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Microbial Communities Associated With Cave Systems In Meghalaya, India

S.R. Joshi*, Purabi Saikia and Maya H. Pyngrope

Microbiology Laboratory Department of Biotechnology & Bioinformatics North-Eastern Hill University, Shillong 793 022 Meghalaya INDIA.

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Abstract

The caves in Meghalaya, India are diverse and some are among the largest caves in Asia. These caves have so far not yet attracted the attention of geomicrobiologists. The microorganisms prevalent in these caves possibly have some influence in speleothem formations and microbial influence in its genesis. Four different caves over the plateau and rolling hills between Assam and Bangladesh spread over a geographical area of 22,000 sq. km. were chosen for study of composition of microorganisms (bacteria and fungi) at different distances from the caves entrance. The bacteria identified by conventional isolation and evaluation by biochemical techniques include *Bacillus subtilis*, *Bacillus cereus*, *Bacillus stratosphericus*, *Bacillus aerophilus*, *Micrococcus luteus*, *Aeromonas veronii*, *Bacillus* sp., *Micrococcus* sp., *Serratia* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Kluyvera* sp., *Arthrobacter* sp., *Hafnia* sp., *Acinetobacter* sp., *Alkaligenes* sp. The fungal species isolated include *Trichoderma harzianum*, *Penicillium janczewskii*, *Penicillium glabrum*, *Fusarium oxysporum*, *Penicillium algiovense*, *Penicillium pinophilum*, *Penicillium oxalicum*, *Syncephalastrum racemosum*, *Aspergillus niger*, *Penicillium* sp., *Mucor* sp., *Trichophyton* sp. and *Curvularia* sp. Maximum diversity of bacteria and fungi were observed at the entrance of the caves which were frequented by tourists and exposed to disturbances.

INTRODUCTION

Caves have always fascinated mankind as they provided shelter to safe home to early man. They are also natural habitats for a wide variety of life form including microorganisms. Caves offer natural experimental systems for fundamental geomicrobiological studies, because they are relatively easy to access (Culver 1982; Frey 1963). There are reports on calcium carbonate dominated speleothems geomicrobiological studies of known caves of the world specially on stalactites, stalagmites, helictites, moonmilk, pool fingers and cave pearls. Microbial communities impact significantly the environment and geochemistry of the caves. Some of the factors that control the contribution of microbes in the caves have recently been identified as CaCO₃ precipitation (Castanier *et al.*, 2000; Baskar *et al.*, 2005, 2006, 2007). Laboratory experiments support that the microbial species isolated from these minerals can produce similar crystals from organic calcium salts (Rivadenera *et al.*, 1993; Baskar *et al.*, 2006). Caves are mostly resource limited due to the prevailing aphotic conditions. There is no photosynthesis and cave ecosystems depend on autochthonous organic materials for energy (Poulson and Lavoie 2000; Simon *et al.* 2003). They are generally considered as extreme environments for life with the seeping and flowing water bringing energy into the caves. Microorganisms control biogeochemical cycles and their diverse metabolism convert minerals into chemical forms that are readily usable by other organisms (Eliu, 2002). Groundwater discharges as springs into the passages of the caves also serve as high energy yielding substrates for some microorganisms (Sarbu *et al.*, 1996; Hose *et al.*, 2000). Microbial communities in the caves are known to acquire energy by transforming available compounds, fixing gases and oxidising reduced metals within rocks. Microbial species play an important role in creating mineral environments of caves and assist in formation of form features like stalactites, stalagmites and cell wall deposits. Explored caves are prevalent in India and Meghalaya caves are among the largest caves reported in Asia which have so far not yet attracted scientific attention with regard to biogenic composition and activities. The present study is the first of its kind on caves of Meghalaya and reports the first geomicrobiological composition from four caves in Meghalaya (India). Some of these microbes are known to play a significant role as biogeochemical agents in the genesis of speleothems (Baskar *et al.*, 2009). The investigation

reports the nature and composition of microbes in these caves at different length from their entrances.

