https://doi.org/10.1595/205651322X16482034395036 *Johnson Matthey Technol. Rev.*, 2023, **67**, (2), 159–170

JOHNSON MATTHEY
TECHNOLOGY REVIEW

technology.matthey.com

Screening of Bioactive Compounds for Biomedical and Industrial Uses from *Actinobacteria* Isolated from the Parsık Cave, Turkey

Identifying novel compounds from extreme environments

Nahdhoit Ahamada Rachid*
Institute of Graduate Studies in Sciences, Istanbul University, Balabanaga Mah. Sehzadebasi Cd., 34134 Vezneciler, Fatih-Istanbul, Turkey

Nihal Doğruöz Güngör
Department of Biology, Faculty of Science, Istanbul University, 34134 Vezneciler, Istanbul, Turkey

***Email:** nahdhoit7@gmail.com

PEER REVIEWED

Received 3rd January 2022; Revised 7th March 2022; Accepted 24th March 2022; Online 25th March 2022

The need to avoid health issues and pollution of the environment from the use of chemicals and synthetic materials inspires scientists to search for new biological compounds beneficial to human beings. Caves, being extreme environments, might be potential sources of these compounds. *Actinobacteria*, one of the main groups that colonise these environments, are known to generate natural bioactive compounds. To investigate the potential uses of Parsık Cave *Actinobacteria*, identification of this group of isolates and the investigation of their secreted biological compounds constituted the principal aim of the present study. The identification was achieved by sequencing 16S rRNA genes of 41 selected bacteria of which 28 species were identified as *Actinobacteria*. *Microbacterium* (21%) and *Pseudarthrobacter* (14%) were the most identified *Actinobacteria* genera. Antimicrobial effects of the isolates P1 and P16 were observed against standard microorganisms like *Candida albicans*. The gas chromatography-mass spectrometry (GC-MS) analysis of their broth showed compounds with known antimicrobial, antioxidant or anticancer properties as well as unknown compounds. Polyketide synthase (PKS) and non-ribosomal peptide synthases (NRPS) respectively were amplified in 32.1% and 53.5% of the identified *Actinobacteria* while 25% were found to have both NRPS and PKS amplified. Amylase, gelatinase, cellulase, deoxyribonuclease (DNase), urease and casein hydrolysing activities were observed in the identified *Actinobacteria*. These results show that *Actinobacteria* from Parsık Cave might be good sources of industrial and biotechnological compounds. Furthermore, discovery of new bioactive compounds from these bacteria is promising due to the many unknown compounds observed in the GC-MS analysis and the high percentage of NRPS and PKS gene amplification.

1. Introduction

Identification of novel bioactive compounds is a big challenge in biotechnology and industry. The problems of drug resistance may be improved by innovation and the discovery of new antibiotic, anticancer, antiviral or other biomedical products. Furthermore, the costs, health and environmental issues associated with using chemical catalysts

may be mitigated by use of biocatalysts (enzymes). Microorganisms are thought to be the main candidates to produce such compounds. New or unculturable species are highly promising for the possibility of obtaining new compounds; unexplored environments which remain generally located far away from human activities are a likely source of such novel microorganisms (1–3).

Caves constitute one of these interesting environments. They can be defined as natural underground openings large enough for human access. These dark chambers are considered extreme ecosystems usually characterised by a constant low temperature, high relative humidity, low oxygen and poor nutrient availability (4–6). Despite their extreme conditions, microorganisms are thought to play an important role in the formation of caves through the formation of cave speleothems like 'moonmilk' and accumulation of mineral oxides (7). In fact, caves are found to be colonised by extremophile organisms such as psychrotolerant, psychrophiles, halophiles and basophiles (8, 9).

Cave microbiology consists of studying cave microbial diversity, defining the biogenic role of this microbiota in different niches of the cave and exploring their potential uses in industrial and biotechnological fields. Both cultural-dependent and culture-independent methods are used in cave microbiological studies (7, 10, 11). Through these two strategies, bacteria appear as the most identified cave microorganisms (10, 11). Actinomycetes are high guanine and cytosine content, gram-positive bacteria which constitute a large group of cave bacteria. They are mainly isolated from moonmilk deposits and other surface formations. They also inhabit cave sediments and can colonise cave waters albeit in low proportions (10–13). These filamentous bacteria are well recognised for their resistance under extreme ecological conditions due to their metabolic versatility (14).

In general, *Actinobacteria* are well known for their secretion of primary and secondary metabolites which are valuable bioactive compounds with biomedical (antibiotic, anticancer, antioxidant) and biotechnological (enzymes, siderophores, bioremediation, self-healing concretes) properties (15, 16). It is reported that approximatively 66% of antibacterial compounds in the world are from bacteria belonging to this phylum. 55% of those compounds are from species of *Streptomyces* while the remaining 11% are from other *Actinobacteria* (15, 17). A wide range of industrial enzymes such as amylases, proteases and cellulases have been positively screened in *Actinobacteria* including *Nocardiopsis* sp., *Streptomyces pactum* and *Thermomonospora* sp. (18).

Microbes inhabiting caves have unique metabolisms to cope with specific conditions like poor nutrient availability. The oligotrophy in caves can induce nutrient competition between bacteria and hence, development of antimicrobial compounds in these bacteria (11). Caves are also mineral-rich environments. During biomineralisation, actinomycetes species secrete extracellular enzymes that are active under extreme conditions: for example, ureolytic activity (urease) in *Streptomyces* species has been reported during microbial-induced calcium precipitation (19). Some bioactive compounds can be produced in small amounts and specific inducers (physical or chemical) are sometimes required for their synthesis. For this reason, screening microorganisms for microbial bioactive compounds under laboratory conditions may sometimes be complicated (1, 20).

In this context, screening for the presence of genes encoding the compounds is also required. Furthermore, the characterisation and identification of these compounds requires additional tests other than phenotypic and genotypic analyses. PKS and NRPS are groups of enzymes coded by genes to produce important secondary metabolites (21, 22). The presence of these genes in microorganisms is thought to be highly related to their biosynthetic activities. Therefore, targeting these genes is an important way to screen the bioactive potential of these bacteria.

