European Journal of Biology
Eur J Biol 2023; 82 (2): 186–195
DOI: 10.26650/EurJBiol.2023.1349885

RESEARCH ARTICLE

Bacterial Biodiversity of the Kapova Karst Cave as a Source of Hydrolases Producers

Willam Kurdy[1], Galina Yakovleva[1], Olga Ilinskaya[1]

[1]Kazan Volga-Region Federal University, Kazan, Tatarstan, Russian Federation

ABSTRACT

Objective: Recent studies have revealed the biodiversity of both cultivated and uncultivated microbiomes in extreme environments. It has been shown that terrestrial subsurface ecosystems contain vast metabolic potential. Heterotrophic bacteria living in karst caves with an organic substrate deficit represent a special reserve for the isolation of metabolite producers. Here, we cultivated a bacterial community collected from biofilms in Kapova Cave (Shulgan–Tash Nature Reserve, Bashkortostan), and assessed its ability to synthesize secreted hydrolytic enzymes including RNases, proteases, and amylases.

Materials and Methods: Isolated bacteria were identified by V3-V4 16S rRNA region sequencing. Enzymatic activities were assessed by measuring transparency zones around colonies grown on the appropriate substrate (RNA, casein, starch). Functional profiles of the communities were predicted using the Global Mapper module on iVikodak. Taxonomic, structural, and compositional diversity were calculated using Shannon–Wiener and Bray–Curtis indices.

Results: Eighty-nine percent of 102 bacterial isolates were *Proteobacteria*, whereas other isolates were divided into three other phyla, *Actinobacteria, Firmicutes*, and *Bacteroidetes* that comprised 5%, 4%, and 2% of the isolates, respectively. Genus Pseudomonas was predominant with 42 isolates. Six isolates showed no extracellular enzymatic activity at all, 73 isolates expressed protease, 57 isolates expressed amylase, and 71 isolates had RNase activity. All three extracellular enzymes were expressed by 39 isolates.

Conclusion: The biodiversity of cultivated microbiota from Kapova Cave was characterized. Bacteria that produce large amounts of protease, RNase and amylase were identified as *Stenotrophomonas rhizophila, Lysinibacillus fusiformis*, and *Pseudomonas stutzeri*, respectively.

Keywords: Karst cave, biodiversity, cultivated bacteria, RNase, protease, amylase

INTRODUCTION

Karst caves are underground cavities that encounter the earth's surface or are closed. They form as rainwater seeps into soluble rocks like limestone or gypsum. These nutrient-limited ecosystems feature constant low temperature, high humidity, darkness, low pressure, and low oxygen concentration. Caves create their own microclimatic and physicochemical conditions, giving rise to living organisms that exist in relative isolation from surface ecosystems. Comparative metagenomic analysis shows that cave microbial communities are specialized terrestrial communities that differ from communities found in oceans, soil, or the rhizosphere.[1] The poorly characterized microbial world found in caves is a potential source of antimicrobial and anticancer drugs[2] as well as microorganisms that produce novel agents. However, caves containing fossils, artifacts, Paleolithic paintings, and mineral deposits are prone to microbial damage, particularly during unregulated tourist visits. For example, only tourists have changed Morca Cave in Turkey after only a few years. Once dominated by *Thermoplasmata (Euryarchaeota), Gammaproteobacteria* and *Alphaproteobacteria*, the cave now features more bacteria belonging to *Bacilli* and *Bacteroidia*.[3] Therefore, understanding the microbial diversity in caves is a prerequisite to cave conservation, restoration, and safe cave tourism. It is believed that microbes in caves are most active on rocky surfaces, as most caves lack a significant layer of soil and sediment. In addition, microorganisms constantly move between the walls of the cave and sediments. Removing microbial groups from walls is often futile because soil and sediments are reservoirs of these microbial groups.[4]

These microorganisms not only should be preserved but also

Corresponding Author: Willam Kurdy **E-mail:** william.m.kurdy@hotmail.com
Submitted: 25.08.2023 • **Revision Requested:** 16.10.2023 • **Last Revision Received:** 20.10.2023 • **Accepted:** 10.11.2023 • **Published Online:** 21.12.2023

may secrete novel enzymes that can be used in industry, agriculture, and medicine.

Shulgan–Tash (or Kapova) Cave is located in the basin of the Belaya River in the Southern Urals within the Shulgan–Tash State Nature Reserve of the Republic of Bashkortostan; this karst cave is known worldwide for its rock art dating back to the Upper Paleolithic.[5] Seventeen main morphotypes have been identified in Kapova Cave. Communities isolated from the walls of the aphotic part of the cave include prokaryotes (bacteria, including *Actino-* and *Cyanobacteria*) and eukaryotes (yeasts and microscopic fungi).[6] Both metagenomics and traditional culturing methods have been performed to identify representatives belonging to *Proteobacteria, Actinobacteria, Firmicutes, Nitrospirae, Bacteroidetes, Verrucomicrobia* and *Acidobacteria* phyla as well as to isolate new Pseudomonas strains.[7]

Although karst caves present similar oligotrophic habitats, their microbial communities differ and are poorly understood. The bacterial taxa of Kapova Cave and the karst caves of China are dominated by two phyla, *Proteobacteria* and *Actinobacteria*.[8–10] In the Oylat Cave in Turkey, *Proteobacteria* dominate, followed by *Actinobacteria, Acidobacteria*, and *Nitrospirae*.[11] In Pertosa–Auletta Cave in southern Italy, *Proteobacteria* and *Acidobacteria*, dominate, followed by *Actinobacteria*.[12]

Elucidating the biodiversity of karst caves serves will inform the future discovery of useful microbial metabolites. Microorganisms secrete enzymes that benefit industry, agriculture, and medicine. For example, proteases are widely used in the food, leather, and feed industries as well as the production of detergents.[13] As proteases are degradative enzymes with high specificity and selectivity,[14] they can be used to process waste and optimize detergents. Moreover, proteases are widely used in medicine to treat burns, carbuncles, and wounds.[13] Amylase comprises approximately 25% of the world enzyme market[15] and is widely used in food applications like baking and brewing.[16] Extracellular bacterial RNases are also promising antiviral[17–20] and antitumor enzymes.[21–23]

Thus, work aimed to characterize the bacterial community collected by scraping visible colonies or biofilms from the walls of Kapova Cave and assess the ability of isolates to synthesize secreted hydrolytic enzymes, namely RNases, proteases, and amylases.

