Unveiling of Unique Microbiome Resource Having High Antimicrobial Peptide Activity Endowed With Agriculture and Industrial Applications From Pukzing Cave

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Abstract

Cave ecosystems are considered as a specific niche for the presence of the endemic and extremophilic microorganism. In this study, Pukzing cave documented as the biggest cave of Mizoram, Northeast India was explored for bacterial diversity using both culture-based and culture-independent methods. Culture dependent method revealed 235 bacterial isolates using three different treatments i.e. heat, cold and normal. The highest bacterial population was recovered from heat treatment (n=97; 41.2%) followed by normal (n=79; 33.6%) and cold treatment (n=59; 25.1%) which clearly indicates the dominance of extremophiles. The antimicrobial potential of all the isolates was checked and found that 48 (20.4%) isolates showing antimicrobial ability against all tested pathogens. Phylum Actinobacteria was found to be dominant and showed antimicrobial potential. Modular biosynthetic genes like PKS type II, PKS type I and NRPS genes were detected in 37, 46 and 57 respectively. Similarly, the community-based analysis also predicted the dominance of phylum Actinobacteria using the sequencing of both hyper-variable regions (HVR) i.e. V3 and V4. Subsystem annotation using the V4 region predicted two functions associated with amino acid and its derivatives and cofactors, vitamins, prosthetic groups, pigments functions. A positive strong correlation in relative abundance at the phylum level using Spearman correlation was observed in the V4 region. We conclude that the cave environment harbors unique microbial flora and hypervariable region V4 is more informative as compared to hyper-variable region III. These six microbial pathogens i.e. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis*, and *Candida albicans* was selected for screening as they are well known for different disease cause organism in various fields such as agriculture, domestic animals and human health. Higher activity of AMP assay against these microbes indicates that cave microbial communities could be a potential source of future genomic resources.

1. Introduction

Cave ecosystem is one of the poorly explored ecosystems on earth and is considered as an extreme environment, not appropriate for the development of life due to extreme abiotic conditions (Karolina and Urszula, 2016; Yasir, 2018). Though, majority of caves possess oligotrophic ecosystem with less than 2 mg of total organic carbon (TOC)/liter, availability of very less light, low temperature and high humidity, the cave environment is considered as specific niche for specialized group of microorganisms including endemic as well as extremophiles (Engel et al., 2009; Schabereiter-Gurtner et al., 2002). Being oligotrophic conditions, the microbial population associated with caves is 10^6 cells/g of rock (Barton et al., 2007). Studies predicted the potential of cave microbial flora and their role in geological processes with significant potential to produce bioactive molecules of biotechnological importance (Yasir, 2018). At the same time, the cave associated microbial communities also influence the formation and preservation of cave deposits and serve as primary producers for the complex organisms (Barton and Northup, 2007). Microbial interactions with their environment also play an important role in determining the shape and help in the deposit of wall deposits like stalactite (hanging rock from the roof of the cave), stalagmite (a shape arises from the floor of the cave), etc. (Banerjee and Joshi, 2014).

Exploration of caves is been gaining interest from the last two decades and several unique microbial strains having secondary metabolites production potential have been reported (Yasir, 2018; Cuezva et al., 2012; Portillo et al., 2008). Caves are located worldwide, and the available literature suggests the existence of noble genera in the caves located in Mexico (Quintana et al., 2013), Northern Thailand (Nakaew et al., 2009) and Spain (Jurado et al., 2005 and 2006).

Although it is predicted that the microorganisms associated with caves could play an important contribution in understanding cave ecology, mineral formation and ecosystem bioenergetics (Chen et al., 2009; Chimienti et al., 2016) Cave microbiology from Mizoram, India and its importance is still scantily reported and has given very less attention (De Mandal et al., 2017). Among the several caves located in Mizoram, Northeast India, Pukzing cave is recorded as the largest cave of the state with stretches of 25 meters which is located in Pukzing village near Marpara in the western hills of Aizawl district (<http://www.incredible-northeastindia.com/mizoram/caves.html>). So far, no systematic study is been carried out to understand the microbial population associated with one of the largest caves of Mizoram, Northeast India. This study was carried out for the first time to determine the microbial population of this cave using culture-dependent and culture-independent methods. The present study was planned to have a clear-cut estimation of the bacterial population using the metagenomics approach as the culture-dependent techniques leads to a high underestimation of microbial biodiversity due to the inability to grow most of the organisms on nutritional media (Torsvik and Overeas, 2002). As we know that the advancement in sequencing technologies and the use of high-throughput sequencing technologies has made the studies of microbial diversity easier and more informative. However, using metagenomics approaches to study the microbial diversity of any location has chances to lose some low abundant taxa as stated earlier (Stefani et al., 2015). Hence, the present study targeted both culture-based and culture-independent methods to gain the most appropriate representation of the microbial population associated with the studies cave. The bacterial isolates obtained through culture-dependent methods in this study were screened for their antibacterial activities and the biosynthetic modular genes (PKS I, PKSII and NRPS) were detected in the potential isolates. This study indicated that the use of V4 region in culture-independent method is more appropriate as compared to V3 as the V4 region has given more diverse group of bacterial population and also subsystem annotation of V4 region predicted two functions associated with amino acid and its derivatives and cofactors, vitamins, prosthetic groups, pigments functions which was not shown in case of V3 region.

2. Materials And Methods

2.1 Samples collection and physiochemical analysis

The fresh cave sediment sample (approximately 150g) were collected randomly from four different places within the Pukzing cave, situated at Pukzing village near Marpara in the western hills of Mamit District of Mizoram (23°21'44.2"N 92°25'53.6"E). The Pukzing cave which is the biggest cave of the state of Mizoram is about 25 m wide and is located at around 2100 m above sea level (Ray, 1993; Sati and Vangchhia, 2016). The collected samples were then mixed together to make a composite sample, placed

in sterile Himedia Polythene Bags, brought into the laboratory and stored at 4°C in the refrigerator until use for the culturable study. While for the non culturable metagenomic DNA extraction work the sediment samples were stored at -80°C. The pH and temperature of the cave sediment sample was measured using pH meter thermometers respectively. Further, total carbon, total hydrogen and total nitrogen were determined using CHN analyzer at SAIC, Tezpur University.

2.2 Culturable bacterial diversity estimation

2.2.1 Isolation of bacteria from a cave sediment sample

One gram of soil sample was taken and mixed with 10 ml of sterile distilled water. The mixture solution was then separated into normal or pretreated using either cold treatment (15°C), and hot treatment (55°C). After treatment, the sample was serially diluted up to 10^{-1} to 10^{-5} dilution. 100 µl of each dilution was taken from each treatment and spread on six nutritional media plates. The plates were incubated at 28°C and 37°C for 2–3 weeks to observe the colonies of bacteria. The obtained cultures were re-streaked in their respective media and purified cultures were maintained at 4°C.

2.2.2 Media Composition

The nutritional media was used as follows: **1.** Luria Bertani Agar media (10 g of Peptone; 10 g of sodium chloride; 5 g of yeast extract and 20 g of agar); **2.** Tryptic Soya Agar Media (17 g of tryptone; 3 g of soya peptone; 5 g of sodium chloride; 2.5 g of dipotassium hydrogen phosphate; 2.5 g of dextrose and 20 g of agar); **3.** Starch Casein Agar (10 g of starch; 1 g of casein powder; 37 g of seawater and 20 g of agar); **4.** Tyrosine Agar Media; **5.** Tap Water Yeast Extract Agar Media (5 g of yeast extract; 2 g of dipotassium hydrogen phosphate and 20 g of agar) and **6.** Glycerol Asparagine Agar media.

2.2.3 Genomic DNA extraction and PCR amplification using 16S rRNA gene

Total genomic DNA of the culturable bacteria isolates was extracted by using the Genomic DNA purification kit (Invitrogen Life technologies). The purity of the obtain DNA (µg/ml) was verified using µ-Drop™ Plate (Thermo Scientific™ Multiskan™ GO Spectrophotometer). PCR amplification of 16S rRNA gene was performed by using universal primers PA: 5'-AGA GTT TGA TCC TGG CTC AG-3') and PH: 5'-AAG GAG GTG ATC CAG CCG CA-3' (Qin et al., 2009). The PCR reaction mixture preparations and its process were carried out as denoted in Passari et al. (2017). The amplified PCR product was run on 1.5% of agarose gel and visualized under gel documentation system XR⁺ (Bio-Rad). The amplified product was purified using Pure-link PCR Purification Kit (Invitrogen) and was sequenced commercially at Sci-Genome Labs Pvt. Ltd, India.