In our study, we focused on the screening of bioactive compounds (enzymes and antimicrobial compounds) of potential *Actinobacteria* strains from our previous study (11). Here, a group of industrially interesting enzymes have been phenotypically screened and the antimicrobial potential of the isolates has been studied through both phenotypic and genotypic analyses. Some of the bioactive compounds have been identified through GC-MS analysis.

2. Material and Methods

2.1 Molecular Identification of Species

A total of 41 isolates were selected according to the medium used for their isolation including actinomycetes-specific media: actinomycetes isolation agar, starch casein agar, international

Streptomyces project-2 medium and Reasoner's 2A agar. DNA of these isolates was isolated using the Invitrogen™ PureLink™ Genomic DNA Mini Kit K182001 (Invitrogen™, USA) according to the manufacturer's procedure. To amplify the 16s rRNA gene, the polymerase chain reaction (PCR) was run using universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- ACG GCT ACC TTG TTA CGA CTT-3') under these conditions: initial denaturation at 94°C for 30 s, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s, then a final extension step was performed at 72°C for 10 min. The resulting PCR products were run on 1% agarose gel by electrophoresis before sequencing using the Sanger DNA sequencing method.

2.2 Screening for Non-Ribosomal Peptide Synthases and Polyketide Synthase Genes in *Actinobacteria* from Parsık Cave

Primers coding the NRPS and PKS gene regions of bacteria were used to screen the enzyme-encoding gene clusters in the identified *Actinobacteria.* They consist of A7R (5'-SASGTCVCCSGTSCGGTAS-3') / AF3 (5'-GCSTACSYSATSTACACSTCSGG-3') for NRPS gene cluster and DegKS2R (5'-GTICCIGTICCRTGISCYTCIAC-3') / DegKS2F (5'-GCIATGGAYCCICARCARMGIVT-3') for PKS gene cluster (23). PCR was run following the methods proposed by Amos *et al*. (23) with some modification. For the A7R/AF3 primers, PCR was run under the following conditions: primary denaturation at 95°C for 5 min followed by 35 cycles (denaturation at 94°C for 35 s, annealing at 55°C for 40 s and extension at 72°C for 1 min) and final elongation at 72°C for 8 min. PCR for the PKS region was run under the following conditions: primary denaturation at 94°C for 4 min followed by 35 cycles of (denaturation at 94°C for 35 s, annealing at 55.6°C for 40 s and primary extension at 72°C for 75 s) and elongation at 72°C for 8 min. The PCR products were run in a 1.2% gel electrophoresis to visualise the bands.

2.3 Antimicrobial Production Potentiality of the Isolates

Identified *Actinobacteria* were tested against selected standard microorganisms constituted of pathogens or opportunistic pathogens of Gram-negative and Gram-positive bacteria and one fungi strain: *Escherichia coli* (ATCC® 8739™), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC® 33591™), *Pseudomonas aeruginosa* (ATCC® 9027™), *Staphylococcus aureus* (ATCC® 6538™), *Staphylococcus epidermidis* (ATCC® 12228™) and *C. albicans* (ATCC® 10231™). Fresh culture of each isolate was used to prepare 0.5 McFarland and 100 µl of each suspension was spread onto 1/2 tryptic soy agar. The incubation was at 30°C for 48 h. 0.5 McFarland of each standard microorganism was also prepared and 100 µl of each suspension was spread to tryptic soy agar medium and incubated at room temperature for 3–5 min. After that, discs of the isolates (6 mm) were cut off from the 48 h cultures and inversely placed onto the plates containing standard microorganisms. After 24 h at 30°C, every standard Petri plate was observed for the inhibition zone around the isolate's discs. Isolates with phenotypical antimicrobial activities were selected for characterisation of bioactive compounds.

2.4 Characterisation of Bioactive Compounds

Fresh cultures were inoculated on tryptic soy broth for 24 h. The broth was chromatographically separated using an Agilent 1260 Infinity II LC System (Agilent Technologies Inc, USA) instrument and Agilent Poroshell 120 EC-C18 Threaded Column (3.0 mm × 150 mm, 2.7 µm particle size). A gradient elution of 5 mM ammonium formate in water (A) and methanol (B) was used to construct the mobile phase system. This was as follows: 0–0.5 min, 10% B; 0.5–5 min, 70% B; 5–7 min, 95% B; 7–10 min, 95% B; 10–15 min, 10% B. Sample injection was 2 µl with a flow rate of 0.5 ml min^{-1} and column oven maintained at 25°C.

Mass spectroscopy was performed using an Agilent 6550 iFunnel equipped with the high-resolution accurate mass quadrupole time of flight/mass spectroscopy sheath gas flow (nitrogen 12 l min^{-1}) and the selected scanning range m/z was between 50 and 1800. Samples were screened using Agilent MassHunter METLIN Metabolite Personal Compound Database and Library (Metlin_AM_PCDL.cdb).

2.5 Screening of Enzymatic Activities of Parsık Cave *Actinobacteria*

The isolates were screened for seven enzymatic activities: amylase, lipolytic activity (Lip.A),

cellulase, casein hydrolase (Cas.Hydrolase), DNase, urease, proteinase and gelatinase. Except for DNase and gelatinase activities, a fresh culture of each isolate was inoculated in media composed of minimal salt agar medium and enzyme specific substrates. 1000 ml of minimal salt agar medium was composed of 0.1 g NaCl, 1 g KH_2PO_4, 0.1 g $MgSO_4$, 5 g $(NH_4)_2SO_4$ and 15 g agar. For DNase and gelatinase activities, respectively, DNase test agar and gelatine were directly used. During the incubation period (3–10 days) at 30°C, the growth of isolates was checked daily and clear zones, growth or change in the colour were checked for positive or negative activities (9).

