J Speleol. 2019 Sep;48(3):295-303. doi: 10.5038/1827-806X.48.3.2272
51. Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Klenk H-P, et al. Taxonomy, physiology, and natural products of actinobacteria. Microbiol Mol Biol Rev. 2016;80:1-43. doi: 10.1128/MMBR.00019-15
52. Monciardini P, Iorio M, Maffioli S, Sosio M, Donadio S. Discovering new bioactive molecules from microbial sources. Microbial Biotechnol. 2014 May;7(3):209-20. doi: 10.1111/1751-7915.12123