2.2.4 Phylogenetic analysis

The obtained sequences were trimmed using Finch TV 1.4.1 version and then compared with the NCBI database using the BlastN search program. After that, the sequences were aligned using the Clustal W software packaged in MEGA 5.05 (Thompson et al., 1997). The aligned sequences were used to select a

phylogenetic model based on using BIC scores (Bayesian Information Criterion) and AICc value (Akaike Information Criterion, corrected) (Nei and Kumar, 2000). A maximum-likelihood tree was constructed using MEGA 6.0 with Jukes-Cunter model for actinobacteria; Kimura 3-parameter model for gram-positive bacteria and Tamura 3-parameter model for gram-negative bacteria (Kimura et al., 1980). The robustness of the phylogenetic tree was tested by bootstrap analysis (1,000 replicates) using *p*-distance model (Felsenstein 1985).

2.2.5 Antimicrobial and biosynthetic potential of culturable bacterial isolates

Antimicrobial screening was performed against *Staphylococcus aureus* (MTCC-96), *Pseudomonas aeruginosa* (MTCC-2453), *Escherichia coli* (MTCC-739), *Micrococcus luteus* (MTCC-5262), *Bacillus subtilis* (MTCC-2097) and *Candida albicans* (MTCC-3017) obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and maintained in Molecular Microbiology and Systematic Laboratory, Department of Biotechnology, Mizoram University. These six microbial species were selected for screening as they are well known for different disease related to agriculture, domestic animals and human health. The bacterial sample was inoculated in Tryptone yeast extract broth medium (ISP medium1: 5 g of Casein Enzymic hydrolysate; 3 g of Yeast Extract) and incubated at 28⁰C, 150 rpm for 7–10 days. Cells were harvested by centrifugation at 8,000 rpm and the supernatant was collected into a fresh tube for testing the antimicrobial activity by agar well diffusion method (Saadoun and Muhana, 2008). The test pathogenic microbes were inoculated on a modified nutrient agar plate (5 g of glucose; 5 g of peptone; 3 g of beef extract; 5 g of sodium chloride and 20 g of agar in one liter of sterile distilled water) and wells of 6 mm diameter were prepared by using sterile cork borer. In each of the plates, wells were filled with 50 µl of clear supernatant of various bacterial sample isolates (test) and the plates were incubated at 37 ± 2⁰C for 24 h. The antimicrobial data were analyzed in replicates (mean ± standard deviation of mean replicates) using Microsoft Excel XP 2007, while one-way ANOVA was used to determine the difference between antimicrobial activities among the bacterial isolates by using SPSS software version 20.0.

The potential isolates based on antimicrobial activity were selected to detect antimicrobial biosynthetic genes. Polyketide synthase genes (PKS I and PKS II) were amplified using degenerate primers: (K1F:5'-TSAAGTCSAACATCGGBCA-3' and M6R:5'-CGCAGGTTSCSGTACCAGTA-3' and KS ∞ :5'-TSGCSTGCTTGGAYGCSATC-3' and KS β : 5'-TGGAANCCGCCGAABCCTCT-3') (Ayuso-Sacido et al., 2005). Non ribosomal peptide synthetase (NRPS) adenylation domain was amplified with a set of degenerate primers (A3F: 5'-GCSTACSYSAT STACACSTCSGG-3' and A7R: 5'-SASGTCVCCSGTSGCGTAS- 3') (Metsa-Ketela et al., 1999). The reaction was carried out in the Veriti thermal cycler (Applied Biosystems, Singapore) in a final volume of 50 µl containing 50 ng of genomic DNA, 2.0 U of Taq DNA polymerase, 1mM MgCl₂, 0.5mM of dNTPs, 2.0 µM of each primer and 10% DMSO. PCR conditions consisted of one denaturation step at 96⁰C for 5 min., followed by 35 cycles of denaturation at 96⁰C for 60 s, annealing at 59⁰C for 60 s, and extension at 72⁰C for 2 min. The final extension step was done at 72⁰C for 10 min. A

negative control reaction mixture without DNA template of actinomycetes was also included with each set of PCR reactions. The PCR products were visualized as stated above.

2.3 Non-Culturable bacterial diversity

2.3.1 Bacterial community profiling using Illumina paired-end sequencing with V3 and V4 variable regions

The total DNA of the composite sample mix collected from Pukzing Cave was obtained by Fast DNA spin kit (QIAGEN, USA) as per the protocol. Paired-end Illumina sequencing (250p x 2) of the variable regions V3 and V4 was carried out at SciGenome Pvt. Ltd., Cochin, Kerela, India. Initially pre-processing of pair-end reads in each sample was done with the Fastq-join method (Aronesty, 2011) to filter out any unpaired reads with uncertain bases. Demultiplexing with quality Phred score of $\geq Q20$ is performed to remove bases with poor quality sequences. The taxonomic analysis was carried using Quantitative Insights into Microbial Ecology (QIIME) analysis pipeline (Caporaso et al., 2010). UCLUST algorithm (Edgar, 2010) was implemented for mapping, processed reads into operational taxonomic units (OTU's) with a similarity threshold of 97%. Further aligning of representative sequences and taxonomic classification was achieved with open reference picking method against the Greengenes reference database (DeSantis et al., 2016) and RDP classifier (Cole et al., 2009) respectively. Based on identified OTU's phylogenetic tree was obtained using FastTree method (Price et al., 2009). Before farther in-depth study on OTU's, low-abundance OTU's that are OTU's with minimum sample count, here those OTU having sample count 0 were identified and removed.

2.3.2 Taxonomic, statistical and functional analysis

The relative abundance of taxa at each taxon level is calculated using the in-build Perl script. Heat-map at the Phylum level was generated by calculating Spearman Correlation (Babicki et al., 2016). Weighted and Unweighted UniFrac distances (Lozupone and Knight, 2005) were calculated for filtered OTUs in all samples. MedCalc software was used to perform one-way ANOVA with a post-hoc test (MedCalc, 2018). To study the compositional similarity between samples, Bray-Curtis similarity score was calculated using diversity indices in QIIME (Caporaso et al., 2010) based on comparison of pairwise taxonomic abundances from each sample against other samples followed by Non-metric Multi-Dimensional Scaling using PASTv3.11 software (Hammer et al., 2001). The phylogenetic tree predicted from FastTree was processed and visualized as phylogenetic cladogram with MEGAN software (Ondov et al., 2011). Functional annotation studies including ontology were performed with MG-RAST server (Wilke et al., 2015).

3. Result

3.1 Physiochemical analysis of the cave sediments

The pH values of the Pukzing cave sediment sample were found 7.5 whereas the temperature of the cave was recorded as 27°C. Moreover, total organic matter and phosphorus content of cave sediment samples were determined $1.5 \pm 0.05\%$ and 67.8 ± 0.2 ppm respectively. Further, total carbon (0.93%) and total hydrogen (0.50%) of cave samples were calculated using CHN analyzer.

3.2 Isolation of bacteria from the Pukzing cave sediment sample

In this study, totally 235 bacterial strains were selectively isolated based on macroscopic morphological characteristics. The maximum number of bacterial isolates was obtained from 10^{-2} dilution ($n = 81$; 46.3%) followed by 10^{-4} dilution ($n = 61$; 26.8%), 10^{-3} dilution ($n = 54$; 12.1%) and 10^{-5} dilution ($n = 39$; 9.7%) respectively. The highest bacterial population was recovered from heat treatment ($n = 97$; 41.2%) followed by untreated ($n = 79$; 33.6%) and cold treatment ($n = 59$; 25.1%). Moreover, the maximum number of bacterial isolates was isolated from SCA media ($n = 92$; 39.1%) followed by ISP7 ($n = 57$; 24.2%), ISP5 ($n = 26$; 11.0%), LB ($n = 24$; 10.2%), TSA ($n = 21$; 8.9%) and TH₂O media ($n = 17$; 7.2%). Morphologically, most of the bacterial isolates spread out over the plate appeared as flat colonies, with some exhibiting spores as rough-surfaced, sticky, smooth, and with pigment production. On incubation, the bacterial colonies were observed as white, yellow, orange and pale-yellow colors. Gram test revealed that 142 of the bacterial isolates were gram-positive ($n = 142$; 60.4%) while 93 of them were gram-negative ($n = 93$; 39.5%).

3.3 Antimicrobial activity screening of culturable bacterial isolates

All the 235 isolates were tested for their antimicrobial activities against six bacterial pathogens *P. aeruginosa*, *S. aureus*, *E. coli*, *M. luteus*, *B. subtilis* and yeast *C. albicans*. Out of 235 isolates screened, 136 (57.87%) isolates (58 bacterial and 78 actinobacterial isolates) showed significant antimicrobial potential against at least five of the tested pathogens. Interestingly, almost all the bacterial isolates exhibited antimicrobial activity against *Bacillus subtilis* (235 isolates) and *Staphylococcus aureus* (227 isolates) as indicated in Table 1.