3. Results and Discussion

3.1 Identification of Isolates

The identified species in our study are presented in **Table I**. From the 41 selected isolates, 28 have been identified as *Actinobacteria*. The remaining isolates are constituted of *Proteobacteria, Firmicutes* and three others which were not identified in the National Center for Biotechnology Information (NCBI, **Table I**). As previously mentioned, the bacterial diversity of sediment and surface samples from different karstic caves has been reported with a high rate of *Actinobacteria* (10–12). Some *Actinobacteria* strains like *Streptomyces* species are known for their role in the formation of caves and cave speleothems (14, 17). This can be achieved through their metabolic reactions in the presence of some chemicals in the cave ecosystems (14). Genera like *Nocardia* and *Rhodococcus* identified in our study have been reported with potential for degradation of hazardous chemicals and organic matter (24, 25). They may play an important role in the bioremediation of natural ecosystems and polluted environments.

3.2 Screening of Antimicrobial Production Potential of the Isolates

Our study aimed to determine the bioactive potentials of *Actinobacteria* isolated from Parsık Cave. 53% of the isolates showed bands for NRPS gene after running their PCR products on an agarose gel electrophoresis. 32% of the identified *Actinobacteria* showed bands for PKS gene after running their PCR products on an agarose gel electrophoresis. Isolate P1 showed a band for both regions while neither region was amplified in P16. These genes encode enzyme groups that play an important role in the synthesis of different bioactive compounds including some with antimicrobial, antioxidant and anticancer activities (26–28). These observations bring us back to the potential uses of *Actinobacteria* isolated from the Parsık Cave in the biotechnological and pharmaceutical industries.

However, further tests are recommended to identify the different biological activities of these isolates. One of these tests is the antimicrobial production assay. This test was done by testing the phenotypical response of the isolates against some standard bacteria and one *Candida* species. Only two isolates showed antimicrobial activity. These were the P1 and P16 isolates. The isolate P1, identified as *Streptomyces exfoliatus*, showed antimicrobial activity against the fungal strain *C. albicans*. Antimicrobial potential in *Streptomyces* species has been observed previously in different studies with *Streptomyces* isolated from different environments (29–32). Sharma *et al*. showed through *in vitro* analyses the antifungal potential of *Streptomyces exfoliates* MT9 against wood rotting fungi (33). Inhibition zones were observed around isolate P16, identified as *Micrococcus* sp. TM4_2, when tested against *S. aureus* and *S. epidermidis*. The growth inhibition of the pathogen *S. aureus* and the opportunistic pathogen *S. epidermidis* by isolate P16 shows the antibacterial potential of this isolate and promises biomedical uses for this isolate and its extracts.

Results observed through the genotypic test, contrary to the antimicrobial tests, showed high potential bioactive compound production in our isolates. This great difference between the phenotypical and genotypical results may be explained through different hypotheses such as the laboratory conditions (culture medium or temperature) which may not be favourable to phenotypical expression in the other isolates. Besides that, these isolates may show antimicrobial activities against other standard microorganisms which were not used in our study. The presence of biological activities other than antimicrobial activity like anticancer or antioxidant activity may also explain this observation.

3.3 Gas Chromatography-Mass Spectrometry Analysis of P1 and P16

The two gene clusters observed in the isolate P1 brings us back to the high potential of bioactive compounds that can be secreted by *Actinobacteria*

Table I Enzymatic Activities of Parsık Cave *Actinobacteria*

Isolates	Name	Similarity, %	Amylase	Lip.A	Gelatinase	Urease	Cas. Hydrolase	DNAase	Cellulase
P1	*Streptomyces exfoliatus*	100	(+)	(+)	(–)	(+)	(+)	(–)	(+)
P2	*Agromyces cerinus subsp. Cerinus*	99.00	(–)	(–)	(–)	(–)	(–)	(–)	0
P3	*Nocardioides caeni*	93.41	(–)	(–)	(+)	(+)	(–)	(–)	0
P4	*Propionicimonas paludicola*	91.89	(–)	(–)	(–)	(+)	(–)	(–)	(–)
P5	*Microbacterium paraoxydans*	99.00	(–)	(–)	(–)	(+)	(+)	(–)	0
P6	*Arthrobacter humicola*	96.08	(+)	(–)	(–)	(+)	(+)	(+)	(–)
P7	*Nocardia globerula*	97.99	(–)	(–)	(–)	(+)	(–)	(–)	(–)
P8	*Microbacterium maritypicum*	98.15	(–)	(–)	(–)	(–)	(–)	(–)	0
P9	*Microbacterium esteraromaticum*	91.13	(–)	(–)	(–)	(+)	(+)	(–)	0
P10	*Pseudarthrobacter oxydans*	98.02	(+)	(–)	(–)	(+)	(+)	(–)	0
P11	*Pseudarthrobacter oxydans*	93.54	(–)	(–)	(–)	(+)	(+)	(–)	(–)
P12	*Microbacterium paraoxydans*	99.21	(–)	(–)	(–)	(–)	(–)	(–)	0
P13	*Streptomyces spororaveus*	97.51	(+)	(–)	(–)	(–)	(–)	(–)	(–)
P14	*Arthrobacter halodurans*	91.00	(–)	(–)	(–)	(+)	(+)	(–)	0
P15	*Micrococcus antarcticus*	96.09	(–)	(–)	(–)	(–)	(+)	(+)	(–)
P16	*Micrococcus* sp. TM4_2	98.15	(–)	(–)	(–)	(+)	(–)	(–)	0
P17	ND	ND	(–)	(–)	(–)	(+)	(+)	(–)	(–)
P18	ND	ND	(–)	(–)	(–)	(–)	(–)	(–)	0
P19	*Pseudarthrobacter polychromogenes*	94.48	(–)	(–)	(–)	(–)	(+)	(–)	0
P20	*Rhodococcus degradans*	88.06	(–)	(–)	(–)	(+)	(–)	(–)	(–)
P21	*Kocuria rosea*	86.28	(+)	(–)	(–)	(+)	(–)	(–)	(+)
P22	*Nocardia coeliaca*	96.38	(–)	(–)	(+)	(+)	(+)	(–)	(–)
P23	ND	ND	(–)	(–)	(–)	(–)	(+)	(+)	0
P24	*Microbacterium paraoxydans*	97.11	(+)	(–)	(–)	(–)	(+)	(–)	0
P25	*Oerskovia turbata* NBRC 15015	93.79	(+)	(+)	(–)	(–)	(+)	(–)	(–)
P26	*Kocuria rosea*	99.10	(+)	(+)	(–)	(+)	(+)	(–)	(+)
P27	*Kocuria rosea*	99.27	(+)	(–)	(–)	(+)	(+)	(–)	(–)
P28	*Microterricola gilva*	99.31	(–)	(–)	(–)	(–)	(–)	(–)	0
P29	*Pseudarthrobacter siccitolerans*	94.76	(–)	(–)	(–)	(–)	(–)	(–)	(–)
P30	*Streptomyces nojiriensis*	96.29	(–)	(–)	(–)	(–)	(–)	(–)	(–)

