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Microbial diversity of Gumki cave and their potential role in enzyme production

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Abstract

The work presents microbial community structure of Gumki cave for bioactive compound production. This cave represents a unique atmosphere which is totally different from outer atmosphere. Total 49 isolates were recovered from cave samples. Morphological and biochemical characterization revealed a community that contains nine genera of bacteria: *Bacillus* (27%), *Paenibacillus* (21%), *Staphylococcus* (20%), *Streptococcus* (16%), *Salimicrobium* (8%), *Lysisnibacillus* (2%), Aeromonas (2%), *Proteus* (2%) and *Clostridium* (2%). All these microbes were screened for different enzyme production and about 90% isolates displayed positive results for these enzymes. 75.51% recovered isolates were lipase producers, 47% were producing amylase and 24% and 12% bacteria produced protease and cellulase, respectively.

Keywords: Amylase, Bacterial diversity, Cave, Cellulase, lipase, Protease

Introduction

Cave ecosystem is a nutrient poor environment which represents the reservoir of novel isolates. Interaction between the microorganisms and their environment plays a crucial role in reshaping the structures of caves and helps in formation of wall deposits such as stalactite (a column of rock that hangs from the roof of a cave), stalagmite (a column of rock which rises from the floor of a cave formed over a very long period of time) etc (Banerjee and Joshi, 2013**).** The microbiological study of subterranean environments is limited to caves found in Italy, Spain, Romania, France and USA (Tomoval *et al.,* 2013). There are 1545 caves throughout India (Deshmukh, 1994) but due to infancy stage of cave microbiology in India the majority of Indian caves have yet not been explored. Gumki cave is situated in Nandakini river valley of Chamoli district Uttrakhand (India) at 1400 meter above the sea level. A water stream running along the cave might be serving as nutrition source for the living creatures. Colorful wall patches inside the cave indicate presence of microbes and their active role in mineralization. Fig 1 shows the microbial mat and colourful patches inside the cave. This cave is unexplored from microbiological point of view so it is of interest to explore the microbial diversity of the cave and to reveal various industrial applications of these microbes in various fields. In current decades due to rapid development of biotechnology, enzyme industries are growing speedily. In the present investigation the cave microflora have been screened for various industrially important enzyme products. Amylase enzyme is starch hydrolyzing enzyme which is generally used in textiles, bakery, beer, liquor, infant feeding cereals and animal feed industries. Lipase enzyme is an important group of enzyme associated with degradation of lipids. Cellulase enzyme is another important enzyme generally used in drug, food, cosmetic, textiles and detergent industries. This enzyme is also use in biofuel production and wood pulp/paper industry. Protease enzyme has important role in brewing and baking industries and also used in the production of various oriental foods (Soares *et al*., 2012). The present study was aimed at isolating bacteria inhabiting Gumki cave and to qualitatively screen these microorganisms for amylase, lipase, protease and cellulase production.

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Material and Methods

Stalactite, white mat (microbial mat), brown mat (Iron crust), water and wall scrapping was collected

aseptically. Sampling was conducted during premonsoon season 2016. The temperature and humidity of cave recorded at the time of sampling. The samples were immediately transferred aseptically into ice bucket and carried to laboratory. The samples were air dried for 24 hrs and then crushed to form fine powder. Samples were serially diluted and pour plated on nutrient agar medium and incubated at temperature 26°C for two days. After incubation the isolated colonies were purified and stored on slant and in glycerol stock. The morphological identification was done by gram staining. The isolates were also characterized by biochemical and functional tests following standard procedures.The physicochemical parameter viz. conductivity and pH of the sample were also recorded. To determination of moisture content of the samples were oven dried till constant weight and percent moisture content was calculated by following formula.

$W= [(M_{cms} - M_{cds})/(M_{cds} - M_c)] *100$

Where, W= water content, %, M_{cms} = mass of container and moist specimen, $M_{cds=}$ mass of container and dry specimen, M_c = mass of container. The various biochemical characterization tests of recovered bacterial flora have been done viz., Indole prouction, Methyl Red-Voges Proskauer Test, Citrate Utilization Test, Triple sugar iron agar test, Nitrate reduction test, Oxidase, Catalase and Urease production according to Cappucino and Sherman (2007).

