

MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF CULTURABLE EPILITHIC AND ENDOLITHIC BACTERIA FROM ROCKS OF AYUBIA (MURREE), LOWER HIMALAYA, PAKISTAN

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Abstract. Murree and Ayubia hills are parts of the lower Himalaya Mountain range in Pakistan. The rocks of these hills are younger and mostly limestone in nature. In this study, geomicrobiological investigation of the selected samples collected from Murree and Ayubia hills was carried out. The rock samples were spread on nutrient agar plates and endolithic and epilithic bacteria were isolated on the basis of colony morphology and studied further. All the isolates were screened for different industrially important hydrolytic enzymes. Study isolates were identified through 16S rRNA gene sequencing. These endoliths and epiliths were assayed for antimicrobial activities against clinical isolates *S. aureus*, *P. aeruginosa* and *E. coli*. Total 31 bacterial isolates were recovered of which 15 were endolithic and 16 were epilithic. 16S rRNA gene sequencing revealed major culturable groups colonizing these rocks were clustered in four major groups, Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes which include *Alcaligenes*, *Lysinibacillus*, *Actinobacteria*, *Pseudomonas*, *Pusillimonas*, *Streptomyces*, *Fluviicola*, *Serratia*, *Flavobacterium*, *Stenotrophomonas* and *Brevindomonas* species. The sequences were deposited in NCBI for acquisition of accession numbers. The bacterial isolates were efficient producers of oxidase, catalase, protease, amylase and gelatinase. The endolithic isolates N4 (*Pseudomonas* sp. KT223616) and N28 (*Streptomyces* sp. KT004386) exhibited good activity against all the three clinically isolated target strains. The study revealed the rocks of Murree and Ayubia hills have a rich microbial ecology that besides having an important role in weathering and mineralization processes may also be potential source of biotechnological applications.

Keywords: Murree hills, Pakistan, antimicrobial production, endolith, epilith, microbial mineralization, weathering

Introduction

Many microorganisms can survive and thrive in physically and geochemically extreme conditions that were previously considered hostile to life. These microorganisms are known as extremophiles (Sharma et al., 2012) and grow inside the rocks and on the outer surface of rocks. Such rock inhabiting organisms are categorized as endolithic, epilithic and hypolithic (Golubic et al., 1981) on basis of position of their colonization on the rocks.

Several types of rocks have been studied for microbial diversity such as rocks of igneous origin (both glassy and crystalline) by Thorseth et al. (1992), Villar et al.

(2006), Herrera et al. (2009), sandstones, salts and limestones (sedimentary rocks) by Matthes et al. (2001), Wierzchos et al. (2006), Weber et al. (1996), and gneisses and granites (metamorphic rocks) by Ríos et al. (2005) and Cockell et al. (2002).

These rock dwellers are reported from different habitats on earth, those dwelling in mountains (Walker and Pace, 2007; Horath and Bachofen, 2009), in deserts (Friedmann and Galun, 1974; Friedmann and Ocampo, 1976), from deposits of tsunami (Cockell et al., 2007), from the floor of sea (Mason et al., 2007), from the impact crater (Cockell et al., 2002, 2005) from springs having temperature above 60°C (Walker et al., 2005), from the subsurface of deep sea (Amy et al., 1992; Pedersen, 1997), from monuments of cultural heritage (Scheerer et al., 2009), Daughney et al. in 2004 also reported bacteria thriving in deep hydrothermal vents. The colonization of microbes has been reported in glassy and crystalline igneous rocks (Thorseth et al., 1992; Villar et al., 2006; Herrera et al., 2009); in sandstones, salts and limestones of sedimentary rocks (Weber et al., 1996; Matthes et al., 2001; Wierzchos et al., 2006) and gneisses and granite of metamorphic rocks (Cockell et al., 2002; De-los Ríos et al., 2005).

Endoliths and epiliths, inhabiting the extreme environments, produce molecules which help microbes in their adaptation to extreme life conditions and are considered as a new important source of moieties of biological origin (Sánchez et al., 2009). Accordingly, these microorganisms are important targets as a source of variety of bioactive compounds such as secondary metabolites and other industrially important enzymes like amylase, protease and gelatinase. Microbes play key role in weathering and mineralization of rocks. They attach to mineral surfaces and bring about physical and chemical changes in the structure. They create microenvironment of the complex nature at mineral-water interface, where they produce organic acids, metabolically catalyzed redox reactions and complexing agents lead to change in pH and concentration gradient, which is different from bulk solution. This helps in thermodynamic state which enhances weathering. The extracellular polysaccharides (EPS) also serves as a site for secondary mineral precipitation, which is different in morphology from those precipitated in bulk solution inorganically (Konhauser, 2007). The antimicrobial compound production seems to be the general phenomenon for most bacteria. A commendable array of defense, i.e. broad-spectrum antibiotics, lytic agents such as lysozyme and metabolic byproducts such as organic acids are produced by many types of bacteria. In addition, several other types of bacteriocins, proteins and exotoxins are also produced which are biologically active peptide moieties, with bactericidal mode of action (Riley et al., 2002; Yeaman et al., 2003). Secondary metabolites are produced in response to nutrient exhaustion, biosynthesis or addition of an inducer and growth rate decrease (Demain, 1998). The misuse of antibiotics lead to drug resistance, so the only solution is to explore new environments in terms of its diversity that will not only lead to the discovery of new forms of life, but also the secondary metabolites and other industrially important enzymes. Currently, extremozyme production is the demand of recent markets. These extremozymes can withstand more tough conditions in various industrial processes. The microbes inhabiting such dry and hard environments may be a good source of different hydrolytic enzymes.

In the current research our focus was to isolate and characterize the extremophiles (endoliths and epiliths) from natural rocks of Ayubia (Murree), Khyber Pakhtunkhwa (KPK) Pakistan. All the isolates were characterized on molecular basis by 16S rRNA gene sequencing. The isolated endoliths and epiliths were screened for industrially important hydrolytic enzymes and also for antibacterial activity against clinical isolates.