Table I (*Continued*)

Isolates	Name	Similarity, %	Amylase	Lip.A	Gelatinase	Urease	Cas. Hydrolase	DNAase	Cellulase
P31	*Microbacterium paraoxydans*	97.30	(–)	(–)	(–)	(+)	(–)	(–)	(–)
P32	*Exiguobacterium undae*	98.69	(–)	(–)	(–)	(–)	(+)	(–)	0
P33	*Paenibacillus* sp. CT–7	98.62	(+)	(–)	(–)	(–)	(–)	(–)	0
P34	*Pseudomonas putida*	98.08	(–)	(–)	(–)	(+)	(–)	(–)	(–)
P35	*Bacillus aryabhattai* B8W22	99.23	(+)	(–)	(–)	(+)	(+)	(–)	(–)
P36	*Acinetobacter guillouiae*	96.94	(–)	(–)	(–)	(+)	(–)	(–)	(–)
P37	*Stenotrophomonas rhizophila*	99.00	(–)	(–)	(–)	(+)	(+)	(–)	(–)
P38	*Pseudomonas psychrophila*	99.41	(+)	(–)	(–)	(+)	(–)	(–)	0
P39	*Pseudomonas* sp.	99.80	(–)	(–)	(–)	(+)	(+)	(+)	0
P40	*Citrobacter freundii*	99.00	(–)	(+)	(–)	(+)	(–)	(–)	0
P41	*Pseudomonas azotoformans*	96.92	(–)	(–)	(–)	(+)	(+)	(–)	0

0 = No growth; (+) = positive results; (–) = negative results; ND = non-determined

species. In addition, the antimicrobial activity phenotypically observed in the isolate P16 shows the capacity for biological compound secretion in this isolate. The GC-MS analysis of P1 and P16 isolates revealed multiple products of which some were unknown. Some of the known products revealed in this study are shown in **Table II** and **Table III**. These results show a high rate of amino acids as well as different secondary metabolites. Among the bioactive compounds observed in the isolate P1 (**Table II**), compounds with antimicrobial functions like azaserine, adefovir dipivoxil, valclavam and leucomycin A7/A4 have been identified. Leucomycin A7/A4 are macrolide antibiotics biosynthesised by PKS enzymes (26). They are previously known to be secreted by *Streptomyces* species (26, 27). The insecticidal wilfordine and the anticancer agent pectenotoxin-2 have been also revealed in our study. Neomycin A, identified in the isolate P16, has been reported with high antibacterial activity against tuberculosis agent (28). The antifungal peptide coccinin has been also identified in the isolate P16 and was reported in previous studies with leukaemia cell proliferation inhibition activity and reduced activity of human immunodeficiency virus 1 (HIV-1) reverse transcriptase (34).

GC-MS analysis of the compounds released by our isolates also revealed different amino acids. In 2017, the amino acid market worldwide was reported at US$8 billion and was projected to reach US$24.4 billion by 2020 (35). This shows the high demand for these compounds in different biotechnological and industrial fields. Some of these compounds are reported to be synthesised by a diversity of microorganisms. For example, *Serratia marcescens* produces L-threonine (35). Glutamate is reported to be synthesised in bacteria such as *Micrococcus* and *Microbacterium* (35). Among the amino acids identified in our study, we can enumerate L-tryptophan, L-lysine, L-phenylalanine, L-arginine and DL-pipecolic acid (**Table II** and **Table III**). Lysine, tryptophan and phenylalanine are used as food and feed supplements and in the production of pharmaceuticals and cosmetics (35). L-arginine is known for its activity in secretion of hormones like insulin, prolactin, growth hormones and in muscle mass promotion and wound healing enhancement (35). It is used as a food supplement and in pharmaceuticals and cosmetics (35). In our study, some unknown compounds were recorded from the GC-MS of the P1 and P16 isolates. This may suggest the potential for new biological compounds from Parsık Cave *Actinobacteria*.