Table 1
Antimicrobial activity of bacterial isolates against human bacterial pathogens

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
1	BPSAC001	10	8.0	10.0	4.0	-	7.0
2	BPSAC002	7.5	-	8.0	6.0	-	7.0
3	BPSAC003	8.0	-	10.0	6.0	-	8.0
4	BPSAC004	8.0	-	10.0	6.0	-	8.0
5	BPSAC005	8.5	6.0	10.0	-	6.0	6.0
6	BPSAC006	11.0	-	11.0	7.5	-	10.0
7	BPSAC007	13.0	7.0	10.0	10.0	6.5	10.0
8	BPSAC008	10.0	-	11.0	9.0	-	7.0
9	BPSAC009	10.0	7.0	12.0	7.5	-	9.0
10	BPSAC010	10.0	5.0	10.0	5.0	5.0	8.0
11	BPSAC011	9.0	-	10.0	5.0	-	7.0
12	BPSAC012	6.0	7.0	10.0	6.0	-	5.0
13	BPSAC013	7.5	5.0	10.0	6.0	5.0	9.0
14	BPSAC014	10.0	-	10.0	-	6.0	10.0
15	BPSAC015	10.0	8.0	11.0	7.5	-	7.5
16	BPSAC016	10.0	-	12.5	7.0	5.0	-
17	BPSAC017	11.5	3.0	13.5	7.5	-	8.0
18	BPSAC018	7.5	-	8.5	6.0	-	-
19	BPSAC019	10.0	6.5	12.5	7.5	5.0	10.0
20	BPSAC020	8.5	-	10.0	5.0	5.0	6.0
21	BPSAC021	3.5	-	10.0	5.0	5.0	6.0
22	BPSAC022	12.0	-	10.0	7.0	-	8.5
23	BPSAC023	10.0	7.5	12.0	7.5	5.0	9.0
24	BPSAC024	11.0	-	11.5	7.5	5.0	8.0
25	BPSAC025	10.0	4.0	10.0	7.0	5.0	9.0
26	BPSAC026	10.0	-	12.5	7.0	-	8.0

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
27	BPSAC027	10.0	6.0	10.0	7.5	-	8.0
28	BPSAC028	11.0	-	11.0	6.5	-	7.0
29	BPSAC029	10.0	5.0	10.0	4.0	-	8.0
30	BPSAC030	12.5	-	11.0	-	-	10.0
31	BPSAC031	12.5	10.0	11.0	7.0	-	10.0
32	BPSAC032	12.0	10.0	12.5	7.5	6.5	10.0
33	BPSAC033	12.5	-	12.5	8.0	6.0	9.0
34	BPSAC034	10.0	5.0	11.0	7.5	-	10.0
35	BPSAC035	7.5	-	10.0	7.5	-	7.5
36	BPSAC036	10.0	5.0	12.5	6.0	7.0	10.0
37	BPSAC037	11.0	-	11.0	6.5	-	9.0
38	BPSAC038	12.5	5.0	10.0	7.5	-	7.5
39	BPSAC039	12.5	-	12.5	6.5	-	-
40	BPSAC040	7.5	6.0	10.0	-	5.5	6.5
41	BPSAC041	12.0	-	11.0	6.0	6.0	-
42	BPSAC042	10.0	7.0	10.0	10.0	7.5	7.5
43	BPSAC043	6.0	-	5.5	4.0	5.0	4.0
44	BPSAC044	12.5	-	12.5	7.5	6.0	8.0
45	BPSAC045	11.0	6.0	11.0	6.5	5.0	8.5
46	BPSAC046	10.0	-	10.0	5.0	-	7.5
47	BPSAC047	11.0	-	10.0	7.5	7.0	8.0
48	BPSAC048	12.0	5.5	11.0	-	6.7	7.5
49	BPSAC049	6.0	-	7.5	5.0	-	-
50	BPSAC050	9.0	4.0	8.0	5.0	5.0	9.0
51	BPSAC051	10.0	4.0	12.5	7.5	6.5	8.5
52	BPSAC052	8.5	-	11.0	6.5	-	7.5
53	BPSAC053	6.0	5.5	10.0	-	6.0	8.0

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
54	BPSAC054	8.0	3.0	7.5	6.0	-	7.0
55	BPSAC055	10.0	7.5	12.5	-	6.5	7.5
56	BPSAC056	10.0	3.0	8.5	5.0	4.0	-
57	BPSAC057	8.0	-	10.0	-	-	8.0
58	BPSAC058	10.0	6.0	12.5	7.5	-	9.5
59	BPSAC059	9.0	6.0	10.0	-	6.0	7.0
60	BPSAC060	8.5	-	10.0	6.0	-	7.5
61	BPSAC061	4.5	-	7.0	5.0	-	4.5
62	BPSAC062	12.5	7.0	10.0	7.5	5.0	-
63	BPSAC063	10.0	-	11.0	7.5	-	8.5
64	BPSAC064	12.5	8.0	10.0	6.5	6.5	9.0
65	BPSAC065	8.0	2.0	7.5	-	4.0	8.0
66	BPSAC066	12.0	-	12.5	-	-	8.5
67	BPSAC067	11.0	6.0	12.5	7.5	-	9.0
68	BPSAC068	11.0	3.0	10.0	6.0	-	10.0
69	BPSAC069	10.0	9.0	10.0	7.5	5.0	8.0
70	BPSAC070	12.5	-	12.5	7.5	5.0	-
71	BPSAC071	10.0	-	15.0	-	-	12.5
72	BPSAC072	10.0	6.0	12.5	-	6.0	6.0
73	BPSAC073	10.0	6.0	10.0	6.5	5.0	8.0
74	BPSAC074	8.5	-	5.0	6.0	-	4.0
75	BPSAC075	12.5	-	10.0	9.5	-	7.0
76	BPSAC076	11.0	6.0	11.0	10.0	-	8.0
77	BPSAC077	10.0	-	12.5	7.0	-	7.5
78	BPSAC078	7.0	-	5.5	7.0	-	7.5
79	BPSAC079	12.0	-	6.0	4.0	-	7.5
80	BPSAC080	10.0	4.0	10.0	5.0	5.0	-

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
81	BPSAC081	12.5	5.0	12.0	6.0	6.5	8.5
82	BPSAC082	9.0	-	7.5	7.5	5.0	-
83	BPSAC083	11.0	7.0	12.5	7.0	8.0	8.0
84	BPSAC084	8.0	-	12.5	6.5	5.0	7.5
85	BPSAC085	8.5	6.0	10.0	6.0	-	8.0
86	BPSAC086	-	-	8.5	6.0	-	6.5
87	BPSAC087	10.0	3.0	12.5	6.0	6.0	7.5
88	BPSAC088	-	-	10.0	7.5	-	7.0
89	BPSAC089	11.0	10.0	10.0	7.5	-	10.0
90	BPSAC090	10.0	5.0	10.0	5.0	-	7.5
91	BPSAC091	15.0	-	16.0	10.0	6.0	-
92	BPSAC092	10.0	4.0	7.5	5.0	-	8.0
93	BPSAC093	13.0	-	12.5	7.5	-	-
94	BPSAC094	7.5	5.0	7.5	5.0	-	7.5
95	BPSAC095	8.0	6.5	10.0	-	6.0	6.0
96	BPSAC096	10.0	-	8.5	-	-	7.0
97	BPSAC97	8.5	5.0	10.0	-	5.0	7.0
98	BPSAC98	13.0	-	12.5	7.5	-	7.5
99	BPSAC099	10.0	4.0	12.5	7.5	6.5	8.0
100	BPSAC100	10.0	-	12.0	-	7.5	8.0
101	BPSAC101	10.0	6.0	11.0	-	6.5	9.0
102	BPSAC102	8.0	-	9.0	-	-	-
103	BPSAC103	8.0	6.0	11.0	5.0	-	8.5
104	BPSAC104	9.0	4.0	10.0	6.0	-	8.0
105	BPSAC105	12.5	-	12.5	6.0	5.0	-
106	BPSAC106	10.0	6.5	12.5	-	7.0	10.0
107	BPSAC107	6.0	-	9.5	6.0	-	-