MATERIALS AND METHODS

Sampling Sites

Kapova Cave is located in the Shulgan–Tash State Nature Reserve of the Bashkortostan Republic; it was formed in a karst massif on the right slope of the river valley and is composed of massive limestones of the Viséan stage of the Lower Carboniferous. The cavity lies in the limestones of the lower part of the Carboniferous period. The cave is a weakly branched, three-story, gallery-type cavity that is 3328 m long, of which 782 m are underwater cavities.[5] Here, we used material collected by the Laboratory of Extreme Biology of Kazan Federal University in July 2019. Seven samples of visible colonies or biofilms were sampled from walls of different areas of the cave, whose locations are indicated in Figure 1.

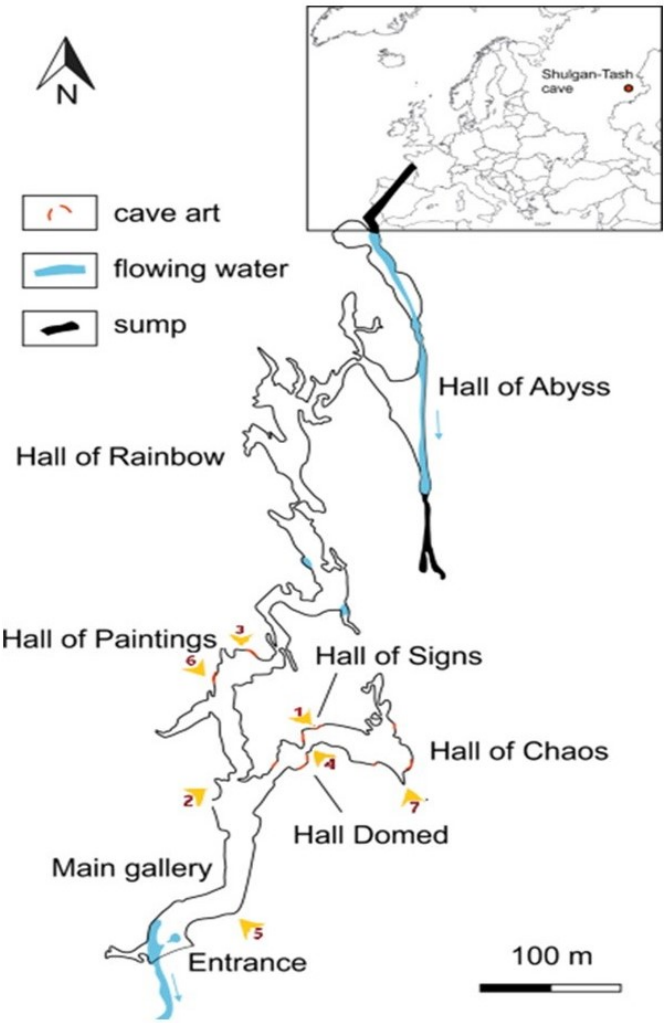

Figure 1. Kapova cave map with sampling locations labeled 1-7.

Pure Bacterial Culture Isolation

Samples were thoroughly mixed in 0.2 ml of a 0.5% NaCl solution and diluted tenfold in triplicate to minimize the bacterial count to use as an inoculation source. To isolate individual bacteria, inoculations, both deep and surface, were sewn on Luria-Bertani (LB), Reasoner's 2A (R2A) medium, and Gauze agar media to capture a spectrum of bacteria from the inoculates. LB is a nutrient-rich medium commonly used to cultivate bacte-

ria, especially members of *Enterobacteriaceae*. R2A Agar is a low-nutrient medium that stimulates the growth of stressed and chlorine-tolerant bacteria at lower incubation temperatures and longer incubation times. Gauze agar media is used to cultivate *Actinomycetes*. Here, microorganisms were cultivated at 30º for 48 h. To obtain pure cultures of microorganisms, colonies with similar morphologies were chosen for subsequent culture using the streak plate method on the appropriate medium twice to check colony purity. Pure cultures were stored in a 10% glycerol solution and frozen at -80°C.

16s rRNA Sequencing

A total of 102 bacterial pure cultures were identified according to their V3-V4 16S rRNA region sequence. Bacterial biomass was collected and placed in a 100°C hot bath for 5 min to disrupt cell integrity. V3 to V4 regions of 16S rRNA genes were then amplified using universal primers (515F and 806R), and an individual index was ligated for labeling isolates. The forward primer sequence was 5'-GTGCCAGCMGCCGCGGTAA-3', and the reverse primer sequence was 5'-GGACTACHVHHHTWTCTAAT-3'.[24] Reaction mixtures (10 µl) comprised 5 µl Hot Start High-Fidelity 2X Master Mix (BioLabs, New England), 0.5 µl primer mix with 5 µM forward and reverse primers, 2 µl gDNA, and 2.5 µl H_2O were used for amplification. The PCR cycles were as follows: (a) 95°C for 5 min; (b) 40 cycles, within each cycle 95°C for 30 s and 60°C for 30 s; and (c) 4°C hold. The concentration of DNA was determined using a Qubit 3.0 fluorometer (Life Technologies, USA) and calibrated to 50 ng/ml. Agencourt AMPure Beads (Beckman Coulter, USA) were used to clean the DNA according to the manufacturer instructions. Sequencing was performed with the MiSeq system (Illumina, USA) using paired-end 2 × 300 (for 16S rRNA genes). Resulting sequences were compared to the NCBI genomic database using the BLAST algorithm for nucleotides (BLASTn).[25] A maximum-likelihood phylogentic tree was created using MEGA-X software.[26]

Isolates with the highest metabolic activity were selected for further sequencing of the nearly full-length 16S rRNA gene following the previously described protocol with some modifications. The V1-V9 regions of the 16S rRNA gene were amplified with the following primers: forward primer (S-D-Bact-0008-c-S-20) with anchor sequence 5'-TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG-3' and reverse primer (1492R) with anchor sequence 5'-CTTGCCTGTCGCTCTATCTTCCGGYTACCTTGTTACGACTT-3'.[24]

Hydrolase Activity Measurement

Bacteria were screened for their ability to synthesize secreted ribonucleolytic, proteolytic and amylolytic enzymes on the following synthetic media, respectively:

(a) Phosphorus-free medium (pH = 8.5, 1L) of the following composition (g/L): Tris basic (hydroxylmethylaminomethane): 6.05; KCl: 5.0; NaCl: 1.0; $(NH_4)_2SO_4$: 2.0; $Na_3C_6H_5O_7$: 1.0; agar-agar: 20.0. Separately prepared: $MgSO_4.7\ H_2O$: 2 g/100 ml; yeast extract: 5 g/100 ml. Both solutions were added to the medium under sterile conditions at a rate of 10 ml/L. Before inoculation, a 40% sterile glucose solution was added to the medium at a rate of 12.5 ml/L as well as yeast RNA (Vector, Novosibirsk, Russia) to a final concentration of 5 mg/mL.