Table II Gas Chromatography and Mass Spectroscopy of the Isolate P1

Compounds	Retention time, min	Charge, m/z	Score	Formula
Valclavam	3.983	330.1661	98.83	$C_{14}H_{23}N_3O_6$
Azaserine	1.092	191.0768	84.80	$C_5H_7N_3O_4$
Adefovir Dipivoxil	3.802	519.2313	95.44	$C_{20}H_{32}N_5O_8P$
Apramycin	4.728	562.2693	83.59	$C_{21}H_{41}N_5O_{11}$
Maculosin	5.405	261.1235	96.12	$C_{14}H_{16}N_2O_3$
Posaconazole	6.298	718.3628	91.28	$C_{37}H_{42}F_2N_8O_4$
Glycobismine A	9.844	620.2767	84.92	$C_{37}H_{34}N_2O_6$
Bouillonamide B	20.356	645.3439	89.11	$C_{32}H_{45}N_5O_6S$
Stigmatellin Y	24.354	507.2711	97.17	$C_{29}H_{40}O_6$
Betaine	1.205	118.0868	96.73	$C_5H_{12}NO_2$
Aminocaproic acid	1.724	132.1020	87.32	$C_6H_{13}NO_2$
Salivaricin A	1.770	706.2533	63.10	$C_{28}H_{48}O_{12}Si_4$
Aconitine	2.786	646.3237	91.49	$C_{34}H_{47}NO_{11}$
Benomyl	3.203	308.1713	94.78	$C_{14}H_{18}N_4O_3$
Pectenotoxin 2	4.265	881.4623	83.96	$C_{47}H_{70}O_{14}$
Wilfordine	4.446	906.2810	85.75	$C_{43}H_{49}NO_{19}$
Leucomycin A7	5.801	780.4154	80.22	$C_{38}H_{63}NO_{14}$
Leucomycin A4	7.246	814.4556	78.50	$C_{41}H_{67}NO_{15}$
Lobeline	7.370	360.1932	97.34	$C_{22}H_{27}NO_2$
Jamaicamide C	8.110	491.2690	40.77	$C_{27}H_{39}ClN_2O_4$
Thapsigargin	8.658	668.3658	91.24	$C_{34}H_{50}O_{12}$
Leukotriene C4-d5	9.290	631.3447	73.40	$C_{30}H_{42}D_5N_3O_9S$
Trilobolide	9.301	523.2546	97.08	$C_{27}H_{38}O_{10}$
Malyngamide C	10.182	473.2768	65.40	$C_{24}H_{38}ClNO_5$
Avermectin A2a aglycone	10.814	639.3510	90.08	$C_{35}H_{52}O_9$
Daphnoline	12.124	598.2905	92.37	$C_{35}H_{36}N_2O_6$
Melleolide K	12.565	452.1825	59.39	$C_{23}H_{27}ClO_6$
Spinetoram	12.667	770.4805	97.90	$C_{42}H_{69}NO_{10}$
Apigenin	14.078	271.0601	92.55	$C_{15}H_{10}O_5$
Spectinomycin	18.154	333.1659	47.34	$C_{14}H_{24}N_2O_7$
Fumitremorgin C	19.464	397.2238	47.11	$C_{22}H_{25}N_3O_3$
L-Arginine	1.160	175.1197	84.63	$C_6H_{14}N_4O_2$
DL-pipecolic acid	1.036	130.0863	98.87	$C_6H_{11}NO_2$
D-Lysine	1.047	147.1128	97.62	$C_6H_{14}N_2O_2$
L-Phenylalanine	2.741	166.0868	92.21	$C_9H_{11}NO_2$
L-Tryptophan	4.491	205.0980	97.13	$C_{11}H_{12}N_2O_2$
Valclavam	3.955	328.151	98.05	$C_{14}H_{23}N_3O_6$
Leonurine	6.044	370.161	99.63	$C_{14}H_{21}N_3O_5$

3.4 Parsık Cave as a House of *Actinobacteria* with High Diversity of Enzymes

The enzymatic activities of microorganisms indicate which organic compounds are present in the studied ecosystem. In addition, studying these activities allows us to understand the potential use of these microorganisms for human or other applications. Enzymes can be used in environmental bioremediation and in other industries such as detergents, cosmetics, leather and textiles production. Different enzymatic activities from cave bacteria have been reported in different studies. Using enzymes secreted by psychrotolerant bacteria is thought to be

Table III Gas Chromatography and Mass Spectroscopy of the Isolate P16

Compounds	Retention time, min	Charge, m/z	Score	Formula
Azaserine	1.089	191.0770	96.75	$C_5H_7N_3O_4$
Dioscorine	1.224	244.1300	97.40	$C_{13}H_{19}NO_2$
Flutrimazole	1.891	364.1608	87.50	$C_{22}H_{16}F_2N_2$
Senkirkine	3.139	366.1897	42.28	$C_{19}H_{27}NO_6$
Bestatin	3.765	326.2074	85.57	$C_{16}H_{24}N_2O_4$
Adefovir Dipivoxil	3.856	519.2316	96.62	$C_{20}H_{32}N_5O_8P$
Apramycin	5.160	562.2695	94.66	$C_{21}H_{41}N_5O_{11}$
Tigecycline	5.572	603.3146	98.00	$C_{29}H_{39}N_5O_8$
Maculosin	5.606	261.1237	85.47	$C_{16}H_{16}N_2O_3$
Diprotin B	5.730	328.2235	99.00	$C_{16}H_{29}N_3O_4$
Posaconazole	6.295	718.3636	92.09	$C_{37}H_{42}F_2N_8O_4$
Coccinin	13.646	551.2465	97.38	$C_{26}H_{40}O_{11}$
Mycinamicin VI	7.717	668.3985	92.27	$C_{35}H_{57}NO_{11}$
Chromafenozide	7.695	417.2147	82.45	$C_{24}H_{30}N_2O_3$
Samandarone	9.129	326.2083	96.68	$C_{19}H_{29}NO_2$
Amprenavir	9.502	523.2564	86.26	$C_{25}H_{35}N_3O_6S$
Glycobismine A	9.841	620.2764	85.91	$C_{37}H_{34}N_2O_6$
Lanceotoxin B	10.078	622.3245	91.62	$C_{32}H_{44}O_{11}$
Vindesine	10.360	776.3983	98.01	$C_{43}H_{55}N_5O_7$
Torvoside G	10.823	609.3971	85.88	$C_{34}H_{56}O_9$
Mycinamicin III	13.680	699.4441	93.42	$C_{36}H_{59}NO_{11}$
Lanceotoxin A	15.204	621.2912	91.39	$C_{32}H_{44}O_{12}$
Neomycin A	17.068	323.1940	76.74	$C_{12}H_{26}N_4O_6$
Dithiazanine	17.937	409.1636	47.06	$C_{23}H_{23}N_2S_2$
Myrsinone	19.846	312.2177	82.26	$C_{17}H_{26}O_4$
Bouillonamide B	20.365	645.3458	84.72	$C_{32}H_{45}N_5O_6S$
Stigmatellin Y	26.497	507.2727	97.22	$C_{29}H_{40}O_6$
Alpha-Chlorohydrin	35.700	111.0208	47.55	$C_3H_7CLO_2$
L-Arginine	1.146	175.1190	99.60	$C_6H_{14}N_4O_2$
DL-pipecolic acid	1.033	130.0865	93.04	$C_6H_{11}NO_2$
D-Lysine	1.044	191.0770	92.46	$C_6H_{14}N_2O_2$
L-Phenylalanine	2.738	166.0863	93.68	$C_9H_{11}NO_2$
L-Tryptophan	4.488	205.0981	90.60	$C_{11}H_{12}N_2O_2$
Valclavam	3.972	328.151	80.19	$C_{14}H_{23}N_3O_6$
Leonurine	6.050	370.161	99.44	$C_{14}H_{21}N_3O_5$

economically valuable to save energy in large scale applications (9). Our isolates showed different enzymatic activities (**Table I**).

