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Advances in Applied Science Research, 2015, 6(4):236-244

ISSN: 0976-8610
CODEN (USA): AASRFC

Bacterial diversity of a sulphur spring in Uttarakhand, India

Seema Rawat

Department of Botany and Microbiology, H. N. B. Garhwal (Central) University, Srinagar, Uttarakhand, India

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ABSTRACT

Sulfur springs have long been credited with unique healing powers for certain diseases both in the early eastern and western medical sciences. In these springs the concentration of elemental sulfur, sulfate, thiosulfate and dissolved sulfide is high and both oxic as well as anoxic conditions are maintained in the water and underlying sediments through the spring. Enrichment culture technique was employed to study the diversity of sulphur spring. The population profile varied significantly. Maximum structural diversity was observed in the soil sample mixed with sulphur water which harboured an assemblage of soil and surface sulphur water microflora. Thiobacillus was dominant microflora of sulphur water (34%) and soil mixed with sulphur water (20%). Pseudomonas was documented to be the dominant microflora of normal water (41%), normal water mixed with sulphur water (41%), cave water (50%) and stalactite (29%). Proteus was predominant in soil sample (25%). Pseudomonas was recovered from all water and soil samples. The diversity indices were employed to study the functional diversity of spring. Maximum diversity of protease producers was observed in soil mixed with sulphur water (H'=2.01; E1=0.64) while that of xylanase producers, maximum diversity was observed in normal water mixed with sulphur water (H'=0.95; E1=0.90). Cellulolytic microflora was most diverse in stalactite (H'=0.98; E1=0.93) while amylolytic microflora was most diverse in sulphur water (H'=0.96; E1=0.88). Amongst pectinolytic microflora, maximum diversity was observed in normal water mixed with sulphur water (H'=0.64; E1=1.80) while phosphate solubilizers were most diverse in sulphur water (H'=0.66; E1=0.96) and maximum diversity amongst siderophore producers was observed in cave water (H'=0.72; E1=0.63).

Key words: Sulfur springs, Thiosulfate, Enrichment culture technique, *Thiobacillus*, *Pseudomonas*, Stalactite.

INTRODUCTION

Sulphur springs have long been credited with unique healing powers for certain diseases both in the early eastern and western medical sciences. These springs are spread world-wide. In the spring, concentration of sulphur, sulfate, thiosulfate and dissolved sulfide concentration in the emergent water is high and maintains oxic as well as anoxic conditions in the water and underlying sediments through the spring. These springs generally have acidic pH but few springs have neutral pH; can be a hot water spring or cold water spring [1, 2, 3].

The prevailing ecological conditions in these springs like pH, temperature, sulfide, sulphur or sulphate concentration, redox conditions, presence of other electron acceptors, light availability and organic content influence the community succession in these springs. The carbonate caves associated with these springs also harbours complex micobial communities [4].

In sulphur- and sulphide-rich environments viz., springs, hydrothermal vents, anaerobic zones of lakes, and shallow marine and intertidal systems, utilization and cycling of sulphur species play a major role in energy production and the maintenance of microbial community [5].

The wide range of ecological conditions existing in these springs make them an interesting ecosystem which has always drawn attention of microbial ecologists. The structural diversity of microbial form alongwith genetic, physiological and functional diversity is believed to provide a complete understanding of these springs. The microbial community has been extensively studied in several springs by many workers [6, 7, 8]. They harbour a number of mesophilic, thermophilic and hyperthermophilic microflora. These springs comprise of a wide assemblage of bacteria belonging to alpha, beta, gamma and delta subdivisions of proteobacteria.

Springs comprise of sulfur oxidising bacteria, sulfur and sulfate reducing bacteria. The former comprises of aerobic bacteria viz., species of genera *Alcaligens, Beggiatoa, Paracoccus, Pseudomonas, Thiothrix, Thioploca, Thiobacillus Thiomicrospira, Thiosphaera* and *Xanthobacter* [4] while latter comprises of anaerobic bacteria viz., *Desulfovibrio, Desulfatomaculum, Desulfuromonas, Desulfobulbus, Desulfobacter, Desulfococcus, Desulfosarcina* and *Desulfonema* [2, 6, 7].