Methodology

Sampling

The rocks and soil samples were collected from Ayubia, District Abbottabad, Khyber Pakhtunkhwa (KPK), Pakistan, (Elevation 2600 meters), (34°4'20"N, 73°23'55"E) (Figure 1). The rock samples were collected in pre-sterilized (ultra violet treated for 20 minutes) polyethylene zipper bags. The temperature at the time of sampling was 20°C while pH of both samples was 5. All the samples were proceeded to laboratory and stored at 4°C.

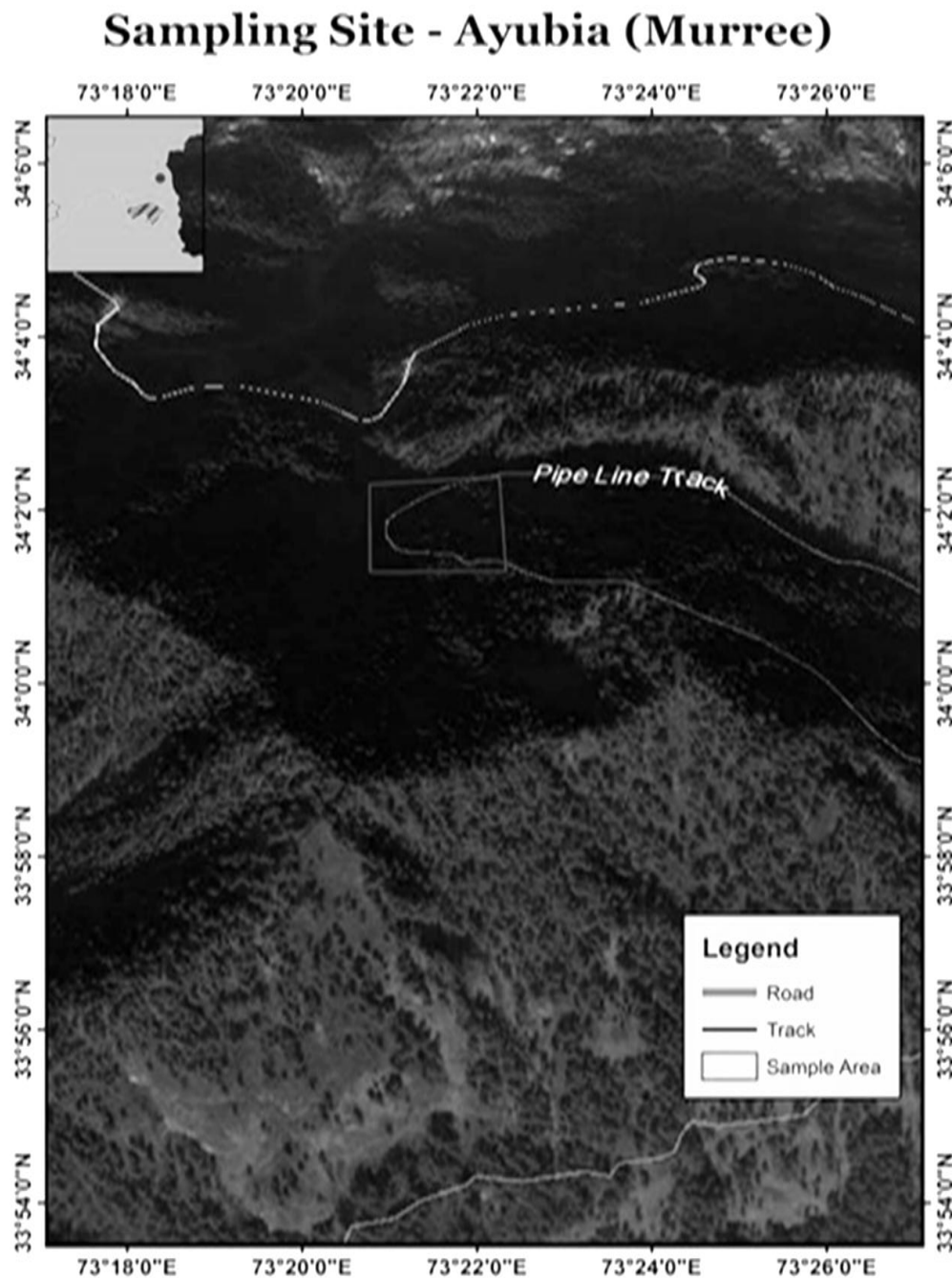


Figure 1. Sampling site: Ayubia Muree lower Himalaya, Pakistan

Rock analysis

The collected rocks belong to Lockhart formation/Lockhart Limestones, which are derived from Paleocene era and are around 65.5 million years old (Akhtar and Butt, 2000). Along Murree-Nathiagali road, near Changlagali the Lokhart limestones are very well exposed, and there it overlies the early to middle Paleocene Hangu Formation and underlain by Patala Formation. Lithology of the rock sample consists of highly fossiliferous limestone with infrequent calcite filled fractures followed by 0.8 meter thick band of dark gray colored calcareous shale. Bedded in between is thick fossiliferous nodular limestone of gray to dark gray in color.

Isolation of endolithic and epilithic bacteria

For the isolation of endolithic and epilithic bacteria, Nutrient Agar/Broth and Luria-Bertani Agar/Broth were used. For epilithic bacterial isolation, outer surface of rocks were directly swabbed with sterile cotton swabs. While for isolation of endolithic bacteria, rocks were broken down with sterile hammer under aseptic conditions. Then the freshly exposed inner surface was swabbed with sterile cotton swabs. The swabs were then immersed and shaken in 1 ml sterile water, the suspensions were then spread on nutrient agar plates (Difco, Detroit, USA) and incubated at 30°C for 3 days and cell count (CFU/ml) was determined for all samples. For purification, visible colonies were picked on the basis of morphological differences and cultured on nutrient agar plates separately.

Identification of bacteria by morphological and biochemical analysis

Identification of isolated strains was carried out morphologically and biochemically. Bergey's Manual of Determinative Bacteriology (9th Edition) was used for identification of isolates. Culture characteristics of the isolated strains were observed. While the microscopic analysis was done through Gram staining procedure.