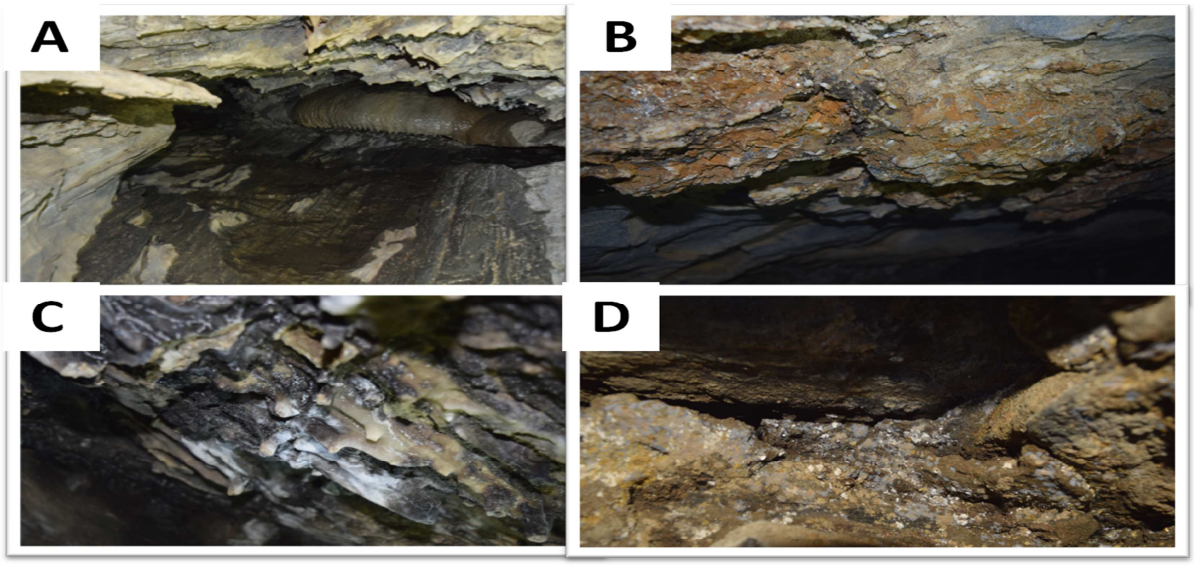

Fig.1: Structures inside the Gumki cave Garhwal Himalaya. A) cave passage B),C),D) Microbial mat.

The various functional characterization tests viz., amylase, cellulase, protease and lipase were performed to determine the functional potential of recovered microflora. Amylase activity was assayed by Chada *et al.,* (1997) using starch as substrate. Extracellular protease production was determined by using skim-milk according to Ladd and Butler (1972). Cellulase assay was performed according to Tether and Wood (1982) using CMC as a substrate and tributyrene agar was used for lipase producing bacteria (Cappucinno and Sherman, 2007). The identification of bacteria was done by ABIS online software. All bacterial cultures were qualitatively screened on agar plates. After spot inoculation on different media the plates were incubated at 22 °C for two days. After incubation the plates were examined for halozone formation. Clear zone of hydrolysis was observed in tributyrene and skim milk agar plates. In CMC (carboxy methyl cellulose), plates were flooded with 1% Congo red dye for 15 minutes followed by distaining with 1 M NaCl solution for 15 minutes. Clear orange colour zone was observed in case of cellulase production. In starch agar plate, grams

iodide was poured into the plates and zone of clearance was observed. Relative enzyme index of all enzymes were calculated by following formula. All the readings were taken in triplicates,

$$\text{Relative enzyme index} = \text{zone diameter} \div \text{colony diameter}$$

Results and Discussion

The temperature of the cave and water was recorded 15.0 °C and 14.4 °C respectively. The humidity of the cave was 50% at the time of sampling. The other physicochemical parameters of different samples are mentioned in Table 1. Yun *et al.,* 2016 worked on dripping water of Heshang Cave in Central China and according to them the conductivity of dripping water ranged from 383 µS/cm to 689 µS/cm. The conductivity of cave water sample was observed to be 236µS/cm. The pH of all cave samples had been slightly alkaline.

Table.1. Physicochemical characteristics of cave samples.