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
108	BPSAC108	4.0	-	5.5	5.0	-	-
109	BPSAC109	-	-	8.0	-	5.5	8.0
110	BPSAC110	10.0	6.0	10.0	10.0	6.5	-
111	BPSAC111	10.0	7.0	10.0	6.0	-	7.0
112	BPSAC112	12.5	5.0	10.0	8.0	6.0	7.0
113	BPSAC113	15.0	-	12.5	-	-	8.0
114	BPSAC114	15.0	8.0	12.5	7.0	6.0	-
115	BPSAC115	10.0	6.0	11.0	7.0	-	8.0
116	BPSAC116	13.5	-	15.0	9.0	-	-
117	BPSAC117	12.5	5.0	12.5	8.5	-	10.0
118	BPSAC118	8.5	4.0	10.0	6.0	-	7.0
119	BPSAC119	11.5	8.0	15.0	7.5	6.0	9.0
120	BPSAC120	10.0	6.5	11.0	10.0	7.0	7.5
121	BPSAC121	-	-	8.0	6.0	-	6.0
122	BPSAC122	15.0	5.0	15.0	-	7.5	9.0
123	BPSAC123	9.0	8.0	9.0	-	6.0	7.5
124	BPSAC124	6.0	6.0	8.0	-	5.0	6.5
125	BPSAC125	10.0	6.0	12.0	-	6.0	10.0
126	BPSAC126	15.0	6.0	15.0	10.0	8.0	10.0
127	BPSAC127	12.0	8.0	12.5	-	7.5	8.0
128	BPSAC128	11.0	6.0	8.5	-	6.5	6.5
129	BPSAC129	13.5	8.0	12.5	-	7.0	10.0
130	BPSAC130	12.5	6.0	12.5	8.5	6.5	10.0
131	BPSAC131	11.5	-	11.0	7.0	5.0	-
132	BPSAC132	8.5	4.0	13.0	6.5	6.0	7.5
133	BPSAC133	7.5	-	7.5	7.5	-	-
134	BPSAC134	5.0	-	8.5	6.0	-	7.5

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
135	BPSAC135	10.0	5.0	12.5	6.5	-	8.0
136	BPSAC136	12.0	-	14.0	7.5	5.0	-
137	BPSAC137	9.0	5.0	9.0	-	6.0	8.0
138	BPSAC138	11.0	7.5	12.0	8.5	5.0	8.0
139	BPSAC139	13.0	6.0	11.0	7.5	-	7.5
140	BPSAC140	12.5	-	12.5	7.5	-	7.5
141	BPSAC141	12.5	6.5	15.0	7.5	6.5	10.0
142	BPSAC142	8.5	-	9.0	-	5.5	7.0
143	BPSAC143	10.0	-	12.5	6.5	-	-
144	BPSAC144	9.5	-	8.5	-	-	7.0
145	BPSAC145	10.0	5.0	10.0	-	6.0	6.0
146	BPSAC146	11.0	-	11.0	-	--	7.0
147	BPSAC147	15.0	3.0	15.0	6.0	7.5	10.0
148	BPSAC148	15.0	6.0	15.0	8.0	7.0	8.5
149	BPSAC149	15.0	6.5	15.0	12.5	7.5	10.0
150	BPSAC150	12.5	4.0	12.5	7.5	-	9.0
151	BPSAC151	-	-	7.5	-	-	8.0
152	BPSAC152	11.5	4.0	13.0	7.5	7.0	10.0
153	BPSAC153	12.5	5.0	11.0	-	6.5	9.5
154	BPSAC154	10.0	7.5	9.0	-	6.0	7.5
155	BPSAC155	12.5	-	12.5	7.5	-	7.5
156	BPSAC156	12.5	-	15.0	7.5	5.0	-
157	BPSAC157	15.0	3.0	16.0	12.0	8.5	10.0
158	BPSAC158	12.5	-	12.5	8.0	6.0	-
159	BPSAC159	10.0	6.0	10.0	-	6.0	7.5
160	BPSAC160	12.5	5.0	13.0	7.5	-	8.5
161	BPSAC161	12.5	-	12.5	8.5	-	8.0

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
162	BPSAC162	15.0	-	13.0	7.5	-	-
163	BPSAC163	12.5	8.0	10.0	7.0	6.5	-
164	BPSAC164	9.5	4.0	8.5	6.0	-	7.5
165	BPSAC165	10.0	5.0	10.0	-	4.5	8.5
166	BPSAC166	11.0	-	11.0	-	-	7.0
167	BPSAC167	15.0	5.0	12.5	7.5	-	5.0
168	BPSAC168	12.5	3.0	11.0	-	7.5	10.0
169	BPSAC169	10.0	-	12.0	6.5	-	9.0
170	BPSAC170	10.0	-	8.0	6.5	6.5	-
171	BPSAC171	8.5	7.0	9.0	-	4.0	10.0
172	BPSAC172	12.5	5.0	12.5	7.5	-	7.5
173	BPSAC173	10.0	10.0	8.5	-	6.0	6.0
174	BPSAC174	8.0	-	7.5	-	6.5	10.0
175	BPSAC175	10.0	5.0	12.5	6.5	6.5	7.5
176	BPSAC176	10.0	-	8.5	-	-	7.0
177	BPSAC177	12.0	9.0	12.5	6.0	7.5	10.0
178	BPSAC178	7.5	-	10.0	-	-	8.0
179	BPSAC179	15.0	6.0	15.0	10.0	7.5	12.5
180	BPSAC180	10.0	5.0	15.0	7.5	5.0	10.0
181	BPSAC181	15.0	6.0	15.0	7.5	6.0	-
182	BPSAC182	10.0	6.0	9.5	6.5	7.5	7.5
183	BPSAC183	15.0	-	15.0	8.0	-	8.0
184	BPSAC184	10.0	7.0	10.0	8.5	6.5	10.0
185	BPSAC185	13.0	6.0	15.0	9.0	-	10.0
186	BPSAC186	11.0	-	9.0	-	6.5	6.0
187	BPSAC187	15.0	-	11.0	10.0	-	-
188	BPSAC188	12.5	7.0	15.0	7.5	5.0	10.0

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
189	BPSAC189	13.5	-	15.0	10.0	-	-
190	BPSAC190	10.0	-	10.0	-	6.5	7.5
191	BPSAC191	10.0	7.0	12.0	-	7.0	6.5
192	BPSAC192	10.0	6.0	12.0	-	7.0	7.0
193	BPSAC193	8.5	6.0	10.0	-	6.0	8.0
194	BPSAC194	11.0	4.0	13.0	7.5	-	7.0
195	BPSAC195	-	-	12.5	8.0	5.0	-
196	BPSAC196	12.5	4.0	12.5	10.0	6.0	-
197	BPSAC197	12.5	8.0	8.0	-	7.5	6.5
198	BPSAC198	10.0	7.0	9.0	10.0	6.5	-
199	BPSAC199	-	6.5	8.5	5.0	-	6.0
200	BPSAC200	12.5	-	12.5	7.5	-	7.5
201	BPSAC201	10.0	7.5	12.0	-	6.0	7.0
202	BPSAC202	10.0	-	8.0	6.5	6.5	-
203	BPSAC203	10.0	5.0	10.0	-	5.0	8.5
204	BPSAC204	12.5	-	12.5	8.0	6.0	-
205	BPSAC205	10.0	6.0	10.0	-	6.0	7.5
206	BPSAC206	15.0	6.0	14.0	11.0	7.5	10.0
207	BPSAC207	10.0	7.5	9.0	-	6.0	7.5
208	BPSAC208	8.5	-	9.0	-	5.5	7.0
209	BPSAC209	12.5	-	12.5	8.0	-	8.5
210	BPSAC210	12.0	-	12.5	-	7.5	8.0
211	BPSAC211	10.0	6.5	12.5	-	7.0	10.0
212	BPSAC212	6.0	6.0	9.5	6.0	5.0	7.0
213	BPSAC213	9.0	-	7.0	-	6.0	7.0
214	BPSAC214	11.0	7.5	11.0	-	6.5	7.5
215	BPSAC215	7.5	-	11.0	6.0	-	9.0

Sl No.	Isolate	Bacterial pathogens zone in mm					
		S. aureus	M. luteus	B. subtilis	E. coli	P. aeruginosa	C. albicans
216	BPSAC216	12.5	6.0	12.5	6.0	8.0	7.5
217	BPSAC217	-	-	15.0	-	7.5	10.0
218	BPSAC218	10.0	6.0	12.0	-	6.5	6.5
219	BPSAC219	12.5	-	12.5	7.0	-	10.0
220	BPSAC220	10.0	6.0	9.0	7.0	7.5	7.0
221	BPSAC221	10.0	6.0	11.0	10.0	5.0	7.5
222	BPSAC222	10.0	5.0	12.5	7.5	5.0	7.0
223	BPSAC223	10.0	-	15.0	-	--	10.0
224	BPSAC224	10.0	8.0	8.0	-	5.5	8.0
225	BPSAC225	10.0	-	10.0	6.5	6.0	-
226	BPSAC226	12.5	6.0	13.0	-	6.0	7.5
227	BPSAC227	12.5	-	12.5	7.5	--	-
228	BPSAC228	9.0	-	8.0	7.0	--	-
229	BPSAC229	8.5	5.0	11.0	-	6.0	6.0
230	BPSAC230	10.0	5.0	11.0	7.0	5.0	9.0
231	BPSAC231	9.0	6.5	9.0	-	5.0	6.5
232	BPSAC232	13.5	4.0	13.5	10.0	5.0	10.0
233	BPSAC233	11.5	-	12.0	7.5	-	8.0
234	BPSAC234	12.5	4.0	12.5	8.5	7.0	7.5
235	BPSAC235	10.0	-	9.0	7.5	-	7.5