MATERIALS AND METHODS

2.1 Collection of Samples: A total of 7 samples viz., Nornal Water (NW), Sulfur Water (SW), Cave Water (CW), Normal water mixed with sulfur water (NSW), Stalactite (STC), Soil sample (S), Soil sample mixed with sulfur water (SSW) were aseptically collected in sterile containers from the sulphur spring. These samples were transported on dry ice to the lab.

2.2 Recovery of Isolates: Microbial population dynamics was studied by enrichment culturing on Thiobacillus broth and Starkey's broth. Plating was done on the same medium after 24h, 72h, 120h, 168h and 264h. Different morphotypes were purified by restreaking on Nutrient agar and Starkey's medium and pure culture.

2.3 Biochemical Characterization: The various biochemical tests viz., Indole- Methyl red- Voges Proskaeur-Citrate Utilization Test, Triple Sugar Iron Agar Test and Nitrate Reduction Test, Oxidase Test, Catalase Test and Urease Test were carried out for characterization of isolates according to [8].

2.4 Functional Diversity: The functional diversity amongst isolates was studied by qualitative screening of their xylanolytic, cellulolytic, amylolytic, proteolytic and pectinolytic activity, phosphorus solubilization and siderophore production. Xylanase and Cellulase assay was performed according to [9] using 1% xylan and 1% CMC as substrate, respectively. Amylase was assayed according to [10] using 1% starch as substrate. Protease was assayed according to [11] using 1% skim milk as susbtrate. Pectinase was assayed according to [12] using 1% pectin as substrate. Siderophore was assayed according to [13] on Chromeazurol S agar medium. Phosphorus solubilization was measured on Pikovskya's agar according to [14].

2.5 Diversity indices

Various indices viz., Shannon's index (H') [15], Margalef's Richness index [16] and Evenness index [17] were calculated based on the characteristics of recovered isolates.

Shannon's index

$$H' = \sum_{i=1}^{S} (p_1 \ln p_1)$$

Pi= ni/N

ni = number of individuals of the i^{th} species

N= Total number of all individuals

Richness Index1 (R1) or Margalef`s Index

$$R1 = \frac{(n-1)}{\ln N}$$

Where

n = total number of species

N = total number of individuals of all species.

Evenness index (E1)

$$E1 = \frac{H'}{\ln(S)}$$

Where S is number of total species

RESULTS

During enrichment on *Thiobacillus* broth and Starkey's broth, changes in characteristics of broth were observed with time period. After 3 days in *Thiobacillus* broth, all samples changed colour of broth to dark red except sulfur water in which there was marginal change. Significant change in colour was observed for NSW, which changed the colour of broth to yellow. There was no change in colour of broth of all other samples. No further change in colour of broth was observed with further incubation to 11 days. Yellow colour depositions were observed in NW, SW, NSW and STC samples upto 11 days while black colour in CW and SSW upto 7 days; black and yellow colour in soil sample upto 7 days. Cave water depositions changed to yellow colour after 11 days while that of soil, to yellow and of SSW, to black and yellow. The changes in pH were non-significant. A significant decrease in pH was observed with sulfur water; pH of broth decreased to 3.5 after 11 days.

After 3 days, no change in colour of Starkey's broth was observed in all samples. White colour depositions were observed in SW upto 11 days while brown colour in S, STC and SSW upto 3 days. In other samples no depositions were present upto 3 days. In NW and CW, white colour depositions were observed after 7 days while in NSW, after 11 days.