Study Area and Geology

There are more than 1,000 caves in Meghalaya, a few of them forming one of the longest caves on the Indian subcontinent. The Khasi Hills of Meghalaya Plateau is an uplifted Precambrian crystalline complex and forms the north-eastern extension of the Indian Peninsular Shield. It is an E-W trending oblong horst block elevated about 600-1800 m above the Bangladesh plains in the south and separated from Peninsular India by the Rajmahal-Garo gap (Ghosh *et al.* 2005). The caves investigated in this study are located in the East Khasi Hills (25° 07' - 25° 32' North Latitudes and 91° 21' - 92° 15' East Longitudes) and Jaintia Hills (25° 28' North Latitudes and 92° 25' East Longitudes); bounded by Ri-Bhoi district of the north, Karbi-Anglong district on the north-east, Badarpur district on the east, Bangladesh on the south and West Khasi Hills district on the west. The Shillong Plateau and adjoining Mikir Hills are the only exposures of the Archean-Proterozoic rocks in the entire North-East Indian region, covered on all sides by the Tertiary sediments and the recent alluvium. It is believed that the rocks of the intermediate age may exist in beneath the sedimentary cover of the Brahmaputra Valley (Dasgupta and Biswa, 2000). Physiographically, Meghalaya represents a remnant of an ancient plateau of Pre-Cambrian Indian peninsular shield block uplifted to its present height or about 600-1800m above m.s.l. The Kernel of the plateau is the exposed Archean gneisses and schists covered in the central and eastern parts by Pre-Cambrian quartzites and phyllites intruded later by the younger granites and basic/ ultrabasic suites. The limestone covered country over the Garo, Khasi and Jaintia Hills represent a typical Karst topology. The rocks are composed predominantly of para- and ortho-gneisses, migmatites and meta-sedimentary bands. They comprise mainly biotite-gneiss, biotite-granulite, amphibolite, calc-granulite, banded-magnetite quartzite, sapphirine-cordierite granulite, magnetite-grunerite granulite, quartz-dumortierite-tourmaline schist, etc. Some of the schistose rocks comprising biotite-schist, quartz-sillimanite schist,

* Corresponding author. Address: Microbiology Laboratory, Department of Biotechnology & Bioinformatics, North-Eastern Hill University, Shillong 793 022, Meghalaya, INDIA. E-mail address: srjoshi2006@yahoo.co.in

meta-basite, biotite-cordierite granulite, red schist, etc. occur as detached remnants over the older gneissic complex around Sonapahar. The mildly folded sediments of Shillong Group of rocks have suffered low grade metamorphism and are dissected by numerous faults along which the different blocks apparently moved up and down at various times during the Tertiary period and have significant deposits of limestone rocks (**Nandy** 2001).

Krem Mawmluh:

The cave is situated approximately half a kilometre west of Cherrapunjee adjacent to the small hamlet of Mawmluh. This cave interestingly has a five river passage with impressive proportions. With a length of 4,503 m, it is currently the 4th longest in the Indian subcontinent

Krem Soh Shympi:

It has a large pothole entrance of 20 m deep. The cave passage is very large with numerous formations at one end. This cave has a length of 760 m. It is situated at Mawlong, East Khasi Hills, Meghalaya.

Krem Phyllut:

The cave is relatively long (total length of 1,003 m, width 4.5 m, height 15 m) and has three entrances approximately of 2 m height, 2.5 m width. It is situated in Mawsmi village, South of Cherrapunjee, Meghalaya

Krem Lashiing:

It is a massive cave measuring 50 m wide and 40 m high. It has a huge amount of sticky and slippery mud in the cave. This cave has a length of 2,650 m and is situated at Pdengshakap in Jaintia Hills district of Meghalaya. These caves are located amidst forested zone having varying sizes (length 160m-4503m, height 15m -25m, width 4 -32m) but the inner parts are large enough to facilitate easy movement. The caves are aphotic and have myriads of stalagmites and stalactites. The sporadic fauna that were observed towards the cave entrances were spiders, rodents, snakes and small fishes. The caves were moist and only dripping water could be observed. The average annual temperature of the caves is around 15-20°C (Table. 1)

Although that are some reports on bacterial composition of two caves (**Baskar et al.**, 2009) with reference to biogenic activities, a comprehensive study on the presence of fungi together with the bacteria from caves of Meghalaya have not been reported so far. Thus, the present study investigates the microbial community (fungi & bacteria) with reference to twilight and aphotic aquatic habitats associated with four different caves of Meghalaya, India.