The physiological role of amylase enzyme, which is mainly used in the textile, food, fermentation and paper industries, is to hydrolyse starch molecules into dextrins and glucose molecules (36). It is secreted by different biological sources of which the most important is the *Streptomyces* species from *Actinobacteria* (36). 29% of our isolates showed amylase activity. Different strains of these isolates showed clear zones around their colonies after incubation in starch agar plates and staining with iodide. They include *S. exfoliates, Arthrobacter humicola, Kocuria rosea* and *Oerskovia turbata* NBRC 15015 (**Table I**).

Protease activity was also tested in our study. Proteases are a group of enzymes directed to hydrolyse the peptide bonds of protein molecules into amino acid chains or simple peptide molecules (37). They are synthesised from different sources including plants, animals

and microorganisms. They are used in different industrial and biotechnological areas including waste treatment, pharmaceuticals, detergent production, photographic and food industries (37, 38). Microbial proteases are obtained both from fungi and bacterial sources. Most of the fungi proteases are acidic while the bacterial ones are mainly active in alkaline environments (39). Proteases from *Actinobacteria* are reported from different strains including *Streptomyces* and *Nocardia* isolated from different ecosystems (39).

In our study, protease activity was tested through gelatin and casein hydrolysing profiles of the isolates. 5% of our isolates showed gelatinase activity while 48% showed casein hydrolase activity (**Table I**). The species identified as *Nocardia* and *Nocardioides* showed gelatin hydrolysing activity while those identified as *Streptomyces, Nocardia*, *Nocardioides, Microbacterium, K. Rosea* and *Arthrobacter* showed protease activity by hydrolysing casein molecules. These isolates might be considered candidates for the previously mentioned areas as they can produce cold-tolerant and alkaline protease enzymes in oligotrophic areas.

Among the investigated enzymatic activities in this study is the ureolytic activity. It consists of the degradation of urea molecules to generate ammonia which provides an alkaline environment at the end of the reaction (40). The urease activity of cave microorganisms has been previously demonstrated from karstic cave isolates and they are often studied for their possible significant role in cave and speleothem formation (9, 19, 40). Cave bacteria that show urease activity mostly include strains of *Proteobacteria, Firmicutes* and *Actinobacteria* (4, 9, 19). Some *Streptomyces* species isolated from moonmilk of the Springtails' Cave were reported with ureolytic activity through phenotypical and genotypical analyses (19). *Micrococcus luteus* from Magura Cave, Bulgaria, also showed pink colour after incubation on urea-based agar plates, related to the urease secretion ability of the bacteria (9). The precipitation of calcium by bacteria is thought to take place through different mechanisms which include the urease reaction (9). The isolation of urease positive bacteria and their *in vitro* calcium precipitation ability were shown in previous cave microbiological studies (4).

In our study, 65% of the selected isolates showed positive results for urease activity. They include species of *Streptomyces, Arthrobacter, Propionicimonas, Microbacterium* and *Nocardioides.* These bacteria may play a significant role in construction activities like self-healing concrete production or concrete bioremediation. In addition, they may be used to remove calcium ions from wastewater. The calcium precipitation ability of these isolates should be the subject of future studies for further industrial and biotechnological applications.

Three of the identified *Actinobacteria* (*S. exfoliatus, O. turbata* NBRC 15015 and *K. rosea*), which represent 9.7% of our isolates, showed positive lipolytic activity in the basal agar medium supplemented with 1% Tween 80 and $CaCl_2.2H_2O$ (**Table I**). They formed precipitation zones around their colonies that indicate the complex formed by liberated fatty acids and calcium present in the media. Different *Actinobacteria* isolated from the Hampoeil Cave, Iran, were also reported with esterase activity which is one of lipolytic enzymes (41). In our previous study in Parsık Cave, only strains of *Proteobacteria* were identified with lipolytic activity through the VITEK® test (11). In the present study, species of the same genera show different results. This suggests the lipolytic activity of the bacteria isolated from the same cave (same and different points) may be related to the substrate. Furthermore, it may be necessary to study the activities from different isolation points in order to establish the different hydrolytic activities shown by the ecosystem isolates. Lipolytic enzymes are used in industrial fields such as detergent production (11, 41). Such lipolytic enzymes isolated from cold-tolerant bacteria might be preferred in detergent production to reduce energy use during washing.

Two of our identified *Actinobacteria* showed a clear zone around their colonies incubated on DNA agar plates for five days (**Table I**). This zone was formed after flooding 1 N hydrochloric acid on the agar plate. The clear zone is caused by the reaction of DNase secreted by the isolates on the DNA (depolymerisation) present in the medium. DNase enzymes are known for different functions depending on the microbial sources: they can cause disruption of biofilms or play a role in bacterial predation, inhibition of natural transformation or the recovery of nutrients such as carbon and phosphate (42, 43). DNase activity was previously reported from *Actinobacteria* species isolated from other cave ecosystems (9, 43).

Cellulases are hydrolytic enzymes that hydrolyse cellulose by hydrolysing the B-1,4 glycosidic bonds into sugars (44, 45). These enzymes are important in different applications such as the degradation of cellulosic waste, bioethanol production, textile

polishing and the production of detergent, food and feed (44, 45). A diversity of bacteria including *Actinobacteria* isolated from different ecosystems are reported with cellulase secretion ability (44, 45). In our study, three of the identified *Actinobacteria* (P1, P21 and P26 isolates) showed positive results at the end of a cellulase enzyme test (**Table I**). Their culture on carboxymethylcellulose agar plates were stained with Congo red dye, showing a yellow hallo against a red background. Interestingly, most of our isolates did not grow on the carboxymethylcellulose agar plates. This may be explained by inappropriate incubation conditions for most of the isolates. Furthermore, the medium used was not favourable for most of these oligotrophic isolates which may cause eutrophication.