Interestingly, out of these 136 isolates, 48 isolates (29 actinobacterial isolates [60.41%] and 19 bacterial isolates [39.58%]) exhibited broad-spectrum antimicrobial activities against all pathogens. Among these, BPSCV70 (*Micrococcus luteus*), BPSCV82 (*Streptomyces* sp.), BPSCV83 (*Streptomyces* sp.), BPSCV84 (*Micromonospora* sp.), BPSCV89 (*Actinobacteria bacterium*), BPSCV102 (*Streptomyces* sp.) and BPSCV120 (*Actinomycetales bacterium*) showed significant zone of inhibition accounting for 15 mm against *Staphylococcus aureus* and *Bacillus subtilis* respectively (Fig. 1). Similarly, isolates BPSCV15 (*Virgibacillus* sp.), BPSCV16 (*Staphylococcus saprophyticus*), BPSCV46 (*Kocuria palustris*) and BPSCV99 (*Streptomyces* sp.) exhibited maximum antimicrobial activity against *Micrococcus luteus* (10 mm) whereas strain BPSCV102 (*Streptomyces* sp.) showed the highest antimicrobial activity against *Candida*

albicans (12.5 mm). Given the broad-spectrum antimicrobial nature of 136 bacterial isolates they were all selected as potential candidates for further investigation

3.4 Biosynthetic gene detection vs antimicrobial activity of the selected isolates

From the 136 potent isolates selected, the presence of genes encoding PKS type II were detected in 46 isolates, while a band of the expected size for PKS type I candidate amplicons was detected in only 37 such isolates (27.2%) using their respective degenerate primers.

Moreover, 54 isolates (39.7%) also detected NRPS gene with the expected band size of 600 bp. In 21 isolates (15.44%) positive amplification products were obtained for all three biosynthetic genes (PKS type I, PKS type II and NRPS) and they were BPSCV3, BPSCV10, BPSCV11, BPSCV12, BPSCV16, BPSCV18, BPSCV21, BPSCV22, BPSCV24, BPSCV35, BPSCV43, BPSCV64, BPSCV66, BPSCV70, BPSCV78, BPSCV80, BPSCV82, BPSCV84, BPSCV89, BPSCV99 and BPSCV102.

3.5 Identification and phylogenetic analysis of the selected bacterial isolates

All the potential isolates were identified by 16S-rRNA gene sequencing and amplicon product size was found ~ 1500 bp. All the isolates showed 98–100% sequence similarity to the reference isolates based on BLAST analysis and the sequences of the isolates were deposited in NCBI GenBank to obtain accession number (MK855189-MK855321). The results showed that the isolates were classified into 13 families belonging to *Streptomycetaceae* (n = 66; 49.6%), *Bacillaceae* (n = 19; 14.2%), *Micromonosporaceae* (n = 8; 6.01%), *Microbacteriaceae* (n = 6; 4.51%), *Micrococcaceae* (n = 6; 4.51%), *Staphylococcaceae* (n = 6; 4.51%), *Actinomycetaceae* (n = 5; 3.75%), *Xanthomonadaceae* (n = 5; 3.75%), *Pseudonocardiaceae* (n = 3; 2.25%), *Nocardiopsaceae* (n = 3; 2.25%), *Pseudomonadaceae* (n = 3; 2.25%), *Brevibacteriaceae* (n = 2; 1.5%) and *Alcaligenaceae* (n = 1; 0.75%) respectively.

The phylogenetic tree was constructed based on 16S-rRNA gene sequences values using Mega 6.0. In case of actinobacteria phylogenetic tree was assembled using the maximum likelihood method with Jukes-Cantor model according to lowest BIC (2190.170) and highest AIC (844.878). The gaps were treated by pairwise deletion and the estimated Transition/Transversion bias (R) was 0.50. The tree showed that the entire genus *Streptomyces* group was clustered together in one clade with the bootstrap supported value of 60%. Whereas, the other rare genera like *Micromonospora* was closely clustered with *Pseudonocardia* and *Saccharopolyspora* group with bootstrap supported value 55%. Interestingly, genera *Pseudonocardia* and *Saccharopolyspora* were represented under the same family. Further, very rare genera like *Microbacterium*, *Kocuria*, *Micrococcus*, *Nocardiopsis*, and *Brevibacterium* were clustered together in another clade with the bootstrap supported value of 63% (Fig. 2A).

In case of gram-positive bacteria, the phylogenetic tree was also made with maximum likelihood method, but following the Kimura 2-parameter model according to the lowest BIC (5088.975) and highest AIC

(4558.721) values. Here also, gaps were treated by pairwise deletion while the estimated Transition/Transversion bias (R) was found to be 1.27. The phylogenetic tree revealed that all *Bacillus* genera were clustered together in one clade with the bootstrap supported value of 54% whereas all the *Staphylococcus* genera were clustered separately in another clade with the bootstrap supported value of 88% (Fig. 2B).

For gram-negative bacteria, the phylogenetic tree was also built by maximum likelihood method but by using the model of Tamura 3-parameter according to lowest BIC (3223.764) and highest AIC (2058.828) values. Gaps again were treated by pairwise deletion while here the estimated Transition/Transversion bias (R) was 1.08. The phylogenetic tree indicated that all isolates of genus *Stenotrophomonas* were closely clustered with genera *Achromobacter* in one clade with the bootstrap supported value of 58% whereas all isolates belonging to genera *Pseudomonas* was clustered with their type strains *Pseudomonas oryzae* type strain NBRC10219 having bootstrap supported value of 51% respectively (Fig. 2C).

3.6 NGS based Non-culturable microbial community profiling of the Pukzing Cave samples and its functional analysis

3.6.1 Deciphering of the microbial population: Cave Sample

The high throughput Illumina sequencing from total DNA of the cave sediment samples yielded 554,834 and 208,946 paired-end reads with an average Phred scores of 35.93 and 37.43 for the 16S-rRNA variable gene regions V3 and V4 respectively. Based on relative abundance obtained at a phyla level; a total of 38 phyla (BHI80-139; Chlorobi; Elusimicrobia; Fibrobacteres; Gemmatimonadetes; GN02; MVP-21; OD1; FCPU426; ZB3; Synergistetes; Euryarchaeota; NKB19; TM6; GN04; Thermi; GAL15; WPS-2; Armatimonadetes; Chlamydiae; AD3; TM7; Crenarchaeota; Fusobacteria; WS2; WS3; Nitrospirae; Chloroflexi; Synergistetes; Acidobacteria; Cyanobacteria; Planctomycetes; Proteobacteria; Firmicutes; Verrucomicrobia; Bacteroidetes; Actinobacteria & unidentified isolates) was recorded for V3 and V4 region of cave sediment sample (Fig. 3A). The phylum Archae group under Euryarchaeota and Crenarchaeota was only found in the V4 region of cave sediment sample (Fig. 3B).

The bacterial community analysis exhibited that the phylum Actinobacteria were the most dominant bacteria (93.4%) followed by Proteobacteria (1.82%), Firmicutes (1.34%), Chloroflexi (0.98%) and other (0.89%) respectively in V3 region. Similarly, the most dominant phylum Actinobacteria was recorded (41.4%) followed by Bacteroidetes (11.6%), Verrucomicrobia (10.3%), Firmicutes (9.68%) and other bacteria (21.4%) respectively in V4 region (Fig. 3C). In the Pukzing cave, more than 76% of bacterial species were associated with three major phyla including Actinobacteria, Firmicutes, and Proteobacteria (Supplementary File 1).

Phylum Actinobacteria

In the present study, the identified class under this phylum were *Saccharopolyspora*, *Nocardioideaceae*, *Streptomycetaceae*, *Pseudonocardia*, *Pseudonocardiaceae*, *Nocardiaceae*, *Streptomyces*, *Actinomycetales*, *Actinomycetospora*, *Frankiaceae*, *Actinosynnemataceae*, *Actinomadura*, *Mycobacterium*, *Amycolatopsis*, *Rhodococcus*, *Actinokineospira*, *Corynebacterium*, *Nocardioidea*, *Nocardia*, *Micromonosporaceae*, *Micrococcaceae*, *Intrasporangiaceae*, *Acidimicrobiales*, *Kineosporiaceae*, *Kibdelosporangium*, *Sporichthyaceae*, *Cellulomonas*, *Propionibacteriaceae*, *Geodermatophilaceae*, *Bifidobacterium*, *Euzeybya*, *Gaiellaceae*, 0319-7L14, *Solirubrobacterales* and *Rubrobacter*.