METHODOLOGY:

Sampling

Samples for the studies were taken from minimal contaminated (not disturbed by human) areas of the cave. The water samples were collected under sterile conditions in sterile bottles. Three replicates of spring waters were collected in sterilized bottles and stored at 4C till completion of analysis.

Microbial analysis:

The microbial community structure was characterized and identified using a combination of conventional isolation and biochemical techniques for the most frequent species of microbes. A serial dilution of the collected water sample was performed and different dilutions were placed on Thiosulphate, Sulphite Agar, Nutrient Agar and B-4 agar (**Bouquet et al.**, 1973) were used to isolate bacteria. Potato Dextrose Agar media was used for the isolation of fungi. The inoculated plates were incubated under aerobic conditions between 15C and 28C mimicking cave conditions (photic and aphotic) and cave temperatures. Individual colonies were selected based on colony morphotypes and colours for further analysis. The isolate were preserved at 4C after obtaining pure culture. The isolates were identified using conventional techniques (morphological, biochemical physiological tests and sporulation patterns). Bacterial isolates were characterized by performing biochemical tests like staining reaction, oxidase test, catalase

test, Imvic tests, amylase production test, gelatin hydrolysis test, urease test, H₂S production test, oxidation fermentation test of glucose for carbohydrate utilization, carbohydrate fermentation test etc. Fungal isolates were readily identified by their characteristic morphology such as shape, size and arrangement of spores and hyphae under microscopic observation.

RESULTS & DISCUSSION:

The purpose of the present study was to investigate the microbial community associated with the caves in Meghalaya in the Indian subcontinent. Based on the aerobic isolation procedure 16 isolates of bacteria and 14 isolates of fungi were obtained from four caves. The dominating bacterial isolates were *Bacillus subtilis*, *Bacillus cereus*, *Bacillus stratosphericus*, *Micrococcus luteus*, *Serratia* sp and *Alkaligenes* sp. The dominating fungal isolates were *Trichoderma harzianum*, *Penicillium jancezewskii*, *Penicillium glabrum*, *Penicillium oxalicum*, *Syncephalustrum recemosum*, and *Microsporum* sp. Fungal isolates were predominant towards the cave entrances but bacteria were universally distributed in the caves (Table 2 & 3).

The geological process that are mediated by microbial metabolism appear to be fundamentally related to the distribution of microbes throughout the caves (**Jones**, 2001). It is generally assumed that metabolic diversity is a reflection of the different electron flow paths employed by various microorganisms. Recent studies on stromatolite forming microbial mats indicate that microbial activity in caves can result in calcification, precipitation and production of extracellular compounds that control precipitation in the absence of microbes (Mark et al 2001). The highly weathered nature of the calcite crystals in the speleothems suggests that they have been etched by microbially mediated processes (**Baskar et al.**, 2009). Bacteria may act as highly reactive geochemical interfaces (**Beveridge** 1989; **Fein et al.**, 1997 and their extracellular polymers are especially effective at binding ions from solution and serving as nucleation surfaces for mineral formation (**Ferris et al.**, 1987; **Beveridge** 1989; **Fortin et al.**, 1997). Their metabolic activities can therefore, induce localised conditions that are favourable for mineral precipitation (**Thompson** and **Ferris** 1990; **Merz**, 1992; **Fortin et al.**, 1997; **Barker** and **Banfield**, 1998). Microbes play an active part in changing the nature of limestone rocks, both by dissolving it as well as by contributing to the creation of a range of deposits on the cave walls (**Baskar et al.**, 2009).

The preliminary microbial community reported from these caves confirms to the microorganisms reported from caves (**Cacchio et al.**, 2003, **Baskar et al.**, 2006, **Ehrlich**, 1996). However, culture-independent approaches need to be employed to unravel the total biodiversity of caves and the roles as culture dependant approaches show only a minor fraction of the total microbial diversity (**Amman et al.**, 1996). The microbial diversity characterized in the present study in some of the non-explored caves in Meghalaya, India support the general hypothesis that microbial involvement is part of the general activities taking place in speleothemic cave formation.