Molecular characterization

Further confirmation of the isolated bacterial strains was done by molecular characterization through 16S rRNA gene sequencing. The genomic DNA of all bacterial strains was extracted by DNA extraction kit (QIAGEN). The concentrations of DNA were qualified and quantified by spectrometry, NanoDrop 2000 (Thermo Scientific).

PCR amplification

PCR amplification of extracted DNA was done using universal bacterial primers 27F' (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R' (5'-CTACGGCTACCTTGTACGA-3') bacterial primers. The PCR mixture consisted GoTaq®Green Master Mix Promega (25 µl), primer 27F' (1 µl), primer 1492R' (1 µl), DNA extract (2 µl), and *Nuclease-Free Water* (50 µl). MJ mini Personal Thermal Cycler was used to perform the PCR reaction (BIO RAD) the cycling conditions consisted of an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. At 72°C the final extension was performed for 7 min. Electrophoresis was done for the PCR product analysis with 1% agarose gel.

Sequencing and phylogenetic analysis

Sequencing of the amplified PCR product was done by MacroGen Service Center (Geunchun-gu, Seoul, South Korea). BLAST tool was used for the identification of nearest relatives of the sequences in the NCBI database, and homologues were analysed for phylogeny using Molecular Evolutionary Genetic Analysis (MEGA) version 6. Maximum Likelihood method was used for the construction of phylogenetic tree (Tamura and Nei 1993), and diversity among endoliths and epiliths extremophiles were studied (Tamura et al. 2013). Finally, the sequence was submitted to the National Center for Biotechnology Information (NCBI) GeneBank for assignment of an accession number. The 1000 bootstrap replicates were used to estimate the significance of product tree.

Antimicrobial and biochemical potential

Antimicrobial potential of isolated strains

Three multi-drug resistant clinically isolated pathogens were used as target subjects, i.e. *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. The antimicrobial activity of the strains was evaluated by using the point inoculation method. The test microbial colonies were transferred into sterile tubes containing normal saline and the turbidity was adjusted to 0.5 McFarland standard solution. Homogenous lawns were prepared on Muller Hinton agar plates under aseptic conditions and inoculated with the test strains.

Intracellular and extracellular enzymes

All the isolated bacterial strains were screened for the production of both intracellular and extracellular enzymes. Intracellular enzymes, including catalase, oxidase and hydrogen sulfide production and extracellular enzymes including amylase, protease and gelatinase were screened according to the protocol described by Murray et al. (1981).

Results

A total of 31 different endolithic and epilithic isolates were reported in the present study. All these different strains were isolated on the basis of distinct morphological characteristics. Morphological features such as size, shape, color, margins were checked and recorded.

Epilithic and endolithic bacteria

Among 31 isolates, 15 were epilithic named P3, P4, P5, P10, P10i, P11, P12, P17, P18, P19, P20, P21, P23, P24 and P26. while 16 were endolithic named NI, N3, N4, N5, N6, M6, N10, N12, N14, N21, N22, N26, N27, N28, N30 and N40. Colony morphology features are in *Table 1b*. Gram's staining results show that out of 31 strains, 3 were Gram positive and the rest were Gram negative.

The pH of the site was 5 to 6 and temperature was 20°C. Shrubs and tall pine trees were present around the sampling site. Insects, snakes, monkeys and different birds were seen there. A total of 31 different endolithic and epilithic isolates were reported in the present study. Microscopic, morphological and biochemical characteristics of the isolates were studied by comparing these characteristics with Bergey's Manual of Determinative Bacteriology (9th Edition). 16S rRNA gene sequences of the isolates

were submitted in databank and were assigned the Accession numbers by the NCBI (Table 1a). Isolates N22, N21, P21, N14, N27 and P24 were identified as *Alcaligenes* spp., P17 and N40 were identified as *Lysinibacillus* spp., P20 was identified as *Brevindimonas* spp., P23 and N30 were identified *Bordetella* spp., P4, P10, P10i, P11, P26, P19, N4 and N10 were identified as *Pseudomonas* spp., N12 as *Pusillimonas* spp. N26 as *Fluviicola* spp., N28 as *Streptomyces* spp. and isolate P18 identified as *Parapusillimonas* spp., P12 was identified as *Serratia* spp., P5 belonged to *Stenotrophomonas* spp., and P3 was identified as *Flavobacterium* spp., while N1, N6 and M6 were identified as *Actinobacteria* spp. (Figures 2 a,b).