Phylum Firmicutes

Within Firmicutes, the identified bacterial strains are *Streptococcus*, *Ruminococcaceae*, *Lachnospiraceae*, *Blautia*, *Clostridiaceae*, *Dorea*, *Turicibacter*, *Synergistaceae*, *Ruminococcus*, *Coprococcus*, *Bacillales*, *Lachnospiraceae*, and *Eubacterium*.

Phylum Proteobacteria

Within the Proteobacteria, most phylotypes were classified under the class Alphaproteobacteria and Gammaproteobacteria. These are *Enterobacteriaceae*, *agg27*, *YS2*, *Sutterella*, *JG30-KF-CM45*, *Bacillus*, *Pseudomonas* and *Rhodospirillaceae*.

3.6.2. Statistical tests, taxonomic differences, and analysis of microbial population: V3 and V4 region

Statistical analyses on taxonomic abundance were performed using a hypergeometric test (Fig. 4) with an extended error bar plot. It signifies statistical significance ($p\text{-value} > 0.05$) in species which includes *Saccharopolyspora hirsute* ($p\text{-value } 8.26\text{e-}7$), *Akkermansia muciniphila* ($1.56\text{e-}3$), *Nocardioideaceae* ($4.57\text{e-}3$), *Prevotella copri* ($7.14\text{e-}3$), *Saccharopolyspora* ($9.77\text{e-}3$) and Unassigned taxa ($3.59\text{e-}6$). Other species are showing the difference in proportions including V3 and V4 samples but are not statistical significance.

The similarity in bacterial community structure was inferred from taxonomic data at species level using Bray-Curtis (BC) similarity score and therefore reduced using Non-metric Multidimensional Scaling at 2D space. During processing, taxonomic clades present in at least one sample with a relative OTU count of 100 and above are considered for similarity matrix calculation. Shorter linear distance denotes greater similarity between samples, but in Fig. 5 it is clearly visible that V3 and V4 samples linear distance is larger and distinct.

The phylogenetic tree generated using QIIME is compared for V3 and V4 samples and visualized with the MEGAN tool as cladogram (Fig. 6). MEGAN analysis of Illumina reads showed that Proteobacteria group (alpha, beta, delta and gamma bacteria) were matched together in one group. Similarly, to results from the MEGAN analysis, combination reads of V3 and V4 regions indicated that the PVC group (Planctomycetes and Verrucomicrobia), FCB group (Chlorobi and Bacteroidetes) and Acidobacteria

(Acidobacteriaceae and Solibacterales) was also clustered separately. We have found that the larger group of the Terrabacteria (Actinobacteria, Deinococcus, Cyanobacteria, Chloroflexi, Firmicutes, and Armatimonadetes) was clustered together according to the MEGAN analysis of the Illumina reads (Fig. 6). There is also a group called unassigned which was found as unclassified bacteria in MEGAN analysis.

Rarefaction curve analysis and diversity index are inferred and plotted in Fig. 7. The alpha diversity analysis demonstrated that the V3 region indicating high diversity (35.0) as compared to V4 region (30.0) (Fig. 7A). The rarefaction curves showed that a linear relationship between taxa and species richness in the V3 region whereas no linear curve was found between taxa and species richness in the V4 region (Fig. 7B). Taxonomic and phylogenetic similarity using beta diversity is calculated with Bray-Curtis (B-C) similarity measure and FastUnifrac. Based on the average, we have observed that beta diversity is much higher in both phylogenetic and taxonomic similarity in V3 sample as compared to V4 sample (Fig. 8A-8D). The abundance of taxa in a sample computed describes the OTU richness in a sample with more richness in V4 sample as compared to V3 sample (Fig. 8B). At species level microbial communities that are shared and unique to each sample is described in Fig. 8C with 333 species shared in both V3 and V4 sample, 396 being unique to V3 and 129 species unique to V4 sample.

At the phylum level, heat map analysis is used to understand the taxonomic differences between the two variable regions (V3 & V4). Relative abundance at the phylum level using Spearman correlation (at $P < 0.05$ significance) is generated with a heat map where green signifies strong positive correlation, and pink represents a strong negative correlation (Fig. 9). A positive correlation means when one phylum was increased in one variable region (V3) than the other variable region increases at the same time with vice-versa in negative correlation. We have observed that a positive correlation was much higher in the V4 region as compared to the V3 region.

Network interaction analysis on the microbial taxonomic community at the species level is deciphered (Fig. 10) using Cytoscape where red hexagon shape nodes represent the sample, circular nodes in green represent species in samples V3 and V4, Cyan include species in V3 sample and yellow depicts species in V4 sample. Network analysis indicates all the similar species are very closely clustered together in V3 and V4 region that are visualized in green color. The similar species are as follows *Actinomycetales*, *Pseudonocardia*, *Rubrobacter*, *Solirubrobacterales*, *Streptomyces*, *Saccharopolyspora*, *Actinomycetospora*, *Kibdelosporangium*, *Pseudonocardiaceae*, *Amycolatopsis*, *Streptococcus*, *Solirubrobacteraceae*, *JG30-KF-CM45*, *AKYG1722*, *iii1-15*, *Nocardiaceae*, *Ellin6529*, *Enterobacteriaceae*, *Clostridiaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Ruminococcus* and *Ellin6075* respectively.

3.6.2 Overall functional Insight Prediction

Predicted insights based on functional category include annotated proteins, unknown proteins, and ribosomal RNA genes which range in 0.28%, 0.13% and 99.59% (in V3 sample) respectively. Similarly, in V4, rRNA genes fall in a high percentage level of 99.06% with annotated protein being 0.22% and unknown protein of 0.72%. Functional analysis with V3 sample did not map with any highest functional

category in COG (Clusters of Orthologous Groups), NOG (Non-supervised Orthologous Groups) and KO (KEGG Orthology) category whereas with subsystems-based annotation using SEED database showed only Carbohydrate metabolism in their classification. But in the case of V4 sample, subsystem annotation predicted two functions associated with Amino acid and its derivatives and Cofactors, Vitamins, Prosthetic Groups, Pigments functions. Based on KO, the function is attributed to metabolism and with COG it is involved in metabolism, cellular processes, and signaling.

4. Discussion

Microorganisms' presence inside the cave systems are commonly oligotrophic or chemolithotrophic in nature and impose precise nutrients for their growth. Hence, it is very difficult to isolate some rare or specific bacteria from these sources (Portillo et al., 2008). In our study, the maximum number of bacteria was obtained from SCA media (n = 92; 39.1%) followed by ISP7 (n = 57; 24.2%), ISP5 (n = 26; 11.0%), LB (n = 24; 10.2%), TSA (n = 21; 8.9%) and TH₂O media (n = 17; 7.2%). This finding was similarly reported by Adam et al. (2018) who state that 46 actinobacteria isolates collected from cave moon milk deposits were obtained from starch casein nitrate medium. Moreover, Tomova et al. (2013) reported that the greatest number of heterotrophic bacteria (46 isolates) collected from the Gallery with the drawings in Magura cave, Bulgaria was isolated from nutrient agar (NA) media. We have found that out of 235 isolates, 142 bacterial isolates were gram-positive (60.4%) and 93 of the gram-negative (39.5%). These findings were consistent with Bhullar et al. (2012) who state that out of 93 bacterial strains, 33% Gram-positive and 63% Gram-negative was isolated from Lechuguilla cave, New Mexico.

In this study, we have found significant antimicrobial activity in cave bacterial isolates against a gram-positive and gram-negative bacterial pathogen. In our study, out of 235 isolates, 136 (57.87%) strains showed significant antimicrobial potential at least five of the six tested pathogens whereas 48 (20.4%) isolates exhibited antimicrobial ability against all tested pathogens. This finding is similarly reported by Tomova et al. (2013) who suggested that 50% of the bacterial isolates obtained from Magura cave showed significant inhibitory activity against *Pseudomonas aeruginosa* and the yeast pathogen *Rhodotorula mucilaginosa*. Out of 136 isolates, 29 (21.3%) actinobacterial isolates and 19 (13.9%) bacterial isolates showed potential antimicrobial activities against all tested pathogens. Few researchers reported that mainly actinobacteria isolated from karstic caves showing significant antimicrobial activity against bacterial pathogens (Kim et al., 1998; Yucel and Yamac, 2010). Yucel and Yamac (2010) reported that 62% of actinobacteria isolated from the rock wall, speleothems surface and soil samples of karstic caves obtained from Turkey showed antimicrobial activity against four bacteria, two yeasts, and four filamentous fungi pathogens. Yasir et al. (2018) reported that out of 84 isolates, only 30 strains revealed antimicrobial activity against three pathogens i.e. *Salmonella typhi*, *Staphylococcus aureus*, and *Candida albicans*. Isolates BPSCV70 (*Micrococcus luteus*), BPSCV82 (*Streptomyces* sp.), BPSCV83 (*Streptomyces* sp.), BPSCV84 (*Micromonospora* sp.), BPSCV89 (*Actinobacteria bacterium*), BPSCV102 (*Streptomyces* sp.) and BPSCV120 (*Actinomycetales bacterium*) showed promising activity against *Staphylococcus aureus* (15 mm) and *Bacillus subtilis* (15 mm). This finding was similarly reported by Yasir et al. (2018)