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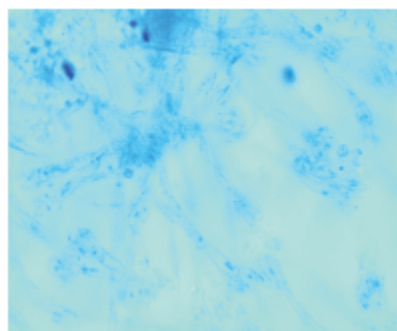
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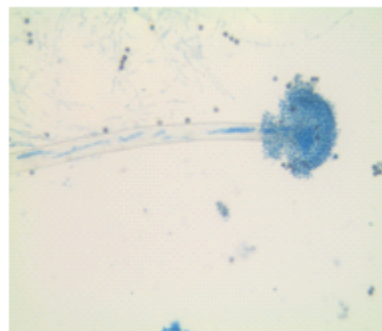
Figure 1: Location map of the study area (Inset shows position of Meghalaya in India)



Figure 1: Aquatic habitats inside the caves

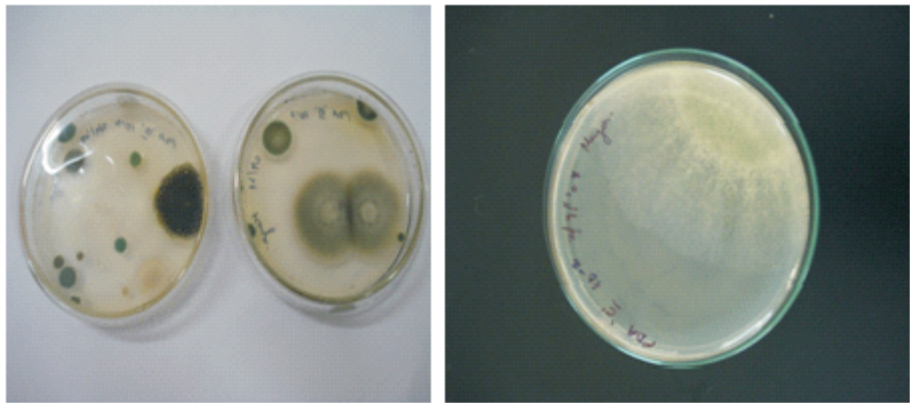


Aspergillus niger



Penicillium sp.

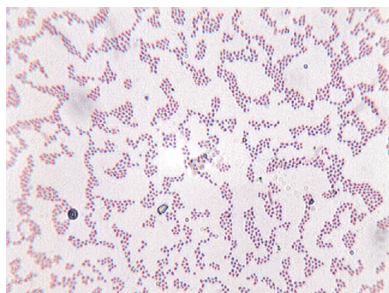
Figure 2 : Continue...



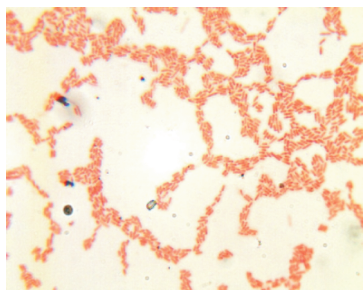
Fungal plates



Casein hydrolysis test



Gram positive cocci



Gram negative rod

SUPPLEMENTARY MATERIALS

Table 1: General characteristics of the four caves.

Caves	Length of the cave (m)	Distance from cave entrance (m)	Zone	P ^H of water sample	Cave Temp°C
Krem Mawmluh	4503	0	Twilight	7.37	15-17
		50	Aphotic	7.2	
		100	Aphotic	7.8	
		150	Aphotic	7.97	
		200	Aphotic	8.0	
Krem Soh Shympi	760	0	Twilight	7.1	16-19
		50	Aphotic	7.25	
		100	Aphotic	7.35	
		15	Aphotic	7.4	
		200	Aphotic	7.74	
Krem Phyllut	1003	0	Twilight	7.3	16-18
		50	Aphotic	7.35	
		100	Aphotic	7.48	
		150	Aphotic	7.59	
		200	Aphotic	7.8	
Krem Lashiing	2650	0	Twilight	7.25	17-20
		50	Aphotic	7.4	
		100	Aphotic	7.45	
		150	Aphotic	7.58	
		200	Aphotic	7.84	

SUPPLEMENTARY MATERIAL

Table 2: Fungal and bacterial isolates and population structure at different distances from the cave entrance.