(b) A medium containing (g/L): yeast extract: 5.0; casein: 5.0; NaCl: 5.0; agar-agar: 20.0 (pH = 6.5).

(c) Nutrient agar supplemented with 8 g/L starch (pH = 6.8).

Bacterial cultures were sown on Petri dishes with three types of medium (a, b, c) and cultivated for 18 h at 30°C. Hydrolase activities were assessed by measuring the transparent region surrounding the colonies grown on the appropriate substrate (RNA, casein, starch) after the dishes were flooded with a 5% solution of 1 N HCl (colonies grown on medium a), trichloroacetic acid (on medium b), or Lugol's iodine solution (medium c) to visualize the hydrolysis zones. Jeffris et al. [27] used this method to assess RNase activity based on the size of clearance zone corresponding to extracellular enzymes production but not colony size, which we measured to measure different bacterial colonies as described by Price et al. for phospholipase activity detection.[28] Therefore, the hydrolase activity coefficient of a bacterial isolate was calculated as the ratio of the colony radius including the transparent zone surrounding it to the radius of the colony itself. The absence of a lysis zone surrounding colonies corresponds to the absence of secreted hydrolase. Larger transparent zones yield greater hydrolase activity coefficients expressed in conventional units; coefficients of 1, 1.5, and 2 indicate no extracellular hydrolase activity, medium activity, and high activity, respectively.

Prediction of Functional Profiles

Functional profiles of the communities were assessed using the Global Mapper module on iVikodak.[29] Values acquired represent the relative abundance of functional genes according to KEGG.

Statistical Analysis

Alpha diversity was assessed by two measures: taxonomic structural diversity using the Shannon–Wiener index and taxonomic compositional diversity. The Shannon–Wiener Index (H) was calculated using the following formula using Excel software:

$H = -å\ P_i\ (\ln P_i)$,

where P_i is the proportion of individuals belonging to the i-th genera in the dataset of interest.

To assess compositional taxonomic beta diversity, the Bray–Curtis index[30] was calculated as follows:

$BC_{ij} = 1 - (2C_{ij}/(S_i + S_j))$,

where C_{ij} is the sum of values only for common genera between two sampling sites; S_i and S_j are the number of isolates counted at sites i and j individually.

RESULTS

Taxonomic Identification of Bacterial Isolates

Of the 102 bacteria isolated from Kapova Cave, 99 were identified at the genus or family level according to their V3-V4 16S rRNA region sequences. 89% are members of the phylum Proteobacteria, whereas the others comprise three other phyla, *Actinobacteria, Firmicutes*, and *Bacteroidetes*, accounting for 5%, 4%, and 2% of the isolates, respectively (Figure 2). The composition of microbial communities did not significantly differ across sampling points. Two isolates were identified only at the family level as *Burkholderiaceae* and *Enterobacteriaceae* (Figure 3). These isolates are included in the phylogenetic tree at this level (Figure 4). Representatives of the genera *Pseudomonas* were predominant, with 42 isolates (Table 1).

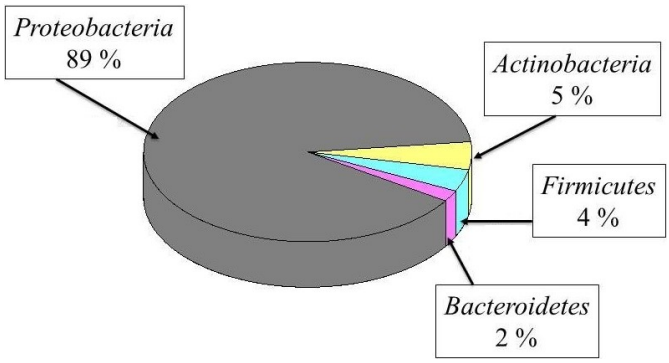

Figure 2. Distribution of bacterial isolates from Kapova cave according to phyla.

Diversity and Potential Functional Activity of Bacteria from Kapova Cave

Sample 7 had the best index of equally represented genera, followed by samples 6 and 1 (Table 2). The Bray–Curtis index of beta diversity ranges from 0 (when communities share the same isolates or phylogenetic lineages) to 1 (when communities do not share common phylogenetic lineages). Table 3 shows that samples 1 and 2 differ significantly from the rest of the samples.

Functional characteristics of the bacterial community from all seven samples were predicted using the Global Mapper module in the iVikodak software. The relative abundance of various metabolic pathways was considered, particularly those related to antimicrobial resistance, xenobiotic destruction, metabolism, and secretory systems. Figure 5 reveals that the community can metabolize different substrates and harbors antibiotic resistance genes.

Enzymatic Activity of Isolates

Six isolates showed no enzymatic activity (amylase, protease, RNase), whereas 73, 57, and 71 isolates expressed protease, amylase, and extracellular RNase activity, respectively. Thirty-nine isolates expressed all three extracellular enzyme activities (Figure 6).

Genera with the highest protease activity were *Pseudomonas, Stenotrophomonas, Bacillus, Acinetobacter*, and *Yersinia*. Genera with the highest RNase activity were *Pseudomonas, Bacillus, Yersinia, Acinetobacter, Lysinibacillus*, *Polaromonas*, and *Caulobacter*. Genera with the highest amylase activity were *Pseudomonas, Serratia, Yersinia*, and *Acinetobacter*.