	Stalactite	Micobial mat	Iron crust	Cave water	Wall scrapping
pH	7.8133 ±0.205067	8.223±0.203054	7.967±0.101181	7.883±0.021884	8.020±0.084537
Conductivity (mS/cm)	1.34 ±0.014142	0.139±0.001871	0.276±0.00324	0.236±0.003082	0.363±0.001414
Moisture content (%)	0.164±0.001225	4.1±0.0707	0.80±0.012903	-	22.38±0.055064

Total 49 bacteria were isolated from the cave samples thus the cave is inhabited by the diverse bacterial population. Most of the bacteria were gram positive bacilli and gram positive cocci. The results of biochemical tests are given in Fig 2. Major genera reported to be *Bacillus, Paenibacillus, Staphylococcus, Streptococcus, Salimicrobium, Lysinibacillus, Aeromonas, Proteus* and *Clostridium.* Microbial flora of this cave has been found to be diverse and also resembled with the findings for other studies. On the basis of culture dependent method bacteria isolated from cave of Slovenia were divided into five groups. First group was motile Gram negative rods, with an oxidative metabolism, but differed in the utilization of sugars and amino acids. Second group was Gram negative non-fermentatives. Third group was Gram negative rods with a fermentative metabolism and the fourth and fifth group was Gram positive cocci and Gram positive irregular rods, respectively (Mulec *et al*., 2002). In the present investigation most of the isolates belonged to group Firmicutes.

Many researchers have studied microbial community structure of different caves and reported *Bacillus* as major genus as present study also reported. Megusar and Sket (1977) worked on microbial mat of Planinska jama cave and reported that majority of bacteria belonged to Gram positive cocci, rods and pleomorphic bacteria and most of bacteria were *Bacillus subtilis*, *Bacillus cereus*, *Proactinomyces polychromogens* and *Bacterium brevi.* Indian cave microbiologists also reported Bacillus genera in their studies. Baskar *et al.,* 2006 studied the microbial community of Sahasradhara cave and concluded that microflora of this cave is dominated by *Eubacteria*, mainly sulphate reducing bacteria. They isolated *Bacillus thuringiensis B. pumilis* from statlactite. Baskar *et al*., (2009) worked on Mawsmai cave, Phyllut caves and Mawmluh cave of Meghalaya and recovered *Bacillus licheniformis, B. cereus* form stalactite of Mawsmai Cave, *Bacillus licheniformis, B. cereus and B. mycoides* from stalactite and wall deposits of Phyllut cave, *Bacillus licheniformi, B. cereus, B. pumilis, Actinomycetes Micrococcus luteus* from moonmilk of Mawmluh cave. We also recovered *Bacillus cereus* form our cave samples. Baskar *et al.* 2014 isolated *Bacillus pumilis, B. cereus, B. anthracis B. lentus, B. circulans B. sphaericus, Actinomycetes* from moonmilk collected from Sahastradhara cave, Dehradun.

The second major genus reported in present investigation was *Paenibacillus*. Bhullar *et al*., 2012 recovered *Paenibacillus lautus* from Lechuguilla cave, New Mexico and revealed their potential role in antibiotic resistance. On the basis of phylogentic analysis Lee, 2016 reported a novel species of the *Penibacillus* from Jeju cave, Republic of Korea which he named *Paenibacillus cavernae.* He also isolated other species of Paenibacillus viz. *P. vulneris, P. rigui, P. chinjuensis, P. filicis, P. ehimensis, P. tianmuensis,*

P. elgii, P. aestuarii,P. chitinolyticus, P. larvae, P. contaminans, P.doosanensis, P. gansuensis P. selenitireducens, P. cineris, P. rhizosphaerae, P. favisporus, P. polymyxa, P. turicensis, P. residui, P. popilliae. Presence of *Staphylococcus* and *Streptococcus* indicates the interruption of external factors in cave environment. The site under investigation is rarely visited by tourists so the water stream and animal visit might be responsible for the presence of these pathogenic bacteria.