who suggested that only 15 isolates exhibited inhibitory activity against *S. typhi* whereas, 20 strains were highly significant against *S. aureus*. Moreover, only six isolates exhibited potent antibacterial activity against both tested bacterial pathogens. Further, genus *Pseudomonas* and *Bacillus* were also showing strong antimicrobial activity against tested pathogenic bacteria as suggested by Yasir et al. (2018). Adam et al. (2018) reported that three moonmilk *Streptomyces* strains (MMun141, MMun146 and MMun156) showed strong antimicrobial activities against all tested gram-positive bacterial pathogens (*Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*) whereas isolates *Kocuria* strain MMun160 and *Amycolatopsis* strain MMun171 showed active against gram-negative bacterial pathogens (*Escherichia coli*, *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Klebsiella pneumoniae*). Cervimycins A-D produced from *Streptomyces tendae* strain HKI 0179, isolated from an ancient cave, the Grotta dei Cervi in Italy showed greatest antibacterial activity against gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and multi-drug-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE) as reported by Herold et al. (2005). Higher activity of AMP assay against these microbes indicates that cave microbial community could be a potential source of future genomic resource discovery especially for wider applications in agricultural, domestic animal and human health (Logan 1988; Saglam et al., 2017; Bottcher et al., 2018; Edelman et al., 2005).

Clarridge (2004) states that the 16S-rRNA gene sequence is the perfect technique to identify the isolates up to species level. In our study, we have found that all the isolates showed 98–100% identity with reference sequences in NCBI GenBank. All the isolates were divided into 13 families indicates that all isolates having a strong relationship with cave soil samples. In our study, the phylogenetic tree showed that actinobacteria were the dominant group followed by Proteobacteria and Firmicutes group. Similarly, Lee et al. (2012) reported that the phylogenetic tree of 16S-rRNA gene sequences obtained from 60 caves around the world. Among them, the most abundant groups belong to *Proteobacteria* followed by *Actinobacteria*, and *Chloroflexi*. The similar findings were reported by Yasir (2018) who state that the cultured isolates from both caves were divided into the phyla *Proteobacteria*, *Actinobacteria*, and *Firmicutes*. Among them, 13 genera were identified from the culturable study including *Bacillus*, *Microbacterium*, *Pseudomonas* and *Staphylococcus*.

Culture dependent is a commonly useful method to isolate microorganisms from any source, but they do not provide perfect identification of microorganisms. Hence, various molecular techniques have been developed to protect cultural heritage. For example, Pinar et al. (2014) has used two PCR based methods for the identification of the cultivable fraction of the halophilic microflora that inhabit the Catacombs of Palermo and used molecular approach to develop non-invasive and perfect sampling methods for DNA extraction for bacterial and fungal diversity analyses (Pinar et al., 2015 and 2016). The next-generation sequencing technologies were used to analyze bacterial diversity on bricks from barracks in the former Auschwitz IleBirkenau Museum and to investigate the microbial population colonizing the medieval church of San Leonardo di Siponto (Italy). Interestingly, they have found three dominant bacterial phyla i.e. Proteobacteria Actinobacteria and Bacteroidetes (Gutarowska et al., 2015; Chimienti et al., 2016). In our study, total of 38 phyla (BHI80-139; Chlorobi; Elusimicrobia; Fibrobacteres; Gemmatimonadetes; GN02; MVP-21; OD1; FCPU426; ZB3; Synergistetes; Euryarchaeota; NKB19; TM6; GN04; Thermi; GAL15;

WPS-2; Armatimonadetes; Chlamydiae; AD3; TM7; Crenarchaeota; Fusobacteria; WS2; WS3; Nitrospirae; Chloroflexi; Synergistetes; Acidobacteria; Cyanobacteria; Planctomycetes; Proteobacteria; Firmicutes; Verrucomicrobia; Bacteroidetes; Actinobacteria & unassigned other) was recorded in V3 and V4 region of cave sediment sample. Interestingly, we have recorded that the phylum Actinobacteria was the most dominant bacteria in both V3 and V4 regions. Previous researchers reported that the phylum Actinobacteria was found highly dominant in all the cave samples under V4 hypervariable region of 16S-rRNA (De-Mandal et al., 2016; Yasir, 2018). In the present study, we have identified under the phylum Actinobacteria were *Saccharopolyspora*, *Nocardiodaceae*, *Streptomycetaceae*, *Pseudonocardia*, *Pseudonocardiaceae*, *Nocardiaceae*, *Streptomyces*, *Actinomycetales*, *Actinomycetospira*, *Frankiaceae*, *Actinosynnemataceae*, *Actinomadura*, *Mycobacterium*, *Amycolatopsis*, *Rhodococcus*, *Actinokineospora*, *Corynebacterium*, *Nocardioidea*, *Nocardia*, *Micromonosporaceae*, *Micrococcaceae*, *Intrasporangiaceae*, *Acidimicrobiales*, *Kineosporiaceae*, *Kibdelosporangium*, *Sporichthyaceae*, *Cellulomonas*, *Propionibacteriaceae*, *Geodermatophilaceae*, *Bifidobacterium*, *Euzebya*, *Gaiellaceae*, *0319-7L14*, *Solirubrobacterales* and *Rubrobacter*. Similarly De-Mandal et al. (2017) reported that cave sediment samples collected from three different caves (Bukpuk [CBP V3], Lamsialpuk [CLP V3] and Reiekpuk [CRP V3]) of Mizoram and found that high abundance of dominant family (>0.01%) under Actinobacteria were *Nocardiaceae*, *Streptomycetaceae*, *Micrococcaceae*, *Frankiaceae*, *Gaiellaceae*, *Pseudonocardiaceae*, *Streptomycetaceae*, *EB1017*, *Mycobacteriaceae*, *Actinosynnemataceae*, *Corynebacteriaceae*, *Rubrobacteraceae*, *Nocardiodaceae*, *Micromonosporaceae*, *Geoderma tophilaceae*, *Sporichthyaceae*, *Actinosynnemataceae*, *Nakamurellaceae*, *Pseudonocardiaceae*, *Cryptospora34ngiaceae*, *Kineosporiaceae*, and *Ruaniaceae*. Moreover, few other genera under Actinobacteria were *Mycobacterium*, *Corynebacterium*, *Rubrobacter*, *Actinoplanes*, *Saccharothrix*, and *Pseudonocardia*. Various studies over the past decade suggested phylum Actinobacteria was highly present in all types of caves because of the favorable condition and environment to sustain inside the caves and are dynamically involved for the formation of crystals in cave walls and biomineralization process (Barton et al., 2001; Jurado et al., 2009). Laiz et al. (2009) also reported Actinobacteria under the genus *Rubrobacter* was isolated from biodeteriorated monuments that can induce crystal formation in caves and produce biofilm on the limestone. Yasir (2018) has isolated number of actinobacteria from two caves i.e. Koat Maqbari Ghaar (KMG) and Smasse-Rawo Ghaar (SG) of North-West region of Pakistan that showed significant antimicrobial activity against various bacterial pathogens.