Isolate 7 had the highest protease activity and was identified as *Stenotrophomonas rhizophila* (99.5%). Isolate 27 had the highest RNase activity and was identified as *Lysinibacillus fusiformis* (99.3%). Isolate 1 had the highest amylase activity and was identified as *Pseudomonas stutzeri* (99.0%). The genus *Pseudomonas* is a prolific producer of several extracellular enzymes, including amylase,[31,32] which is among the most important enzymes for biotechnology.[15]

DISCUSSION

Karst caves are extreme environments brimming with biodiversity; their food web structure,[33] diverse flora,[34] and influence of heavy metal enrichment are widely studied in China.[35] Furthermore, 40% of Turkey's surface area consists of soluble rocks (limestone, dolomite, and gypsum) suitable for karstification.[36] However, the biodiversity of Kapova Cave in the Urals is poorly characterized. Previous studies have estimated the microbial count (1.4×10^3–2.1×10^5 CFU/mL) in Kapova Cave and the possible origin of its microbiota.[6] Emerging technologies like high-throughput sequencing and bioinformatics have enhanced our understanding of microbial diversity.[37] Recent studies have revealed bacterial diversity in caves in Australia, China, Italy, Spain, Turkey, and the United States,[3,38–42] most of which are dominated by nine groups of domain Bacteria: *Proteobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Bacteroidetes*, *Gemmatimonadetes, Firmicutes, Nitrospirae*, and *Actinobacteria*, as well as domain Archaea.[38,42–44] The dominant phylum of Kapova Cave, the karst caves in China, and Oylat Cave is *Proteobacteria*, [8–11] whose members are Gram-negative bacteria. Environmental conditions in karst caves such as humidity and the presence of organic nutrient substrates likely promote the development of non-spore-forming bacteria. Yet the predominant species in the Yarık Sinkhole in

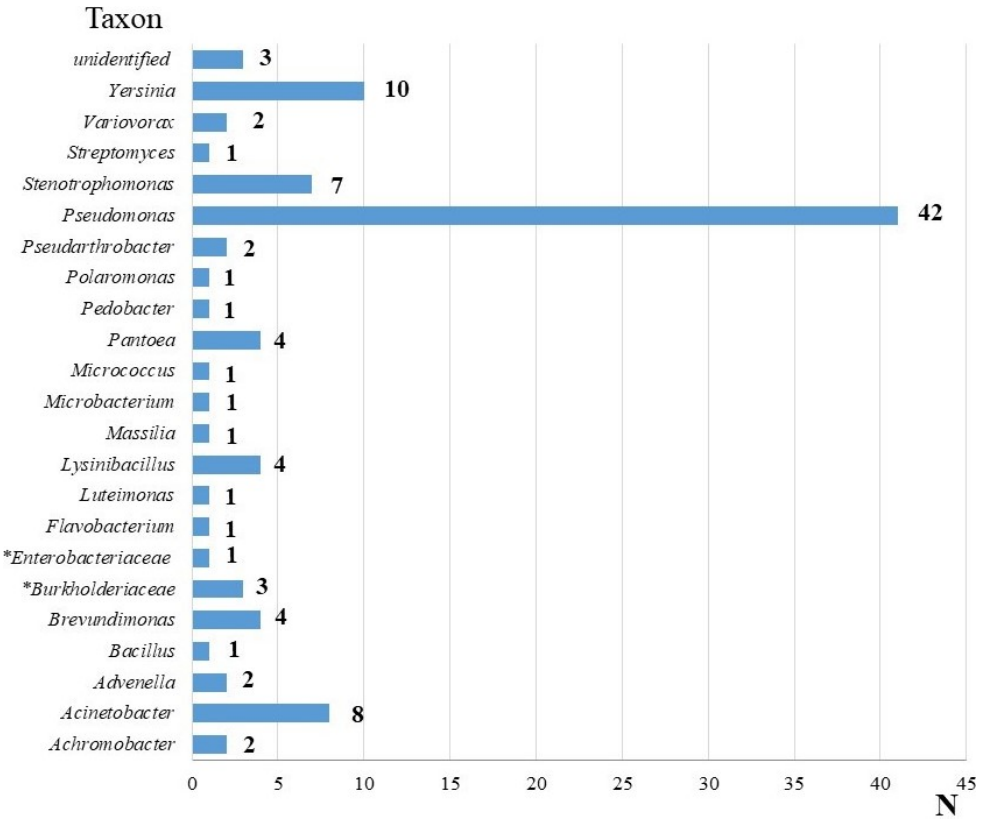

Figure 3. Number (N) of identified bacterial isolates. *Isolate identified only to the family level.

Turkey are *Acinetobacter lwoffii, Methylobacterium tardum*, and *Propionibacterium acnes*.[45] Although the bacteria were sampled during initial exploration, this composition suggests that nearby residents are harming the microbial diversity of the cave. In addition to preserving biodiversity, discovering new and potentially useful bacteria warrants further studies on the diversity of cultivated microorganisms. For example, more than a half of 290 *Actinomycetes* isolates from rock wall and speleothem surfaces of 19 karst caves in Turkey demonstrated antimicrobial activity against antibiotic-resistant bacteria. Strain *Streptomyces* sp. 1492 exhibited bacteriostatic or bactericidal activity against methicillin-resistant *Staphylococcus aureus*, vancomycin resistant *Enterobacter faecium*, and *Acinetobacter baumanii* at bactericidal concentrations lower than that of streptomycin.[46]

Among the isolates collected in this study, *Stenotrophomonas rhizophila* (isolate 7) expressed high levels of protease. This species was also found in the Herrenberg cave in Germany, though it is a plant-associated bacterium[47], as well as in semi-confined caves.[48] *Stenotrophomona* produces keratinases, extracellular proteases, and chitinases.[49] Unlike *S. maltophilia, S. rhizophila* cannot proliferate at 37°C and is therefore not pathogenic.

The isolate with the highest RNase activity (isolate 27) was identified as *Lysinibacillus fusiformis*. The *Lysinibacillus* genus, unlike *Bacillus*, contains peptidoglycan with lysine, aspartic acid, alanine, and glutamic acid.[50] Despite sharing many traits with *Bacillus, Lysinibacillus* is poorly characterized. New *Lysinibacillus* strains were recently isolated from a soil in karst caves in Libo County[51] and Xingyi county in China.[52] *In vitro* and *in vivo* assays showed that strain *Lysinibacillus* S4C11 exerts antifungal activity against various pathogens.[53] Similarly, an antifungal protein with RNase activity isolated from *Bacillus subtilis* inhibited mycelial growth in *Magnaporthe grisease, Sclerotinia sclerotiorum, Rhizoctonia solani, Alternaria oleracea, A. brassicae*, and *Botrytis cinerea*.[54] Bacillar RNases are well-known as antitumor [55] and antiviral [56] agents. Our research shows the potential of microbial genera other than bacilli to secrete RNases that may foster the discovery of new RNases to combat tumors, viruses, and pathogenic fungi.