The isolate P1 which was identified as *S. exfoliatus* exhibited the greatest enzymatic potential (**Table I**). As previously shown, most actinobacterial species that exhibit high bioactive compound potential belong to the genus *Streptomyces* (46). A strain of *S. exfoliates* isolated from a soil environment in Saudi Arabia has been analysed with potential lipase and protease activities (47). In addition, in our study, both PKS and NRPS cluster genes were amplified in this isolate. This result suggests the high potential for bioactive compounds of this isolate. The isolate P1 should be further investigated as a potentially industrially useful *Actinobacteria* from Parsık Cave.

4. Conclusion

For the identification of *Actinobacteria* from Parsık Cave, 41 bacterial isolates were chosen based on their isolation media. The potential bioactive compound secretion of the identified *Actinobacteria* was investigated and antimicrobial effects against standard bacteria and one *Candida* species were phenotypically observed in the isolates P1 and P16. PKS and NRPS gene clusters were amplified in the majority of the identified *Actinobacteria*. GC-MS analysis were run to identify bioactive compounds produced by the P1 and P16 isolates. The identified compounds include compounds with antimicrobial, anticancer and antioxidant activities. In addition, amino acids and various unknown compounds were observed. Amylase, urease, lipase, cellulase, DNase, gelatinase and casein hydrolysing enzymes were observed in different strains of the selected isolates with great enzymatic activity exhibited by isolate P1. Our results show the potential for use of Parsık Cave isolates, especially the identified *Actinobacteria*, in diverse biotechnological and industrial applications. Unknown compounds observed in this study suggest the potential to isolate new bioactive compounds from these sources. In our future work, we plan to investigate novel enzymatic activities, identify biological compounds from the remaining isolates, extract and purify the already identified compounds as well as those that will be identified and propose new industrial and biotechnological products.

Acknowledgements

The author thanks Istanbul University Scientific Project Unit, Turkey (BAP Project No: FHZ-2017-26457) for the financial support.

References

1. D. Bukelskis, D. Dabkeviciene, L. Lukoseviciute, A. Bucelis, I. Kriaučiūnas, J. Lebedeva and N. Kuisiene, *Front. Microbiol.*, 2019, **10**, 2149
2. S. Zada, W. Sajjad, M. Rafiq, S. Ali, Z. Hu, H. Wang and R. Cai, *Microb. Ecol.*, 2021, **84**, (3), 676
3. Z. Cyske, W. Jaroszewicz, M. Żabińska, P. Lorenc, M. Sochocka, P. Bielańska, Ł. Grabowski, L. Gaffke, K. Pierzynowska and G. Węgrzyn, *Acta Biochim. Polon.*, 2021, **68**, (4), 565
4. M. D. Türkgenci, and N. Doğruöz Güngör, *Geomicrobiol. J.,* 2021, **38**, (9), 816
5. H. A. Barton and V. Jurado, *Microbe,* 2007, **2**, 132
6. N. Cheeptham, 'Advances and Challenges in Studying Cave Microbial Diversity', in "Cave Microbiomes: A Novel Resource for Drug Discovery", ed. N. Cheeptham, SpringerBriefs in Microbiology, Vol. 1, Springer Science+Business Media, New York, USA, 2013, pp. 1–34
7. S. Castanier, G. Le Méteyer-Levrel and J.-P. Perthuisot, 'Bacterial Roles in the Precipitation of Carbonate Minerals', in "Microbial Sediments", eds. R. E. Riding and S. M. Awramik, Springer-Verlag, Berlin, Germany, 2000, pp. 32–39
8. H. A. Barton and D. E. Northup, *J. Cave Karst Stud.*, 2007, **69**, (1), 163
9. I. Tomova, I. Lazarkevich, A. Tomova, M. Kambourova and E. Vasileva-Tonkova, *Int. J. Speleol.*, 2013, **42**, (1), 65
10. K. Tomczyk-Żak and U. Zielenkiewicz, *Geomicrobiol. J.*, 2016, **33**, (1), 20
11. B. Çandiroğlu and N. Doğruöz Güngör, *Johnson Matthey Technol. Rev.,* 2020, **64**, (4), 466
12. P. Rangseekaew and W. Pathom-aree, *Front. Microbiol.*, 2019, **10**, 387
13. S. Zada, J. Xie, M. Yang, X. Yang, W. Sajjad,