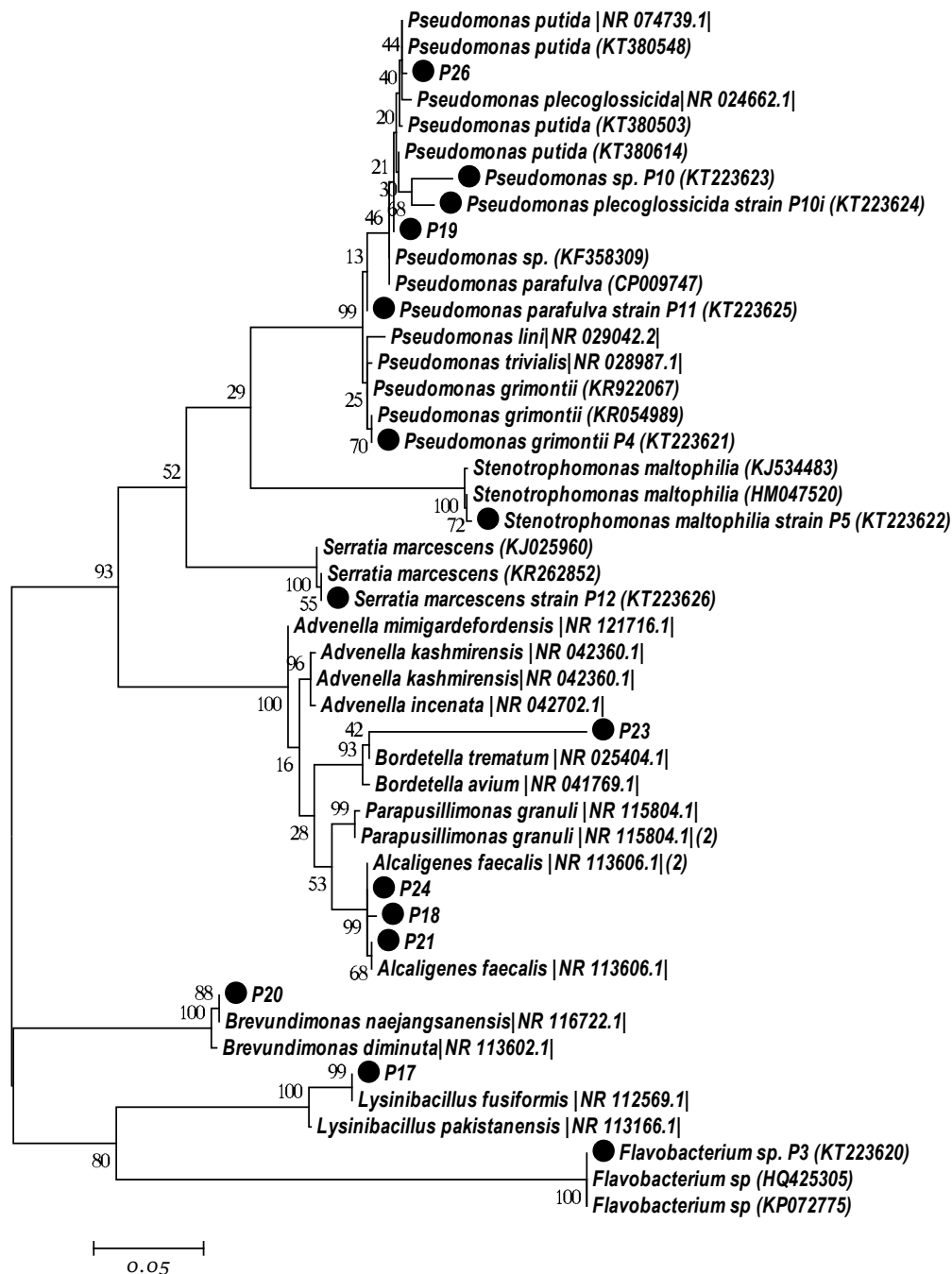


Figure 2 (a). Molecular phylogenetic analysis by Maximum Likelihood method

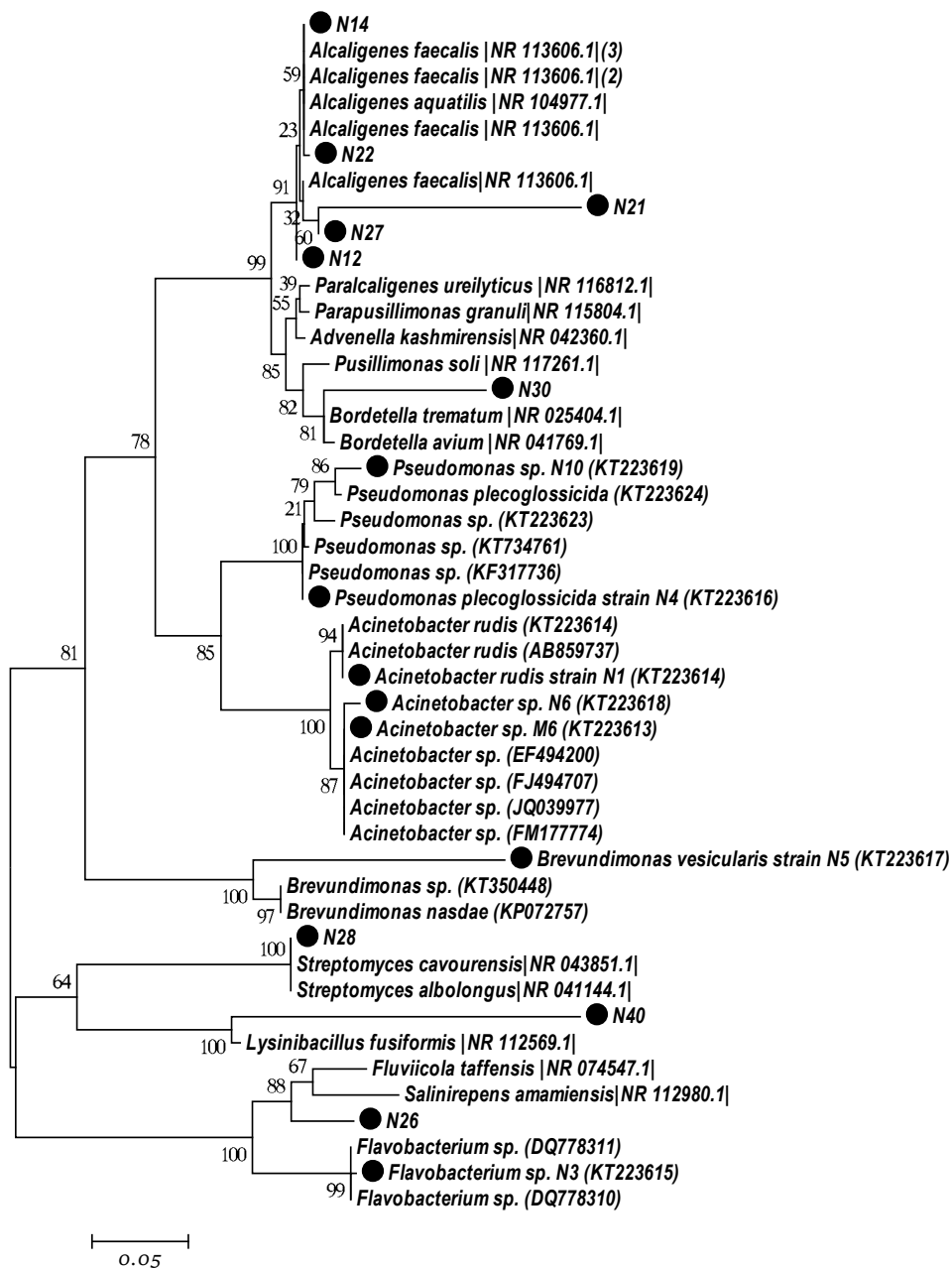


Figure 2 (b). Molecular phylogenetic analysis by Maximum Likelihood method Tamura-Nei model was used for the construction of evolutionary history using the method of maximum likelihood (Tamura K. and Nei M.1993). Next to the branches are the percentage of trees which show the associated taxa clustered together. For heuristic search initial tree(s) were automatically obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated by Maximum Composite Likelihood (MCL) method. MEGA6 was used for evolutionary analysis (Tamura et al. 2012).