Among bacteria, active producers of amylases include some bacilli like *B. macerans, B. polymyxa, B. subtilis*, and *B. stearothermophilus*.[15] Bacterial extracellular proteases and RNases are also predominantly synthesized by members of the genus *Bacillus*.[57] Although microorganisms including *Bacteroides bivius, Bacteroides melaninogenicus, Bacteroides fragilis, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Proteus* sp., and *Propionibacterium*

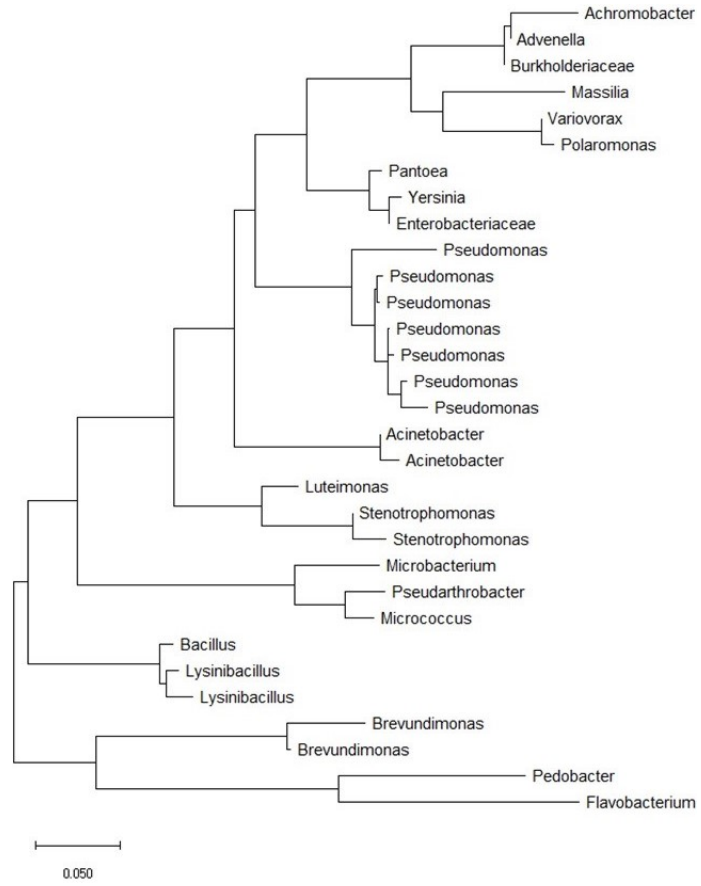

Figure 4. Phylogenetic tree of the isolated bacteria. Isolates with identical 16S rRNA sequences are represented only once.

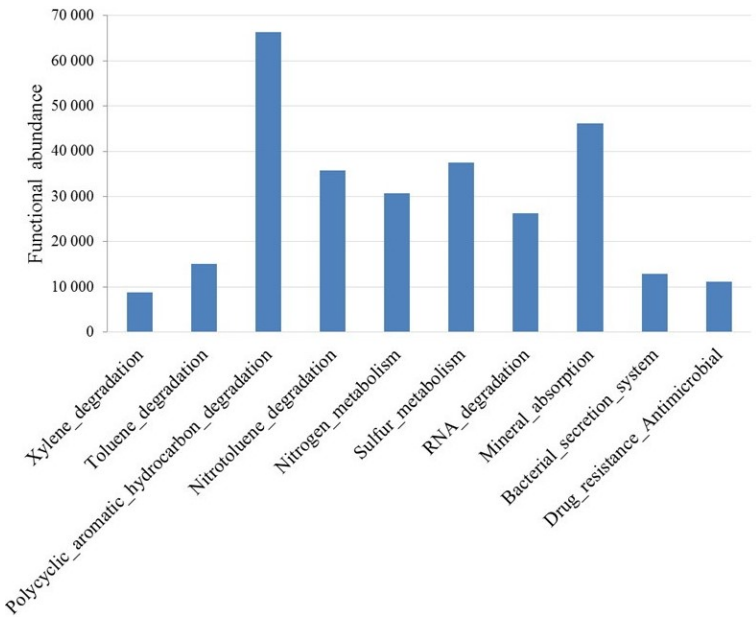

Figure 5. Functional profile of the bacterial community of Kapova cave. Functional abundance represents the number of genes responsible for functions.

Table 1. Identification of analysed isolates based on their 16s rRNA gene sequences.

No	Taxon	No	Taxon	No	Taxon
1	*Pseudomonas stutzeri*	35	*Pseudomonas*	69	*Pseudomonas*
2	*Pseudomonas*	36	*Yersinia*	70	*Pseudomonas*
3	*Pantoea*	37	*Yersinia*	71	*Pseudomonas*
4	*Pseudomonas*	38	Non identified	72	*Pseudomonas*
5	*Pseudomonas*	39	*Pantoea*	73	*Pseudomonas*
6	*Pseudomonas*	40	*Pantoea*	74	*Polaromonas*
7	*Stenotrophomonas rhizophila*	41	*Pseudomonas*	75	*Pseudomonas*
8	*Yersinia*	42	*Brevundimonas*	76	*Pseudomonas*
9	*Yersinia*	43	*Pseudomonas*	77	*Advenella*
10	*Yersinia*	44	*Pantoea*	78	*Pseudomonas*
11	*Achromobacter*	45	*Stenotrophomonas*	79	*Pseudomonas*
12	*Entero bacteriaceae*	46	*Pseudomonas*	80	*Pedobacter*
13	*Pseudomonas*	47	*Stenotrophomonas*	81	*Massilia*
14	*Acinetobacter*	48	*Stenotrophomonas*	82	*Advenella*
15	*Micrococcus*	49	*Yersinia*	83	*Lysinibacillus*
16	*Acinetobacter*	50	*Yersinia*	84	*Stenotrophomonas*
17	*Pseudomonas*	51	*Yersinia*	85	*Stenotrophomonas*
18	*Pseudomonas*	52	*Yersinia*	86	*Stenotrophomonas*
19	*Pseudomonas*	53	*Achromobacter*	87	*Flavobacterium*
20	*Burkholderiaceae*	54	*Acinetobacter*	88	*Pseudomonas*
21	*Burkholderiaceae*	55	*Acinetobacter*	89	Non identified
22	*Pseudomonas*	56	*Acinetobacter*	90	*Pseudarthrobacter*
23	*Pseudomonas*	57	*Acinetobacter*	91	*Pseudomonas*
24	*Bacillus*	57	*Yersinia*	92	*Pseudomonas*
25	*Pseudomonas*	59	*Acinetobacter*	93	*Pseudomonas*
26	*Pseudomonas*	60	*Burkholderiaceae*	94	*Streptomyces*
27	*Lysinibacillus fusiformis*	61	*Pseudarthrobacter*	95	*Brevundimonas*
28	*Pseudomonas*	62	*Microbacterium*	96	Non identified
29	*Pseudomonas*	63	*Acinetobacter*	97	*Pseudomonas*
30	*Pseudomonas*	64	*Pseudomonas*	98	*Pseudomonas*
31	*Lysinibacillus*	65	*Variovorax*	99	*Luteimonas*
32	*Lysinibacillus*	66	*Pseudomonas*	100	*Pseudomonas*
33	*Pseudomonas*	67	*Pseudomonas*	101	*Brevundimonas*
34	*Pseudomonas*	68	*Variovorax*	102	*Brevundimonas*

Table 2. Alpha diversity indices: R is the number of genera, Shannon-Wiener index is genera distribution uniformity.