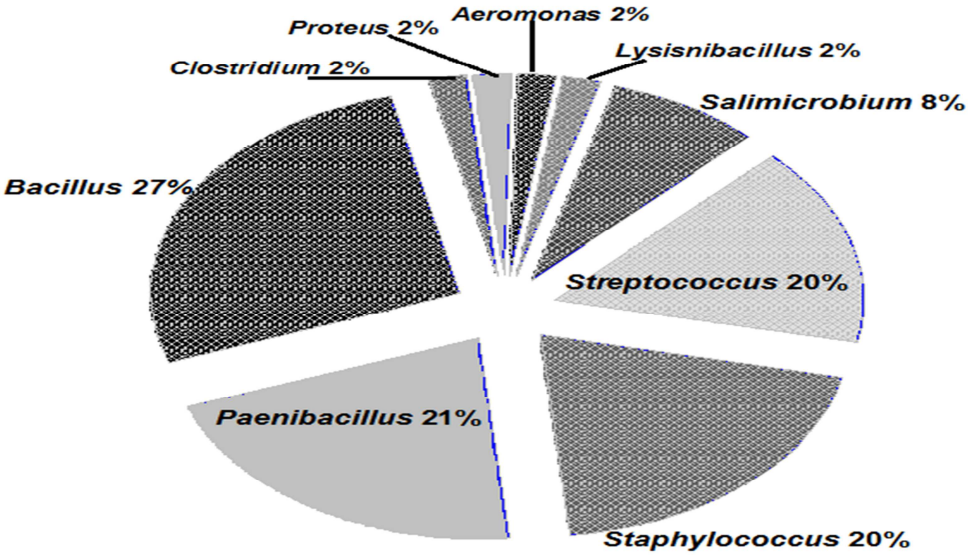

Fig 2: distribution of isolates bacteria genera among the cave samples.

The results of qualitative screening suggested that vast number of bacteria produce protease, lipase cellulase and amylase enzymes. Table 2 summarizes relative enzyme indices of isolates and majority of isolates were lipase producers and the highest relative enzyme index for lipase production was 2.685. Tomoval *et al.*, 2013 screened the actinobacteria of Mugra cave for production of lipase and protease enzymes and reported 40% and 87% bacteria produced lipase and protease enzyme, respectively whereas present study reported 75% lipase and 27% protease producers. Results from the present work depict that there is a great potential of lipolytic activity by cave bacterial community. 46.93% isolates were producing amylase and the highest relative enzyme index for amylase production is 3.533. *Bacillus* and *Paenibacillus* species showed enzyme index >3. Bakri *et al.*, 2012 isolated *Bacillus* species for amylase production and after qualitative screening and found 21 isolates displaying highest enzyme index >3. Present study revealed that only two isolates were exhibiting high amylase enzyme index which can be further used for quantitative screening. 12.24% bacteria are cellulase producers and highest relative enzyme index is 1.822. Gruta do cave studied by Paula *et al.*, 2016 and they screened cave fungi for cellulase enzyme production and found that 90% fungi were positive for cellulase production and the highest enzyme index for enzyme production was 2.46.

The distribution pattern of bacteria for enzyme production is explained using venn diagram (Fig 3). Five isolates were producing all four enzymes in which three isolates belong to *Bacillus* genera and one from *Paenibacillus*. The production of amylase, lipase and protease enzymes by *Bacillus subtilis* and Bacillus *amyloliquefaciens* was studied by Latorre *et al.,* 2016. Many researches also studied *Bacillus spp.* for enzyme production *viz.* for amylase production (Ibrahim *et al.,* 2012) for lipase production (Shah and Bhatt, 2011) , for Protease production (Olajuyigbe and Ajele, 2005), for cellulase production (Dias *et al.,* 2014). *Paenibacillus spp.* was also reported for various hydrolytic enzymes production (Dijksterhuis *et al.,* 1999; Helbig, 2001; Yang *et al.,* 2004).

Table 2: mean enzyme indices of positive isolates for P rotease, lipase, cellulase and amylase enzymes