M. Rafiq, F. Hasan, Z. Hu and H. Wang, *Appl. Microbiol. Biotechnol.*, 2021, **105**, (23), 8921

14. N. Nawani, B. Aigle, A. Mandal, M. Bodas, S. Ghorbel and D. Prakash, *BioMed Res. Int.*, 2013, 687190
15. D. V. Axenov-Gibanov, I. V. Voytsekhovskaya, B. T. Tokovenko, E. S. Protasov, S. V. Gamaiunov, Y. V. Rebets, A. N. Luzhetskyy and M. A. Timofeyev, *PLoS One*, 2016, **11**, (2), e0149216
16. J. W.-F. Law, V. Letchumanan, L. T.-H. Tan, H.-L. Ser, B.-H. Goh and L.-H. Lee, *Prog. Microbe. Mol. Biol.*, 2020, **3**, (1), a0000064
17. "Biotechnology of Antibiotics", ed. W. R. Strohl, 2nd Edn., CRC Press, Boca Raton, USA, 1997, 860 pp
18. D. Prakash, N. Nawani, M. Prakash, M. Bodas, A. Mandal, M. Khetmalas and B. Kapadnis, *BioMed Res. Int.*, 2013, 264020
19. M. Maciejewska, D. Adam, A. Naômé, L. Martinet, E. Tenconi, M. Całusińska, P. Delfosse, M. Hanikenne, D. Baurain, P. Compère, M. Carnol, H. A. Barton and S. Rigali, *Front. Microbiol.*, 2017, **8**, 1181
20. S. Ghosh, N. Kuisiene and N. Cheeptham, *Biochem. Pharmacol.*, 2017, **134**, 18
21. D. H. Amin, N. A. Abdallah, A. Abolmaaty, S. Tolba and E. M. H. Wellington, *Bull. Natl. Res. Cent.*, 2020, **44**, 5
22. S. Jiang, W. Sun, M. Chen, S. Dai, L. Zhang, Y. Liu, K. J. Lee and X. Li, *Antonie Van Leeuwenhoek*, 2007, **92**, (4), 405
23. G. C. A. Amos, C. Borsetto, P. Laskaris, M. Krsek, A. E. Berry, K. K. Newsham, L. Calvo-Bado, D. A. Pearce, C. Vallin and E. M. H. Wellington, *PLoS One*, 2015, **10**, (9), e0138327
24. M. S. Kuyukina and I. B. Ivshina, 'Application of *Rhodococcus* in Bioremediation of Contaminated Environments', in "Biology of Rhodococcus", ed. H. M. Alvarez, Microbiology Monographs, Vol. 16, Springer-Verlag, Berlin, Germany, 2010, pp. 231–262
25. E. M. Rodrigues, K. H. M. Kalks and M. R. Tótola, *J. Environ. Manage.*, 2015, **156**, 15
26. A. R. Johnson and E. E. Carlson, *J. Am. Soc. Mass Spectrom.*, 2019, **30**, (8), 1464
27. C. Vézina, C. Bolduc, A. Kudelsk and P. Audet, *Antimicrob. Agents Chemother.*, 1979, **15**, (5), 738
28. S. A. Waksman and H. A. Lechevalier, *Science*, 1949, **109**, (2830), 305
29. P. Pusparajah, V. Letchumanan, J. W.-F. Law, N.-S. Ab Mutalib, Y. S. Ong, B.-H. Goh, L. T.-H. Tan and L.-H. Lee, *Int. J. Mol. Sci.*, 2021, **22**, (17), 9360
30. W. Jaroszewicz, P. Bielańska, D. Lubomska, K. Kosznik-Kwaśnicka, P. Golec, Ł. Grabowski, E. Wieczerzak, W. Dróżdż, L. Gaffke, K. Pierzynowska, G. Węgrzyn and A. Węgrzyn, *Antibiotics*, 2021, **10**, (10), 1212
31. F. Z. Djebbah, L. Belyagoubi, D. E. Abdelouahid, F. Kherbouche, N. A. Al-Dhabi, M. V. Arasu and B. Ravindran, *J. Infect. Public Health*, 2021, **14**, (11), 1671
32. G. A. Quinn, A. M. Banat, A. M. Abdelhameed and I. M. Banat, *J. Med. Microbiol.*, 2020, **69**, (8), 1040
33. P. Sharma, B. Choudhary, A. Nagpure and R. K. Gupta, *J. Environ. Biol.*, 2016, **37**, (6), 1231
34. P. H. K. Ngai and T. B. Ng, *Peptides*, 2004, **25**, (12), 2063
35. S. Sanchez, R. Rodríguez-Sanoja, A. Ramos and A. L. Demain, *J. Antibiot.*, 2018, **71**, (1), 26
36. R. Salwan, and V. Sharma, ''The Role of Actinobacteria in the Production of Industrial Enzymes', in "New and Future Developments in Microbial Biotechnology and Bioengineering: Actinobacteria: Diversity and Biotechnological Applications", eds. B. P. Singh, V. K. Gupta, and A. K. Passari, Ch. 11, Elsevier BV, Amsterdam, The Netherlands, 2018, pp. 165–177
37. F. J. Contesini, R. R. de Melo and H. H. Sato, *Crit. Rev. Biotechnol.*, 2018, **38**, (3), 321
38. A. K. Sharma, V. Sharma, J. Saxena, B. Yadav, A. Alam and A. Prakash, *Int. J. Sci. Res. Environ. Sci.*, 2015, **3**, (9), 0334
39. A. Razzaq, S. Shamsi, A. Ali, Q. Ali, M. Sajjad, A. Malik and M. Ashraf, *Front. Bioeng. Biotechnol.*, 2019, **7**, 110
40. M. Singh, *Curr. Sci.*, 2019, **116**, (11), 1840
41. J. Hamedi, M. Kafshnouchi and M. Ranjbaran, *Saudi J. Biol. Sci.*, 2019, **26**, (7), 1587
42. H. L. Brown, M. Reuter, K. Hanman, R. P. Betts and A. H. M. van Vliet, *PLoS One*, 2015, **10**, (3), e0121680
43. É. B. de Melo Riceto, R. de Paula Menezes, M. P. A. Penatti and R. dos Santos Pedroso, *Rev. Iberoam. Micol.*, 2015, **32**, (2), 79
44. B. C. Behera, B. K. Sethi, R. R. Mishra, S. K. Dutta and H. N. Thatoi, *J. Genet. Eng. Biotechnol.*, 2017, **15**, (1), 197
45. S. K. Gupta, S. Kataki, S. Chatterjee, R. K. Prasad, S. Datta, M. G. Vairale, S. Sharma, S. K. Dwivedi and D. K. Gupta, *J. Clean. Prod.*, 2020, **258**, 120351
46. N. A. Al-Dhabi, G. A. Esmail, V. Duraipandiyan and M. V. Arasu, *Saudi J. Biol. Sci.*, 2019, **26**, (4), 758
47. N. A. Al-Dhabi, G. A. Esmail, A.-K. M. Ghilan and M. V. Arasu, *Saudi J. Biol. Sci.*, 2020, **27**, (1), 474

The Authors

Nahdhoit Ahamada Rachid is a biologist from Istanbul University, Turkey. In 2021 she obtained her MSc in Microbiology from the Department of Fundamental and Industrial Microbiology, focusing on human impacts on cave microbial diversity. Currently, she is a PhD student in the same department. Her research interests include cave microbiology, bioremediation, microbial ecology and biotechnology.

Nihal Doğruöz Güngör is a Professor in the Department of Fundamental and Industrial Microbiology at Istanbul University. She obtained her doctorate at Istanbul University in 2008, focusing on microbiological corrosion of copper. Her research interests include cave microbiology, antimicrobial activities of bacteria, microbial corrosion and biotechnology.