Sample No	Number of isolates	Richness	Shannon-Wiener index
1	15	4	1.285
2	9	2	0.678
3	11	2	0.678
4	13	3	0.798
5	8	2	0.697
6	26	6	1.36
7	17	6	1.61

Table 3. Bray Curtis index of beta diversity: samples dissimilarity.

Sample No	1	2	3	4	5	6	7
1	0	1	1	1	1	1	1
2	1	0	1	1	1	1	1
3	1	1	0	0.6	0.6	0.83	0.7
4	1	1	0.6	0	0.8	0.63	0.6
5	1	1	0.6	0.8	0	0.82	0.8
6	1	1	0.83	0.6	0.8	0	0.8
7	1	1	0.67	0.6	0.8	0.79	0

acnes produce various proteases, [58] they are pathogenic, and therefore, not suitable for industrial enzyme production. Thus, the isolate *Pseudomonas stutzeri* (number 1) with high amylase activity is promising.

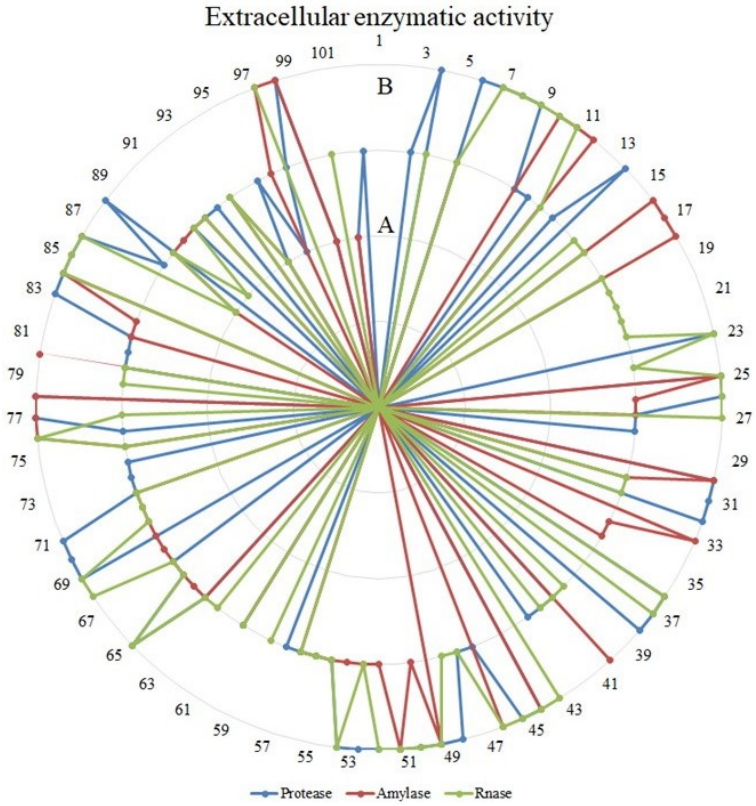

Figure 6. Hydrolytic activity of bacteria isolated from Kapova cave. Hydrolase activity coefficient of a bacterial isolate was calculated as the ratio of colony radius including transparency zone around it to radius of the colony itself. The absence of a lysis zone surrounding colonies indicates the absence of secreted hydrolase. Isolate numbers are circumscribed. Enzymatic activity coefficient value A equals to 1 and is interpreted as isolates not having extracellular hydrolase activity, and value B equals to 2 and is interpreted as possessing high activity.

CONCLUSION

The biodiversity and the ability of bacterial heterotrophic communities harvested from seven biofilms on the walls of Kapova Cave to synthesize secreted hydrolytic enzymes, namely RNases, proteases, and amylases, were explored in this study. Most isolates (89%) belonged to the phylum *Proteobacteria*, and others belonged to three other phyla: *Actinobacteria* (5%), *Firmicutes* (4%), and *Bacteroidetes* (2%). A total of 102 isolates were identified based on sequencing of the V3-V4 16S rRNA gene; among them, 42 belonged to the genus *Pseudomonas*. All three extracellular enzymes were expressed by thirty-nine isolates, and only six of them showed no enzymatic activity. Bacteria producing the highest level of protease, RNase, and amylase were identified as *Stenotrophomonas rhizophila, Lysinibacillus fusiformis, and Pseudomonas stutzeri*, respectively. Overall, these results highlight the importance of discovering microbials that may produce compounds that help develop novel therapeutic and biotechnological agents.

Acknowledgments: We thank the colleagues working within the framework of Program "Priority-2030" for biofilm sampling.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- O.I.; Data Acquisition- W.K., G.Y.; Data Analysis/Interpretation- O.I., W.K., G.Y.; Drafting Manuscript- O.I., W.K.; Critical Revision of Manuscript- G..Y; Final Approval and Accountability- O.I., W.K., G.Y.

Conflict of Interest: Authors declared no conflict of interest.

Financial support: This work was supported by the Russian Science Foundation grant No 22-24-00036.