Isolates code/ enzyme index	Protease	Lipase	Cellulase	Amylase
BWS1	-	1.054±0.045631	-	0.771±0.013096
BWS2	-	0.916±0.030911	-	-
BWS4	-	1.282±0.091397	-	1.667±0.025573
BWS5	-	2.046±0.041695	-	1.300±0.019066
BWS6	-	2.685±0.020137	-	2.137±0.034358
BWS7	-	1.895±0.0143	-	2.159±0.039351
BWS8	-	-	-	1.575±0.034344
BWS9	-	-	-	0.412±0.036885
BWS12	-	0.954±0.039843	-	-
BWS16	-	1.631±0.028311	-	-
Bst1	-	-	0.535±0.022327	-
Bst2	-	-	-	0.750±0.038659
Bst3	-	1.193±0.00495	-	-
Bst6	-	1.654±0.035014	-	-
Bst8	-	1.061±0.047786	-	0.290±0.017507
Bst9	-	1.102±0.004301	-	-
Bst11	0.472±0.001225	1.251±0.033339	-	1.111±0.02514
Bst12	0.217 ±0.001349	1.317±0.00495	-	1.592±0.017277
Bst13	0.312±0.002121	0.500±0.002121	0.217±0.004301	0.809±0.011683
Bst14	0.310±0.001512	1.404±0.003937	0.113±0.019026	0.768±0.034475
Bst15	-	-	-	1.610±0.006964
BI1	-	0.703±0.004899	-	-
BI2	-	0.954±0.006745	-	-
BI3	0.375±0.00535	0.605±0.003536	-	0.375±0.044615
BI5	-	0.514±0.002449	-	-
BI9	-	1.409±0.003742	-	1.00±0.023633
BI10	-	1.707±0.00495	-	-
BI13	-	1.741±0.002828	-	-
BI14	-	1.543±0.003742	-	-
BW1	0.547±0.001871	0.341±0.00255	-	-
BW2	-	0.682±0.00255	-	-
BW3	3.188±0.001414	2.266±0.006745	-	3.533±0.078498
BW4	-	1.364±0.003266	-	0.652±0.025817
BW6	-	-	-	3.211±0.006819
BW8	-	0.909±0.001871	-	-
BMM2	-	1.238±0.119039		-
BMM3	0.720±0.000707	1.008±0.001871	1.822±0.052139	1.872±0.036146
BMM4	0.555±0.003937	2.414±0.003082	-	0.936±0.055817
BMM5	-	0.857±0.008602	1.219±0.020236	0.720±0.020162
BMM7	0.500±0.001225	0.480±0.001225	1.183±0.0343	1.694±0.062498
BMM8	2.250±0.001225	1.001±0.005523	-	-
BMM9	0.333±0.000707	2.296±0.000707	-	-
BMM10	-	2.047±0.002828	-	-

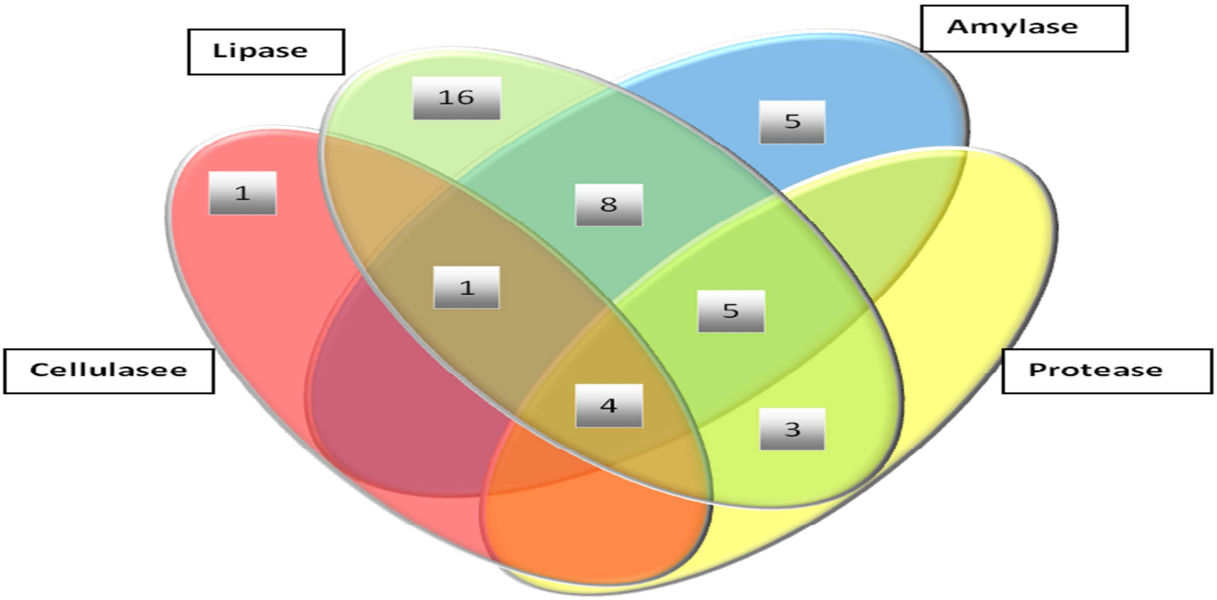

Fig3: Venn diagram showing the distribution of number of isolates producing amylase, lipase, cellulase and protease enzyme.