Evaluation of antimicrobial potential of the isolated strains

Good antibacterial activity was shown by all the isolated bacterial strains. The endolithic bacteria *Pseudomonas* spp. N4 and *Streptomyces* spp. N28 exhibited good activity against all the three clinically isolated strains, i.e. *Pseudomonas aeruginosa*,

Staphylococcus aureus and *Escherichia coli* Among epilithic bacteria *Brevindomonas* sp. P20 and *Bordetella* spp. P23 showed activity against the 3 human pathogenic test strains (Tables 2 a, b).

Table 1a. Isolated strains with their accession numbers, query coverage and identity

S. No.	Isolated strains	Homologous species	Accession number (Assigned)	Query coverage (%)	Identity (%)
1	P17	<i>Lysinibacillus</i> spp.	KT004373	100	99
2	N22	<i>Alcaligenes</i> spp.	KT004374	100	99
3	N30	<i>Bordetella</i> spp.	KT004375	99	89
4	N21	<i>Alcaligenes</i> spp.	KT004376	99	86
5	N40	<i>Lysinibacillus</i> spp.	KT004377	98	82
6	P21	<i>Alcaligenes</i> spp.	KT004378	100	99
7	P23	<i>Bordetella</i> spp.	KT004379	99	90
8	P24	<i>Alcaligenes</i> spp.	KT004380	100	99
9	P26	<i>Pseudomonas</i> spp.	KT004381	100	99
10	N12	<i>Pusillimonas</i> spp.	KT004382	100	97
11	N14	<i>Alcaligenes</i> spp.	KT004383	100	99
12	N26	<i>Fluviicola</i> spp.	KT004384	98	93
13	N27	<i>Alcaligenes</i> spp.	KT004385	100	98
14	N28	<i>Streptomyces</i> spp.	KT004386	100	100
15	P19	<i>Pseudomans</i> spp.	KT004387	100	98
16	P20	<i>Brevundomonas</i> spp.	KT004388	100	100
17	P18	<i>Parapusillimonas</i> spp.	KT004389	100	95
18	M6	<i>Acinetobacter</i> spp.	KT223613	100	99
19	N1	<i>Acinetobacter</i> spp.	KT223614	100	94
20	N3	<i>Flavobacterium</i> Spp.	KT223615	100	99
21	N4	<i>Pseudomonas</i> Spp.	KT223616	100	100
22	N5	<i>Brevundomonas</i> spp	KT223617	100	97
23	N6	<i>Acinetobacter</i> spp.	KT223618	100	95
24	N10	<i>Pseudomonas</i> Spp.	KT223619	99	86
25	P3	<i>Flavobacterium</i> Spp.	KT223620	100	96
26	P4	<i>Pseudomonas</i> Spp.	KT223621	99	96
27	P5	<i>Stenotrophomonas</i> Spp.	KT223622	98	90
28	P10	<i>Pseudomonas</i> Spp.	KT223623	100	100
29	P10i	<i>Pseudomonas</i> Spp.	KT223624	100	100
30	P11	<i>Pseudomonas</i> Spp.	KT223625	99	99
31	P12	<i>Serratia</i> Spp.	KT223626	100	99

Table 1b. Colony morphology and microscopy of isolated strains

S. No.	Strain	Colony Morphology	Microscopic examination
1	P17	Small, circular, cream color, flat, opaque	Gram +ive , long rods
2	P18	Small sized, raised colonies, opaque , pin pointed colonies	Gram -ive, rods
3	P19	Off white in color, flat in shape, small size, circular	Gram –ive, short rods
4	P20	Grows in net form, off white in color, long threads type	Gram –ive, long rods
5	P21	Off white color, flat colonies, small in size	Gram -ive, rods
6	P23	Large in size, sticky, irregular in shape, cream	Gram –ive, short rods

		color, raised colonies with irregular margins	
7	P24	Small in size, circular shape, off white color, flat colonies, opaque	Gram –ive, rods
8	P26	Small in size, shiny appearance, opaque,	Gram –ive , rods
9	N12	Off white color, small in size, flat colonies	Gram –ive, rods
10	N14	Small in size, irregular in shape, cloudy type in appearance, off white in color	Gram -ive, rods
11	N21	Small in size, flat colonies, entire margins, off white color	Gram -ive, short rods
12	N22	Small in size, raised colonies, yellowish in color, circular in shape	Gram –ive, rods
13	N26	Orange color, small in size, flat colonies, irregular in shape	Gram –ive, short rods
14	N27	Transparent type, small in size, irregular in shape, off white in color	Gram –ive, rods
15	N28	Milky white in color, dry, small in size, circular in shape, raised colonies.	Gram +ive, rods
16	N30	Large in size, flat colonies, sticky type, off white color	Gram –ive, rod
17	N40	Medium size, yellowish, circular in shape, flat colonies,	Gram +ive, rods
18	P3	Orange color medium colonies, shiny, raised, round, opaque	Gram –ive, long rods
19	P4	off white color Small in size, irregular , translucent, flat colonies,	Gram –ive, rods
20	P5	Brownish dry large colonies, raised centers, irregular opaque	Gram –ive, rods
21	P10	Small offwhite colonies, round, flat, dry, opaque	Gram –ive, rods
22	P10i	Off white medium colonies, irregular, translucent	Gram –ive, rods
23	P11	Small offwhite colonies, round, flat, dry, opaque	Gram –ive, long rods
24	P12	Cherry red, raised colonies, shiny, round, opaque	Gram –ive, rods
25	N1	offWhite small colonies, hard, raised, round, dry, opaque	Gram –ive, rods
26	N3	Yellowish shiny medium colonies, round, raised, opaque	Gram –ive, rods
27	N4	Off white, small colonies, dry, opaque	Gram –ive, rods
28	N5	Offwhite, spread like a network, fibrous, dry, hard, opaque	Gram –ive, rods
29	N6	Off white small colonies, irregular, flat, shiny, translucent	Gram –ive, rods
30	N10	Brownish small colonies, pin pointed raised at center, dry, irregularly shaped, opaque	Gram –ive, long rods
31	M6	offWhite small colonies, hard, raised, round, dry, opaque	Gram –ive, rods