Caves	Distance from cave entrance (m)	No. of Fungal isolates	No .of Bacterial isolates	Fungal Population (Cfu/ ml H ₂ O)	Bacterial population (Cfu/ ml H ₂ O)
Krem Mawmluh	0	15	10	1.1x10 ³	3.1 x10 ⁶
	50	6	8	1.0x10 ³	2.5 x10 ⁵
	100	3	9	8.0x10 ²	1.8 x10 ⁵
	150	2	7	6.0x10 ²	1.1 x10 ⁵
	200	0	8	Nil	7.5 x10 ⁴
Krem Soh Shympi	0	12	9	1.6x10 ³	5.4 x10 ⁶
	50	4	6	1.4x10 ³	4.5 x10 ⁵
	100	2	7	1.0x10 ²	3.3 x10 ⁵
	150	0	6	Nil	2.8 x10 ⁵
	200	0	4	Nil	2.2 x10 ⁵
Krem Phyllut	0	9	10	9.0x10 ³	4.8 x10 ⁶
	50	4	6	8.0x10 ³	3.5 x10 ⁵
	100	2	6	3.0x10 ³	2.8 x10 ⁴
	150	2	6	3.0x10 ³	2.8 x10 ⁴
	200	0	5	Nil	1.1 x10 ⁴
Krem Lashiing	0	8	8	1.2x10 ³	3.8 x10 ⁶
	50	3	7	1.0x10 ³	3.5 x10 ⁵
	100	2	6	8.0x10 ²	2.2 x10 ⁵
	150	0	7	Nil	1.8 x10 ⁵
	200	0	4	Nil	1.1 x10 ⁵

Table 3: Fungal and bacterial community characterized at different distances from the cave entrance.

Caves	Distance from the cave entrance (m)	Bacterial isolates identified	Fungal isolates identified
Krem Mawmluh	0	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Pseudomonas sp.</i> , <i>Flavobacterium sp.</i> , <i>Kluyvera sp.</i> , <i>Hafnia sp.</i> , <i>Acinetobacter spp.</i> , <i>Alkaligenes sp.</i> , <i>Bacillus stratosphericus</i> , <i>Bacillus sp.</i>	<i>Trichoderma harzianum</i> , <i>Trichophyton sp.</i> , <i>Penicillium janczewskii</i> , <i>Penicillium sp.</i> , <i>Penicillium glabrum</i> . <i>Microsporium spp</i> <i>Syncephalastrum racemosum</i> , <i>Fusarium oxysporum</i> , <i>Penicillium algiovense</i> , <i>Penicillium pinophilum</i> , <i>Penicillium oxalicum</i> , <i>Aspergillus niger</i> , <i>Mucor sp.</i> <i>Trichophyton sp.</i> and <i>Curvularia sp</i>
	50	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Bacillus stratosphericus</i> . <i>Bacillus aerophilus</i> , <i>Micrococcus lutens</i> , <i>Serratia sp.</i> , <i>Flavobacterium sp</i>	<i>Trichoderma harzianum</i> , <i>Trichophyton sp.</i> , <i>Penicillium janczewskii</i> , <i>Penicillium sp.</i> , <i>Penicillium glabrum</i> . <i>Microsporium sp</i>
	100	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Bacillus stratosphericus</i> . <i>Bacillus aerophilus</i> , <i>Micrococcus lutens</i> , <i>Serratia sp.</i> , <i>Arthrobacter sp</i> , <i>Micrococcus sp</i>	<i>Trichoderma harzianum</i> , <i>Penicillium sp.</i> , <i>Microsporium sp</i>
	150	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Bacillus stratosphericus</i> . <i>Micrococcus lutens</i> , <i>Serratia sp.</i> , <i>Micrococcus sp</i>	<i>Penicillium glabrum</i> . <i>Microsporium sp</i>
	200	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Bacillus stratosphericus</i> . <i>Micrococcus lutens</i> , <i>Serratia sp.</i> , <i>Micrococcus spp</i> , <i>Aeromonas veronii</i>	Nil
Krem Soh Shympi	0	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Pseudomonas spp.</i> , <i>Flavobacterium sp.</i> , <i>Kluyvera sp.</i> , <i>Acinetobacter sp.</i> , <i>Alkaligenes sp.</i> , <i>Hafniasp</i>	<i>Penicillium janczewskii</i> , <i>Penicillium glabrum</i> . <i>Microsporium sp</i> <i>Syncephalastrum racemosum</i> , <i>Fusarium oxysporum</i> , <i>Penicillium algiovense</i> , <i>Penicillium oxalicum</i> , <i>Aspergillus niger</i> , <i>Mucor sp</i> <i>Penicillium sp.</i> <i>Trichophyton sp.</i> and <i>Curvularia sp</i>
	50	<i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Bacillus stratosphericus</i> . <i>Bacillus aerophilus</i> , <i>Serratia sp.</i> , <i>Flavobacterium spp</i>	<i>Penicillium oxalicum</i> , <i>Aspergillus niger</i> , <i>Mucor sp</i> <i>Penicillium sp.</i>
	100	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Bacillus stratosphericus</i> . <i>Bacillus aerophilus</i> , <i>Micrococcus lutens</i> , <i>Serratia sp.</i> ,	<i>Aspergillus niger</i> , <i>Mucor sp</i>
	150	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Bacillus stratosphericus</i> . <i>Micrococcus lutens</i> , <i>Serratia sp.</i>	Nil
	200	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus sp.</i> , <i>Serratia sp.</i>	Nil