ORCID IDs of the authors

Willam Kurdy 0009-0005-9405-3019
Galina Yakovleva 0000-0002-8504-3434
Olga Ilinskaya 0000-0001-6936-2032

REFERENCES

1. Ortiz M, Legatzki A, Neilson J W, et al. Making a living while starving in the dark: metagenomic insights into the energy dynamics of a carbonate cave. *ISME J*. 2014;8(2):478-491.
2. Zada S, Sajjad W, Rafiq M, et al. Cave microbes as a potential source of drugs development in the modern era. *Microb Ecol*. 2022;84:676-687.
3. Ahamada Rachid N, Doğruöz Güngör N. Major impacts of caving activities on cave microbial diversity: case study of Morca Cave, Turkey. *Int Microbiol*. 2023;26(2):179-190.
4. Adetutu EM, Ball AS. Microbial diversity and activity in caves. *Microb Australia*. 2014;35(4):192-194.
5. Abdullin SR, Kapralov SA, Kuzmina Y. *Biota of the cave Shulgan-Tash (Kapova)*, State reserve "Shulgan-Tash"; 2012. ISBN 5904555415, 9785904555412. (in Russian).
6. Kuzmina LY, Galimzyanova NF, Chervyatsova OY, Sailfullina NM, Kapralov SA, Ryabova AS. Biogenous fouling in Shulgan-Tash cave (Kapova, Southern Urals) and factors influencing on their expansion. *Ecobiotekh*. 2019;2(2):128-142.
7. Galimzianova NF, Gilvanova EA, Ryabova AS, Guvatova ZG, Kudryavtseva AV, Melentiev AI. Phylogenetic diversity of prokaryotes in microbial communities inhabiting rock surfaces of Shulgan-Tash (Kapova) cave, Southern Urals. *Ecobiotekh*. 2020;3(3):298-304.
8. Wang Y, Cheng X, Wang H, Zhou J, Liu X, Tuovinen OH. The characterization of microbiome and interactions on weathered rocks in a subsurface karst cave, Central China. *Front Microbiol*. 2022;13:909494. doi:org/10.3389/fmicb.2022.909494.
9. Zhu HZ, Zhang ZF, Zhou N, et al. Bacteria and metabolic potential in karst caves revealed by intensive bacterial cultivation and genome assembly. *Appl Environ Microbiol*. 2021;87(6):e02440-20. doi.org/10.1128/AEM.02440-20.
10. Cheng XY, Liu XY, Wang HM, et al. USC dominated community composition and cooccurrence network of methanotrophs and bacteria in subterranean karst caves, *Microbiol Spectr*. 2021;9(1): e0082021. doi:org/10.1128/Spectrum.00820-21.
11. Gulecal-Pektas Y. Bacterial diversity and composition in Oylat cave (Turkey) with combined Sanger/pyrosequencing approach. *Pol J Microbiol*. 2016;65(1):69-75.
12. Addesso R, Gonzalez-Pimentel JL, D'Angeli IM, et al. Microbial community characterizing vermiculations from karst caves and its role in their formation. *Microb Ecol*. 2021;81(4):884-896.
13. Razzaq A, Shamsi S, Ali A, et al. Microbial proteases applications. *Front Bioeng Biotechnol*. 2019;7:110. doi:org/10.3389/fbioe.2019.00110.
14. Rao RR, Vimudha M, Kamini N, Gowthaman M, Chandrasekran B, Saravanan P. Alkaline protease production from *Brevibacterium luteolum* (MTCC 5982) under solid-state fermentation and its application for sulfide-free unhairing of cowhides. *Appl Biochem Biotechnol*. 2017;182:511-528
15. De Souza PM, de Oliveira e Magalhães P. Application of microbial α-amylase in industry – A review. *Braz J Microbiol*. 2010;41(4):850-861.
16. Gopinath SC, Anbu P, Arshad MK, et al. Biotechnological processes in microbial amylase production. *Biomed Res Int*. 2017:1272193. doi:org/10.1155/2017/1272193.
17. Shah Mahmud R, Efimova MA, Ulyanova V. et al. *Bacillus pumilus* ribonuclease rescues mice infected by double-stranded RNA-containing reovirus serotype 1. *Virus Res*. 2020;286:198086. doi:10.1016/j.virusres.2020.198086.
18. Shah Mahmud R, Mostafa A, Müller C, et al. Bacterial ribonuclease binase exerts an intra-cellular anti-viral mode of action targeting viral RNAs in influenza a virus-infected MDCK-II cells. *Virol J*. 2018;15(1):5. doi:org/10.1186/s12985-017-0915-1.
19. Shah Mahmud R, Müller C, Romanova Y, et al. Ribonuclease from *Bacillus* acts as an antiviral agent against negative- and positive-sense single stranded human respiratory RNA. *Viruses Biomed Res Int*. 2017:5279065. doi:org/10.1155/2017/5279065, 2017.
20. 20. Shah Mahmud R, Efimova M, Mostafa A, Ulyanova V, Ilinskaya O. Antiviral activity of bacterial extracellular ribonuclease against single-, double-stranded RNA and DNA containing viruses in cell cultures. *BioNanoSci*. 2016;6:561-563.
21. Mitkevich VA, Pace CN, Koschinski A, Makarov AA, Ilinskaya ON. Cytotoxicity mechanism of the RNase Sa cationic mutants involves inhibition of potassium current through Ca^{2+} -activated channels. *Mol Biol*. 2015;49:933-938.
22. Ilinskaya ON, Shah-Mahmud RS. Ribonucleases as antiviral agents. *Mol Biol*. 2014;48(5):615-623.
23. Khodzhaeva V, Makeeva A, Ulyanova V, et al. Binase immobilized on halloysite nanotubes exerts enhanced cytotoxicity toward human colon adenocarcinoma cells. *Front Pharmacol*. 2017;8:631. doi:org/10.3389/fphar.2017.00631.
24. Walters W, Hyde ER, Berg-Lyons D, et al. Improved bacterial 16S rRNA gene (v4 and v4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems*. 2016;1(1):e00009-15. doi:10.1128/mSystems.00009-15.
25. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: A better web interface. *Nucleic Acids Res*. 2008;36:W5-9. doi:10.1093/nar/gkn201
26. 26. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35(6):1547-1549.
27. 27. Jeffris GD, Holtman WF, Guse D. Rapid method for determining the activity of microorganisms on nucleic acids. *J Bacteriol*. 1957;73:591-601.
28. Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans. Sabouraudia*. 1982;20(1):7-14.
29. Nagpal S, Haque MM, Singh R, Mande SS. iVikodak – A platform and standard workflow for inferring, analyzing, comparing, and visualizing the functional potential of microbial communities, *Front Microbial*. 2019;9:1-15.
30. Bray JR, Curtis JT. An ordination of the upland forest communities of Southern Wisconsin. *Ecological Monographs*. 1957;27:325-349.
31. Klinfoong R, Thummakasorn C, Ungwiwatkul S, BOONTANOM P, Chantarasirid A. Diversity and activity of amylase-producing bacteria isolated from mangrove soil in Thailand. *Biodiversitas* 2022;23(10):5519-5531. doi:10.13057/biodiv/d231064.
32. Khannous L, Jrad M, Dammak M, et al. Isolation of a novel amylase and lipase-producing *Pseudomonas luteola* strain: Study of amylase production conditions. *Lipids Health Dis*. 2014;13:9. doi:10.1186/1476-511X-13-9.
33. Xu C, Zhao L, Du W, Zhang S, Wu Y, Zhou F. Food sources and trophic levels of terrestrial cave fauna in Yuping Town, Libo County, Guizhou Province. *Biodiv Sci*. 2021;29(8): 1108-1119.
34. Fu L, Monro AK, Wei Y. Cataloguing vascular plant diversity of karst caves in China. *Biodiv Sci*. 2022;30(7):21537. doi:org/10.17520/biods.2021537.
35. Liu R, Zhang Z, Shen J, Wang Z. Community characteristics of

bryophyte in Karst caves and its effect on heavy metal pollution: A case study of Zhijin Cave, Guizhou Province, *Biodiv Sci*. 2018;26(12):1277-1288.