Table 2a. antibacterial activity of isolated endolithic strains

S.No	Isolates	Activity against ATCC		
		<i>Pseudomonas aeruginosa</i> (15442)	<i>Staphylococcus aureus</i> (6538)	<i>Escherichia coli</i> (10536)
1	N1	++	+	+
2	N3	+	++	-
3	N4	+++	++	++
4	N5	-	+	++
5	N6	+	-	++
6	M6	-	-	+
7	N10	-	-	-
8	N12	+++	+	++
9	N14	++	-	++
10	N21	++	+++	-
12	N22	+	-	+
12	N26	+++	-	-
13	N27		+++	-
14	N28	+++	+++	+++
15	N30	-	+++	-
16	N40	+	-	-

Key - = No Activity, + = Weak activity, ++ = Moderate Activity, +++ = Strong Activity

Table 2b. antibacterial activity of isolated epilithic bacterial strains

S.No	Isolates	Activity against ATCC		
		<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
17	P3	++	+	+++
18	P4	+	-	++
19	P5	-	++	++
20	P10	+	-	++
21	P10i	-	+	+
22	P11	+	++	-
23	P12	+	-	-
24	P17	++	+	+
25	P18	+++	-	-
26	P19	+++	-	+
27	P20	++	+++	++
28	P21	-	-	++
29	P23	++	++	+++
30	P24	-	-	+
31	P26	+	-	++

Key - = No Activity, + = Weak activity, ++ = Moderate Activity, +++ = Strong Activity

Intracellular and extracellular enzyme production

The bacterial isolates were efficient producers of oxidase and catalase enzymes. Almost all the 31 endolithic and epilithic strains produced catalase and oxidase enzymes. None of the strains produced Hydrogen sulfide gas. Amylase was produced by 7 endolithic and 9 epilithic bacterial strains, gelatinase was produced by 11 endolithic and 11 epilithic strains and 7 endolithic and 5 epilithic strains were positive for protease production (*Tables 3 a, b*).

Table 3a. Production of intracellular and extracellular enzymes by isolated endolithic strains

S. No	Isolates	Extra cellular enzymes activities			Intracellular enzymes activities		
15	N1	+	+	-	-	+	+
16	N3	-	+	+	-	+	+
17	N4	+	-	+	-	+	+
18	N5	+	+	+	-	+	+
19	M6	+	+	-	-	+	+
20	N6	-	-	-	-	+	+
21	N10	-	-	+	-	+	+
22	N12	-	+	-	-	+	
23	N14	+	+	+	-	+	+
24	N21	-	+	-	-	+	+
25	N22	-	-	-	-	+	+
26	N24	+	+	-	-	+	+
27	N26	-	+	+	-	+	+
28	N27	-	-	-	-	+	+
29	N28	-	+	+	-	+	+
30	N30	+	+	-	-	+	+
31	N40	-	-	-	-	+	+

Table 3b. Production of intracellular and extracellular enzymes by isolated epilithic strains

S. No	Isolates	Extra cellular enzymes activities			Intracellular enzymes activities		
1	P3	+	+	+	-	+	+
2	P4	+	+	+	-	+	+
3	P5	-	+	-	-	+	+
4	P10	+	-	+	-	+	+
5	P10i	-	+	+	-	+	+
6	P12	+	+	-	-	+	+
7	P17	+	+	+	-	+	-
8	P18	+	-	-	-	+	+
9	P19	+	+	-	-	+	+
10	P20	+	+	-	-	+	+
11	P21	-	-	-	-	+	+
12	P23	-	+	-	-	+	-
13	P24	-	+	-	-	+	+
14	P26	+	+	-	-	+	+

Discussion

Extreme environment of rocks have long been thought to have limited microbial diversity and activity. Isolation and characterization of endolithic and epilithic bacteria give an insight into Ayubia rocks, and production of secondary metabolites as potential drugs and enzymes for industrial use. In the past century, many endolithic ecosystems were studied which focused on cyanobacterial and algal diversity, using microscopic and culture techniques for identification (Horath and Bachofen, 2009). Variation in morphology, color and size may be induced by different stress factors which are present on endolithic sites, but one cannot rely on morphological properties after cultivation or

in situ. Taxonomic identification is substantially misled by morphological information alone (Norris and Castenholz, 2006).

Using 16S rRNA gene sequencing our isolates were clustered into four different groups that were predominated by Proteobacteria. One possible reason might be the slow metabolic activities of this group in order to prolong its survivability in extreme niches. In addition to this, the endolithic microorganisms differ from the known epilithic ones which is a questionable attribute at the subsurface level. According to the studies conducted by Walker and Pace (2007), the endolithic microbes in the Rocky Mountain range of Antarctica possess restricted diversity and relatively simple systems in comparison to the terrestrial ecosystems, e.g. soil.

The rocks are mostly dominated by Proteobacteria, which includes alpha, beta and gamma Proteobacteria. Other phyla were also found like Firmicutes, Bacteroides and Actinobacteria. The endolithic microbial community of Ayubia is different from that of Tibet the endolithic Bacterioidetes like *Flavobacterium* (Wong et al., 2010). The epilithic and endolithic community reported by (Wierzos et al., 2006) is almost similar to the bacterial community isolated from Ayubia. Which consists mostly of Proteobacteria with Firmicutes and Actinobacteria. Acidobacteria were not found in our study. Endolithic bacterial seven strains of *Pseudomonas* were isolated from Ayubia both endolithic and epilithic and *Pseudomonas grimontii* were also isolated from impact-shocked rocks from Houghton impact structure, Devon Island, Nunavut, Canadian High Arctic (Fike, 2002).