Interestingly, the genera *Streptomyces* was recorded maximum under the family *Streptomycetaceae* that can synthesize various compounds including alcohols, sugars, amino acids, and aromatic compounds and poses abilities to produce clinically important antibiotics (Madigan and Martinko, 2005). Among the Proteobacteria, alpha-proteobacteria were highly dominant followed by gammaproteobacteria, beta proteobacteria, and delta proteobacteria. Previous studies state that few of the species under the Proteobacteria subphylum can subsist under very tremendous environment conditions by utilizing ABC (ATP-Binding Cassettes) and TRAP (Tripartite ATP-independent periplasmic transporters) mechanism (Kumbhare et al., 2015; De-Mandal et al., 2017). Proteobacteria isolated from Koat Maqbari Ghaar (KMG) and Smasse-Rawo Ghaar (SG) caves of Pakistan that showed alpha-proteobacteria (45.6% KMG and

34.1% SRG) and gamma-proteobacteria (35.2% KMG and 32.1% SRG) were dominant in both caves, followed by delta-proteobacteria (12.6% KMG and 16.9% SRG) and beta-proteobacteria (9.2% KMG and 16.7% SRG) as suggested by Yasir (2018). Moreover, the phylum Proteobacteria obtained from well-known Spanish Altamira cave, cosmopolitan was great abundance in dripping waters and cave walls (Portillo et al., 2008; Portillo et al., 2009). Schabereiter-Gurtner et al. (2002) reported that the phylum Proteobacteria was found half of the entire walls in the Tito Bustillo cave in Spain and this phylum can be used for chemolithotrophic energy production due to their versatile metabolic potential live on available ions in the rock contents. Further, the genera *Rhodoplanes* under the subphylum alphaproteobacteria were found in our study that possesses Photo and chemo-organ heterotrophic growth and produce hopanoids and carotenoids (Lakshmi et al., 2009; Lodha et al., 2015; Takaichi et al., 2012). Additionally, another genus *Sphingomonas* under the subphylum alphaproteobacteria was found in nutrient-limited subsurface environments that can metabolize a huge number of diverse aromatic compounds (Balkwil et al., 1997). Jin et al. (2012) reported that gram-negative heterotrophic bacteria *Alteromonas* under the subphylum gammaproteobacteria having abilities to degrade aromatic carbon rings investigated through an oil spill. Further genus *Halomonas* under the same subphylum having the capacity to resist extreme conditions and engross in sandstone formations as reported by Dong et al. (2014). Among the beta proteobacteria, the highly dominant genera were *Achromobacter*, *Burkholderia*, *Neisseria* and *Ramlibacter* were found in our study. Interestingly, the genus *Burkholderia* was diazotrophs bacteria and having abilities to degrade several xenobiotic compounds (Rusch et al., 2015). Moreover, the phylum *Firmicutes* was found dominant in our study. These findings were similarly reported by various researchers who state that the phylum *Firmicutes* obtained from various regions of caves was highly dominant and identified in more extreme ecosystems that are comparatively more resistant to nutrient stress (Chen et al., 2009; Ikner et al., 2007). In our study, we have obtained the *Bacillus* group from the Pukzing cave which can form endospores (Ikner et al., 2007). In addition, the archaea group under mesophilic *Crenarchaeota* and *Euryarchaeota* obtained from Pukzing cave were found in V4 hypervariable region of 16S rRNA. Similarly, both archaea group (mesophilic *Crenarchaeota* and *Euryarchaeota*) was detected in Koat Maqbari Ghaar (KMG) cave of Pakistan and found in the Lechuguilla Cave of United States (Ikner et al., 2007). Moreover, Legatzki et al. (2011) state that the presence of an archaeal community on calcite speleothems from Kartchner Caverns, Arizona, USA. Some important genera *Methylobacterium*, *Rhizobium*, *Kocuria*, *Acinetobacter*, *Renibacterium*, and *Bacillus* were found in our study. Similarly, these genera were reported from another cave and these genera having ability to utilize a carbon substrate as well as play a vital role in nitrogen fixation and calcification (Cuezva et al., 2012; Portillo et al., 2008; Schabereiter-Gurtner et al., 2002; Ikner et al., 2007; Northup et al., 2003). Numerous numbers of oligotrophic and facultative bacteria like *Nitrospira*, *Sphingomonas*, *Bacillus cereus*, *Paenibacillus*, *Streptomyces sp.*, *Brevibacillus*, and *Arthrobacter* were obtained in our culture-independent study and were previously reported from various oligotrophic environments (Hayakawa et al., 2011). The phylogenetic analysis of community study indicated that the tree has divided into two major groups i.e. Bacteria and Archaea group. Among them, the bacteria group is the largest group and is divided into three another sub-groups such as FCB group; PVC group and Terrabacteria group. All the bacterial strains were clustered within these groups in our study. All the

strains epitomize a novel potential isolate in caves biodiversity that indicates us isolated bacterial population properties to having significant discover a new micro-organism from the cave ecosystem (Jurado et al., 2009). Moreover, most of the isolated bacteria showed significant antimicrobial potential against bacterial pathogens. Interestingly, few researchers reported that isolated microbiota from cave ecosystem could be a potential source to discover new microorganisms and antimicrobial agents (Nakaew et al., 2009; Yasir, 2018) having wider applicability in health management of crop, animal and human.

5. Conclusion

This study reveals that, cave ecosystem is a unique source of endemic and extremophilic microorganisms. Diversity can be explored by both culture-based and culture-independent methods. However, culture dependent methods revealed limited 235 bacterial isolates by temperature treatments viz heat, cold and normal. Both extremes, cold and heat treatment led to recovery of the highest bacterial population indicating the dominance of extremophiles Actinobacteria. Study demonstrates that such microbial communities are having antimicrobial potential with biosynthetic genes like PKS type I, PKS type II and NRPS genes. Culture independent, community-based analysis using the sequencing of both hyper-variable regions V3 and V4 also revealed the dominance of phylum Actinobacteria. In comparative analysis, subsystem annotation using the V4 region was found to be more informative for cave metagenomes functional prediction associated with amino acid and its derivatives like cofactors, vitamins, prosthetic groups and pigments functions. AMP evaluation against six microbial pathogen species revealed that cave microbial communities can be an immensely valuable potential source of microbial genomic resources with higher AMP activity. The microbial panel indicates that these findings are promising for new AMP molecules having highly diverse applications in sectors of agriculture, domestic animals and human health.

Abbreviations

PKS: Polyketide synthases

NRPS: Non-ribosomal Peptide Synthetase

HVR: Hyper Variable Regions

AMP: Antimicrobial Peptide

TOC: Total Organic Carbon

DNA: Deoxyribonucleic acid

CHN: Carbon Hydrogen and Nitrogen

PCR: Polymerase chain reaction

NCBI: National Center for Biotechnology Information

BIC: Bayesian Information Criterion

AICc: Akaike Information Criterion, corrected

MEGA: Molecular Evolutionary Genetics Analysis

MTCC: Microbial Type Culture Collection

ANOVA: Analysis of Variance

QIIME: Quantitative Insights into Microbial Ecology

OUT: Operational Taxonomic Unit

MEGAN: MEtaGenome ANalyzer

MG-RAST: Metagenomic Rapid Annotations using Subsystems Technology

BC: Bray-Curtis

COG: Clusters of Orthologous Groups

NOG: Non-supervised Orthologous Groups

KO: KEGG Orthology

NA: Nutrient Agar

KMG: Koat Maqbari Ghaar

SG: Smasse-Rawo Ghaar

ABC: ATP-Binding Cassettes

TRAP: Tripartite ATP-independent periplasmic transporters

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for publication

All authors have gone through the manuscript and approve its submission.

Availability of data and material

All metagenomics data is available at the NCBI gene bank database under the BioProject: PRJNA489154, BioSample: SAMN09949180 and SRA: SRR7867915.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

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Methodology : AKP; JJ; FA; BPS

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Figures

Figure 1

A) Isolation of bacteria using serial dilution method; B) Mixed colony bacterial isolates; C) Morphological characteristics of the isolated pure Bacterial isolates; D) Antibacterial activity of potential isolates where all the isolates showed zone of inhibition against pathogens and disk indicates as control. CA: *Candida albicans*; SA: *Staphylococcus aureus*; BS: *Bacillus subtilis*; EC: *Escherichia coli* and PS: *Pseudomonas aeruginosa*

Figure 2

A) Maximum likelihood phylogenetic tree with Jukes-Cantor model based on 16S rRNA gene sequences of actinobacteria showing the relationship with closest type strain sequences. B) Maximum likelihood phylogenetic tree with Kimura 2-parameter model based on 16S rRNA gene sequences of gram-positive bacteria showing the relationship with closest type strain sequences. C) Maximum likelihood phylogenetic tree with Tamura 3-parameter model based on 16S rRNA gene sequences of gram-negative bacteria showing the relationship with the closest type strain sequences. The number at branches indicate bootstraps value (>50%) from 1000 replicates in all the trees.

Figure 3

Microbial Community Composition including both Bacteria and Archaea at Phylum level (A), only Archaeobacteria (B) and only bacteria (C)

Figure 4

Extended error bar plot up to species level indicating the significant abundance of the microbial population in V3 (red) and V4 (green) region of cave samples.

Figure 5

Cave microbial population showed a Nonmetric multidimensional (NMDS) scaling plot of taxonomic similarity (Bray–Curtis): purple ellipse (V3) and green ellipse (V4).

Figure 6

Phylogenetic analysis of V3 (red) & V4 (purple) region using MEGAN v6.10.11.

Figure 7

A) Describes the rarefaction curve analysis for the observed OTU's B) Alpha diversity was calculated for each community based on taxa distribution at the phylum level using the PAST statistical program.

Figure 8

A) Taxonomic similarity using Bray Curtis B) OTU richness C) Unique and shared OTU's in V3 and V4 samples D) Phylogenetic similarity (Unifrac distance)

Figure 9

Cave microbial population showed a heat map based on Spearman correlation up to species level.