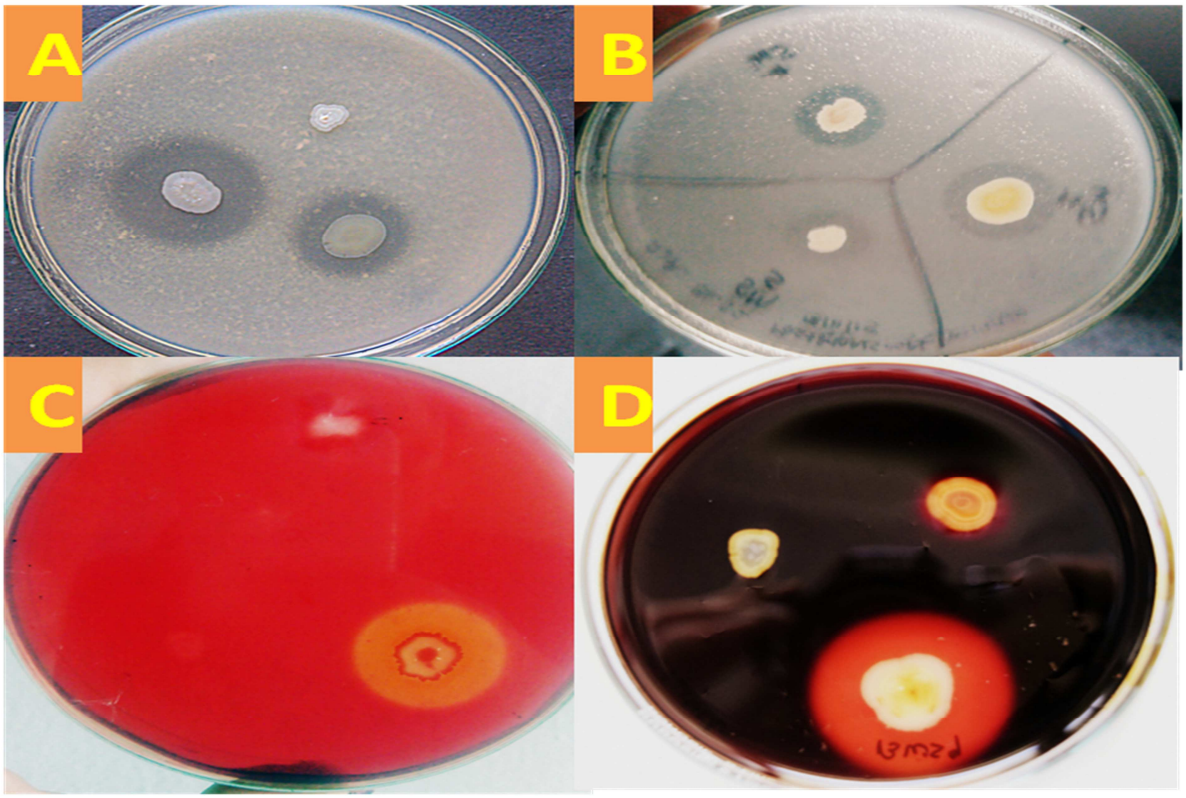

Fig 4: halozone formation in different media A) skim milk agar media for protease production B) tributyrene agar for lipase production C) CMC agar for cellulase production and D) starch agar for amylase production.

The Fig 4 shows the halozone formation in pla tes. Fig 4A shows the potease positive and negative isolates on skim milk agar medium. In Fig 4B positive lipase producers give clear zone of hydrolysis in Tributyrin plate. Fig 4C shows the orange zone on CMC agar plate for cellulase production and Fig 4D shows the amylase positive and negative isolates in Starch agar plate.

Conclusion

Microbial diversity of virgin caves of India needs to be explored because the caves harbor treasurer of many biotechnologically important byproducts. In our study, *Bacillus*, *Paenibacillus* and Staphylococcus were major genera in Gumki cave whereas *Streptococcus, Salimicrobium, Lysinibacillus, Aeromonas, Proteus* and *Clostridium* were also present in few numbers. The presence of microbial community in this cave samples indicates their crucial role in cave structure formation. Qualitative screening of these microbes for four different enzyme viz. lipase, cellulase, amylase and protease indicates that there is vast potential of hydrolytic enzyme production by these cave isolates. This is first time study of microbial diversity of Gumki cave and future perspectives of this investigation involves to quantitative screening, characterization and purification of these enzymes and screening of these isolates for production of other biproducts.

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