The bacterial similarities identified in these diverse environments pave the path to question the definite origin of these communities. The prior question which could arise is that whether these bacteria are truly multicultural or they reside prevalently in a particular environment and they are only transported into other environments.

Bacteria from lower Himalaya rocks have not yet been reported for antimicrobial agents. Secondary metabolites, mainly new and novel antibiotics are in great demand with the increase in multi-drug resistant pathogens. The microbe driven natural products from these environments have largely been ignored by scientists. All the isolated strains were screened for antimicrobial secondary metabolites. Our studies reveal that these extremophiles are good producers of antibiotics, with potent activities against the tested Gram positive and Gram negative clinical isolates. Endoliths and epiliths produce these active metabolites to prolong its survivability in these extreme conditions. *Streptomyces* sp. strain N28 showed maximum activity against all the tested isolates. In our study the endolithic strain N28 *Streptomyces* spp. (KT004386) also able to produce satisfactory zones of inhibition against both Gram positive and Gram negative bacteria. Other strains, i.e. N4 *Pseudomonas* sp. also screened positive against all the three pathogenic strains. *Streptomyces* have greater ability to synthesise and secrete antibiotics. Recent findings showed presence of four volatile compounds which might be responsible for diverse biological activity of the secondary metabolites from *Streptomyces* sp. (Zothanpuia et al., 2017).

In actinobacteria and other microorganisms the biosynthetic systems like PKS, NRPS, and *phzE* are considered to be responsible for the production of a large number of biologically active compounds (Yuan et al., 2014).

Considerable changes occurred in the last decades regarding what scientists consider the limits of habitable environmental conditions. For every extreme environmental condition investigated, a variety of microorganisms have shown they can tolerate these conditions, but they also often require these extreme conditions for survival

(Rampelotto, 2010). The global market for industrial enzymes was nearly US\$ 4.8 billion in 2013, and it is expected to reach US\$ 7.1 billion by 2018, with a compound annual growth rate (CAGR) of 8% over the five-year period, according to BCC Research (BCC Research, 2014). Endoliths and epiliths represent largest reservoir of biodiversity on the planet, and have a great potential for development of new natural products including enzymes. Endoliths and epiliths participate in biogeochemical cycling, polar food web and produce a wide variety of enzymes including amylases, cellulases, peptidases, lipases, xylanases and other classes of enzymes (Gadd, 2007).

Our result demonstrated that these endoliths and epiliths are of great importance in terms of enzyme production. Most of the bacterial isolates were able to produce both intracellular and extracellular enzymes. At extreme conditions the enzymes become more rigid, which is of great importance. *Pseudomonas* sp. N8 was a good producer of protease and amylase, which were according to the findings of Liu et al. (2007). The understanding of specific factors that confer the ability to withstand extreme habitats on such enzymes has become a priority for their biotechnological use (Dalmaso et al., 2015). It is important to study and understand these microorganisms in order to be able to use the biochemical, ecological, evolutionary and industrial potential of these endoliths and epiliths microbes.

Conclusion

According to our results, a total of 31 isolated strains have been analyzed by rRNA sequencing, of which 16 proved to be endolithic and 15 epilithic. This study comes with implication that such unique environment is for the first time explored for bacterial diversity in Himalayan range Pakistan.

In our study the endolithic strain N28 *Streptomyces* spp. (KT004386) also able to produce satisfactory zones of inhibition against both Gram positive and Gram negative bacteria. Other strains i.e. N4 *Pseudomonas* sp. also screened positive against all the three pathogenic strains. *Streptomyces* have greater ability to synthesis and secrete antibiotics. Endolithic bacterial seven strains of *Pseudomonas* were isolated from Ayubia. The bacterial similarities identified in these diverse environments pave the path to question the definite origin of these communities. The prior question which could arise is that whether these bacteria are truly multicultural or they reside prevalently in a particular environment and they are only transported into other environments. In the last decades, substantial changes have occurred regarding what scientists consider the limits of habitable environmental conditions. These endoliths and epiliths have a great potential to produce secondary metabolites and other extremozymes. Further studies are required to produce highly pure compounds of great commercial use.

REFERENCES

- [1] Akhtar, M., Butt, A.A. (2001): The Paleogene of the Kala Chitta Range, northern Pakistan. – N Jb Geol Palaont.Mh 1:43-55
- [2] Amy, P.S., Haldeman, D.L., Ringelberg, D., Hall, D.H., Russell, C. (1992): Comparison of identification systems for classification of bacteria isolated from water and endolithic habitats within the deep subsurface. – Appl Environ Microbiol 58:3367-3373.
- [3] BCC Research. (2014): Global Markets for Enzymes in Industrial Applications; BIO030H; BCC Research: Wellesley. M.A. USA. p. 146.