36. Nazik L, Poyraz M, Karabiyikoglu M. Karstic Landscapes and Landforms in Turkey. In book: *Landscapes and Landforms of Turkey*. Chapter: 5. Springer Nature; 2019:181-196. doi:10.1007/978-3-030-03515-0_5.
37. Gao C, Guo L. Progress on microbial species diversity, community assembly and functional traits. *Biodiv Sci*. 2022;30(10):22429. doi:org/10.17520/biods.2022429.
38. Chelius MK, Beresford G, Horton H, et al. Impacts of alterations of organic inputs on the bacterial community within the sediments of wind cave, South Dakota, USA. *Int J Speleol*.2009:38(1):1-10. doi:10.5038/1827-806X.38.1.1.
39. Holmes AJ, Tujula NA, Holley M, et al. Phylogenetic structure of unusual aquatic microbial formations in Nullarbor caves, Australia. *Environ Microbiol*. 2001;3(4):256-264.
40. Macalady JL, Jones DS, Lyon EH. Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. *Environ Microbiol*. 2007;9(6):1402-1414.
41. Schabereiter-Gurtner C, Saiz-Jimenez C, Piñar G, Lubitz W, Rölleke S. Phylogenetic diversity of bacteria associated with Paleolithic paintings and surrounding rock walls in two Spanish caves (Llonín and La Garma). FEMS *Microbiol Ecol*. 2004;47(2):235-247.
42. Zhou JP, Gu YQ, Zou CS, Mo MH. Phylogenetic diversity of bacteria in an earth-cave in Guizhou province, southwest of China. *J Microbiol*. 2007;45(2):105-112.
43. Gonzalez JM, Portillo MC, Saiz-Jimenez C. Metabolically active crenarchaeota in Altamira Cave. *Naturwissenschaften*. 2006;93(1):42-45.
44. Leuko S, Koskinen K, Sanna L, et al. The influence of human exploration on the microbial community structure and ammonia oxidizing potential of the Su Bentu limestone cave in Sardinia, Italy. *PLoS One*. 2017;12(7):e0180700. doi:org/10.1371/journal.pone.0180700.
45. Doğruöz-Güngör N, Arslan-Aydoğdu EÖ, Dirmit E, Usuloğlu E. Anthropogenic impacts on the bacterial profile of Yarik Sinkhole in Antalya, Turkey. *J Caves Karst Stud*. 2020;82(2):116-124.
46. 46. Yücel S, Yamaç M. Selection of *Streptomyces* isolates from Turkish karstic caves against antibiotic resistant microorganisms. *Pak J Pharm Sci*. 2010;23(1):1-6.
47. Rusznyák A, Akob DM, Nietzsche S, et al. Calcite biomineralization by bacterial isolates from the recently discovered pristine karstic herrenberg cave, *Appl Environ Microbiol*. 2012;78(4):1157-1167.
48. Cuzman OA, Luvidi L, Colantonio C, et al. Biodiversity and conservation correlation in the case of a Roman fresco located in a semi-confined environment. *Int Biodet Biodeg*. 2023;181:105605. doi.org/10.1016/j.ibiod.2023.105605.
49. Pinski A, Zur J, Hasterok R, Hupert-Kocurek K. Comparative genomics of *Stenotrophomonas maltophilia* and *Stenotrophomonas rhizophila* revealed characteristic features of both species. *Int J Mol Sci*. 2020;21(14):4922. doi:org/10.3390/ijms21144922.
50. Ahmed I, Yokota A, Yamazoe A, Fujiwara T. Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. *Int J Syst Evol Microbiol*. 2007;5:1117-1125.
51. Kan Y, Niu XK, Rao MPN, et al. *Lysinibacillus cavernae* sp. nov., isolated from cave soil. *Arch Microbiol*. 2020;202(6):1529-1534.
52. Narsing Rao MP, Dong Z.Y, Niu XK, et al. *Lysinibacillus antri* sp. nov., isolated from cave soil. *Int J Syst Evol Microbiol*. 2020;70(5):3295-3299.
53. Passera A, Rossato M, Oliver JS, et al. Characterization of *Lysinibacillus fusiformis* strain S4C11: *In vitro*, in planta, and in silico analyses reveal a plant-beneficial microbe. *Microbiol Res*. 2021;244:126665. doi:org/10.1016/j.micres.2020.126665.
54. Liu Y, Chen Z, Ng TB, et al. Bacisubin, an antifungal protein with ribonuclease and hemagglutinating activities from *Bacillus subtilis* strain B-916. *Peptides*. 2007;28(3):553-559.
55. Makarov AA, Ilinskaya ON. Cytotoxic ribonucleases: molecular weapons and their targets, *FEBS Lett*. 2003;540(1-3):15-20.
56. Ilinskaya ON, Shah-Mahmud RS. Ribonucleases as antiviral agents, *Mol Biol*. 2014;48(5):615-623.
57. Ulyanova V, Shah Mahmud R, Dudkina E, Vershinina V, Domann E, Ilinskaya O. Phylogenetic distribution of extracellular guanyl-preferring ribonucleases renews taxonomic status of two *Bacillus* strains. *J Gen Appl Microbiol*. 2016;62(4):181-188.
58. McGregor JA, Lawellin D, Franco-Buff A, Todd JK, Makowski EL. Protease production by microorganisms associated with reproductive tract infection. *Am J Obstet Gynecol*. 1986;154(1):109-114.

How cite this article

Kurdy W, Yakovleva G, Ilinskaya O. Bacterial Biodiversity of the Kapova Karst Cave as a Source of Hydrolases Producers. Eur J Biol 2023; 82(2): 186–195. DOI: 10.26650/EurJBiol.2023.1349885