Table 3: Continue ...

0	<i>Bacillus aerophilus, Bacillus subtilis, Bacillus sp., Micrococcus luteus, Arthrobacter sp. Pseudomonas spp., Flavobacterium sp., Kluyvera sp., Acinetobacter sp. Alkaligenes sp</i>	<i>Penicillium glabrum. Microsporium sp Syncephalastrum racemosum, Fusarium oxysporum, Penicillium algiovense, Penicillium oxalicum, Aspergillus niger, Mucor sp Penicillium sp.</i>
50	<i>Bacillus stratosphericus. Bacillus aerophilus, Bacillus subtilis, Micrococcus lutens, Serratia sp., Flavobacterium sp</i>	<i>Aspergillus niger, Mucor sp Penicillium sp. Microsporium sp</i>
100	<i>Bacillus subtilis, Bacillus stratosphericus. Bacillus aerophilus, Micrococcus lutens, Serratia sp., Bacillus sp.</i>	<i>Penicillium sp. Microsporium spp</i>
150	<i>Bacillus subtilis, Bacillus cereus, Bacillus sp., Bacillus stratosphericus. Micrococcus lutens, Serratia sp.</i>	<i>Penicillium spp. Microsporium sp</i>
200	<i>Bacillus subtilis, Bacillus cereus, Bacillus stratosphericus. Micrococcus lutens, Serratia sp.,</i>	Nil
0	<i>Bacillus subtilis, Bacillus cereus, Bacillus sp., Pseudomonas sp., Flavobacterium sp., Acinetobacter sp. Alkaligenes sp., Hafniasp</i>	<i>Trichoderma harzianum, Syncephalastrum racemosum, Fusarium oxysporum, Penicillium algiovense, Penicillium oxalicum, Aspergillus niger, Mucor sp Penicillium sp.</i>
50	<i>Bacillus subtilis, Bacillus cereus, Bacillus sp., Bacillus stratosphericus. Bacillus aerophilus, Micrococcus lutens, Serratia sp.</i>	<i>Trichoderma harzianum, Syncephalastrum racemosum, Penicillium oxalicum</i>
100	<i>Bacillus subtilis, Bacillus stratosphericus. Bacillus aerophilus, Micrococcus lutens, Serratia sp., Bacillus sp.</i>	<i>Penicillium oxalicum, Mucor sp</i>
150	<i>Bacillus subtilis, Bacillus cereus, Bacillus sp., Bacillus stratosphericus. Micrococcus lutens, Serratia sp., Flavobacterium sp.</i>	Nil
200	<i>Bacillus subtilis, Bacillus cereus, Bacillus sp., Bacillus stratosphericus</i>	Nil