- [4] Cockell, C.S., Brack, A., Wynn-Williams, D.D., Baglioni, P., Brandstätter, F., Demets, R., Sancisi-Frey, S. (2007): Interplanetary transfer of photosynthesis: an experimental demonstration of a selective dispersal filter in planetary island biogeography. – *Astrobiology* 7: 1-9.
- [5] Cockell, C.S., Lee, P., Broady, P., Lim, D.S., Osinski, G.R., Parnell, J., Salminen, J. (2005): Effects of asteroid and comet impacts on habitats for lithophytic organisms. A synthesis. – *Meteorit Planet Sci* 40:1945-5100
- [6] Cockell, C.S., Lee, P., Osinski, G., Horneck, G., Broady, P. (2002): Impact-induced microbial endolithic habitats. – *Meteorit Planet Sci* 37:1287-1298.
- [7] Dalmaso, G.Z.L., Ferreira, D., Vermelho, A.B. (2015): Marine extremophiles: a source of hydrolases for biotechnological applications. - *Marine drugs* 13(4):1925-1965.
- [8] Daughney, C. J, Rioux, J.P., Fortin, D., Pichler, T. (2004): Laboratory investigation of the role of bacteria in the weathering of basalt near deep sea hydrothermal vents. – *Geomicrobiol J* 21: 21-31.
- [9] De-los Rios., A., Ascaso, C. (2005): Contributions of in situ microscopy to the current understanding of stone biodeterioration. – *International Microbiology* 8(3): 181-8.
- [10] Demain, A. L. (1998): Induction of microbial secondary metabolism. - *Int. Microbiol.* 1: 259-264.
- [11] Fike, D.A., Cockell, C., Pearce, D., Lee, P. (2002): Heterotrophic microbial colonization of the interior of impact-shocked rocks from Haughton impact structure, Devon Island, Nunavut, Canadian High Arctic. – *Int J Astrobiol* 1:311-23.
- [12] Friedmann, E.I., Galun, M. (1974): Desert algae, lichens, and fungi. – In: Brown, G. W. Jr. (ed) *Desert biology*. Volume 2. New York: Academic Press. pp. 165.
- [13] Friedmann, E.I., Ocampo-Friedmann, R. (1984): Endolithic microorganisms in extreme dry environments: analysis of a lithobiontic microbial habitat. – *Current perspectives in microbial ecology*. pp. 177-185.
- [14] Gadd, G. M. (2007): Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. - *Mycological Research* 111(1): 3-49.
- [15] Golubic, S., Friedmann, I., Schneider, J. (1981):The lithobiontic ecological niche, with special reference to microorganisms. – *J Sediment Res* 51(2):475-478.
- [16] Herrera, A., Cockell, C.S., Self, S., Blaxter, M., Reitner, J., Thorsteinsson, T., Tindle, A.G. (2009): A cryptoendolithic community in volcanic glass. – *Astrobiology* 9:369-381.
- [17] Horath, T., Bachofen, R. (2009): Molecular characterization of an endolithic microbial community in dolomite rock in the Central Alps (Switzerland). – *Microb Ecol* 58:290-306.
- [18] Konhauser, K. O. (2007): *Introduction to Geomicrobiology*. – Blackwell Publishing, UK, pp. 203.
- [19] Mason, O.U., Stingl, U., Wilhelmm, L.J., Moeseneder, M.M., Di Meo-Savoie, C. A., Fisk, M. R., Giovannoni, S.J. (2007): The phylogeny of endolithic microbes associated with marine basalts. – *Environ Microbiol* 9:2539-2550.
- [20] Matthes, U., Turner, S.J., Larson, D.W. (2001): Light attenuation by limestone rock and its constraint on the depth distribution of endolithic algae and cyanobacteria. – *International Journal of Plant Sciences* 162:263-270.
- [21] Norris, T.B., Castenholz, R.W. (2006): Endolithic photosynthetic communities within ancient and recent travertine deposits in Yellowstone National Park. – *FEMS Microbiol Ecol* 57:470–483.
- [22] Pedersen, K. (1997): Microbial life in deep granitic rock. – *Fems Microbiol Rev* 20:399-414.
- [23] Rampelotto, P.H. (2010): Resistance of microorganisms to extreme environmental conditions and its contribution to astrobiology. - *Sustainability* 2(6):1602-1623.
- [24] Riley, M. A., Wertz, J. E. (2002): Bacteriocins: evolution, ecology, and application. - *Ann. Rev. Microbiol.* 56: 117-137.

- [25] Sánchez, L. A., Gómez, F. F., Delgado, O. D. (2009): Cold-adapted microorganisms as a source of new antimicrobials. - *Extremophiles* 13: 111–120.
- [26] Scheerer, S., Ortega-Morales, O., Gaylarde, C. (2009): Microbial deterioration of stone monuments—an updated overview. – *Adv Appl Microbiol* 66:97-139.
- [27] Sharma, A., Kawarabayasi, Y., Satyanarayana. T. (2012): Acidophilic bacteria and archaea: acid stable biocatalysts and their potential applications. – *Extremophiles* 16(1):1-19.
- [28] Tamura, K., Nei, M. (1993): Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. – *Mol Biol Evol* 10:512-526
- [29] Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. – *Mol Bio Evol* 30:2725-2729.
- [30] Thorseth, I.H., Furnes, H., Heldal, M. (1992): The importance of microbiological activity in the alteration of natural basaltic glass. – *Geochim Cosmochim Acta* 56:845-850.
- [31] Villar, S.E., Edwards, H.G., Benning, L.G. (2006): Raman spectroscopic and scanning electron microscopic analysis of a novel biological colonisation of volcanic rocks. – *Icarus*. 184:158-169.
- [32] Walker, J.J., Pace, N.R. (2007): Phylogenetic composition of Rocky Mountain endolithic microbial ecosystems. – *Appl Environ Microbiol* 73:3497–3504.
- [33] Walker, J.J., Spear, J.R., Pace, N.R. (2005): Geobiology of a microbial endolithic community in the Yellowstone geothermal environment. – *Nature* 434:1011-1014.
- [34] Weber, B., Wessels, D.C.J., Büdel, B. (1996): Biology and ecology of cryptoendolithic cyanobacteria of a sandstone outcrop in the Northern Province, South Africa. – *Arch Hydrobiol Suppl Algal Stud* 117:565-579.
- [35] Wierzchos, J., Ascaso, C., McKay, C.P. (2006): Endolithic cyanobacteria in halite rocks from the hyperarid core of the Atacama Desert. – *Astrobiology* 6:415-422.
- [36] Wong, F.K., Lau, M.C., Lacap, D.C., Aitchison, J.C., Cowan, D.A., Pointing, S.B. (2010): Endolithic microbial colonization of limestone in a high-altitude arid environment. – *Microb Ecol* 59:689-99.
- [37] Yeaman, M. R., Yount, N. Y. (2003): Mechanism of antimicrobial peptide action and resistance. - *Pharmacol. Rev.* 55: 27-55
- [38] Yuan, M., Yu, Y., Li, H. R., Dong, N., and Zhang, X. H. (2014): Phylogenetic diversity and biological activity of actinobacteria isolated from the Chukchi Self marine sediments in the Arctic Ocean. – *Mar. Drugs* 12: 1281-97. doi: 10.3390/md12031281
- [39] Zothanpuia, Passari, A. K., Chandra, P., Leo, V. V., Mishra, V. K., Kumar, B., Singh, B. P. (2017): Production of potent antimicrobial compounds from *Streptomyces cyaneofuscatus* associated with fresh water sediment. – *Frontiers in microbiology* 8:68.