Table 1: Changes in characteristics of water and soil samples of Sahastradhara during enrichment on *Thiobacillus* broth

Samples	Characteristics								
	3d			7d			11d		
	pH	Colour change	Depositions	pH	Colour change	Depositions	pH	Colour change	Depositions
NW	7.0	Dark red	Yellow	7.0	Dark red	Yellow	7.0	Dark red	Yellow
SW	6.0	Red	Yellow	5.0	Yellow	Yellow	3.5	Yellow	Yellow
NSW	6.5	Yellow	Yellow	7.5	Dark red	Yellow	7.0	Dark red	Yellow
CW	7.0	Dark red	Black	7.0	Dark red	Black	7.0	Dark red	Yellow
S	8.0	Dark red	Black& Yellow	7.0	Dark red	Black& Yellow	7.0	Dark red	Yellow
SSW	7.0	Dark red	Black	8.0	Dark red	Black	7.0	Dark red	Black& Yellow
STC	8.0	Dark red	Yellow	7.0	Dark red	Yellow	7.0	Dark red	Yellow

Table 2: Changes in characteristics of water and soil samples of Sahastradhara during enrichment on Starkey's broth

Samples	Characteristics								
	3d			7d			11d		
	pH	Colour change	Depositions	pH	Colour change	Depositions	pH	Colour change	Depositions
NW	5.0	-	-	4.0	-	White	4.0	-	White
SW	7.0	-	White	7.0	-	White	7.0	-	White
NSW	5.0	-	-	4.0	-	-	3.5	-	White
CW	5.0	-	-	6.5	-	White	6.5	-	White
S	6.5	-	Brown	6.0	-	-	5.5	-	-
SSW	6.0	-	Brown	6.5	-	-	6.0	-	-
STC	6.5	-	Brown	6.0	-	-	6.0	-	-

\- *No change observed*

3.1 Structural diversity

A significant difference in the population count was observed in water and soil samples of Sahastradhara (Table1). The variation in the population count (log_{10} cfu) of recovered mesophilic bacteria from SW to S was, 6.25± 0.12 to 10.25± 1.46.

The population count of sulfur bacteria on Starkey's medium varied from 4.23± 0.19 (NW) to 8.14 ± 0.22 (SW) while that on *Thiobacillus* agar, varied from 4.10± 0.23 (NW) to 8.65 ± 0.98 (SW).

Distinct bacterial morphotypes were isolated from recovered microbial diversity of different samples of sahastradhara. Bacterial morphotypes were selected on the basis of their colour, chromogenesis, morphological characteristics viz., colony and cell morphology. They were identified on the basis of their morphological and biochemical characteristics according to Bergey's manual of systematic bacteriology. The population varied in different samples (Fig. 2). In sulphur water *Thiobacillus* was the predominant bacteria (34%) followed by *Paracoccus* (16%) and *Xanthobacter* (16%), *Pseudomonas* (12%), *Thiosphaera* (11%) and *Thiomicrospira* (11%). In normal water the predominant population was of *Pseudomonas* (32%) followed by *E. coli* (22%), *Micrococcus* (14%), *Klebsiella* (14%), *Staphyloccus* (11%) and *Proteus* (7%). In normal water mixed with sulphur water the predominant bacteria was *Pseudomonas* (41%) followed by *E. coli* (12%), *Micrococcus* (11%), *Paracoccus* (10%), *Thiomicrospira* (10%), *Klebsiella* (8%) and *Thiobacillus* (8%). In cave water *Pseudomonas* was the most dominant bacteria (50%) followed by *E. coli* (14%), *Klebsiella* (10%), *Micrococcus* (10%), *Alcaligenes* (10%) and *Proteus* (6%). In soil sample *Proteus* was predominant (25%) followed by *Micrococcus* (20%), *Pseudomonas* (18%), *E. coli*

(14%), *Bacillus* (1%) and *Shigella* (10%). In soil mixed with sulphur water *Thiobacillus* was predominant (20%) followed by *Pseudomonas* (14%), *Micrococcus* (14%), *Klebsiella* (10%), *Proteus* (10%), *Paracoccus* (10%), *Xanthobacter* (10%), *Shigella* (7%) and *E. coli* (5%). In stalactite sample *Pseudomonas* and *Micrococcus* were predominant (29%) followed by *Klebsiella* (14%), *Proteus* (14%), *Paracoccus* (7%) and *Serratia* (7%).

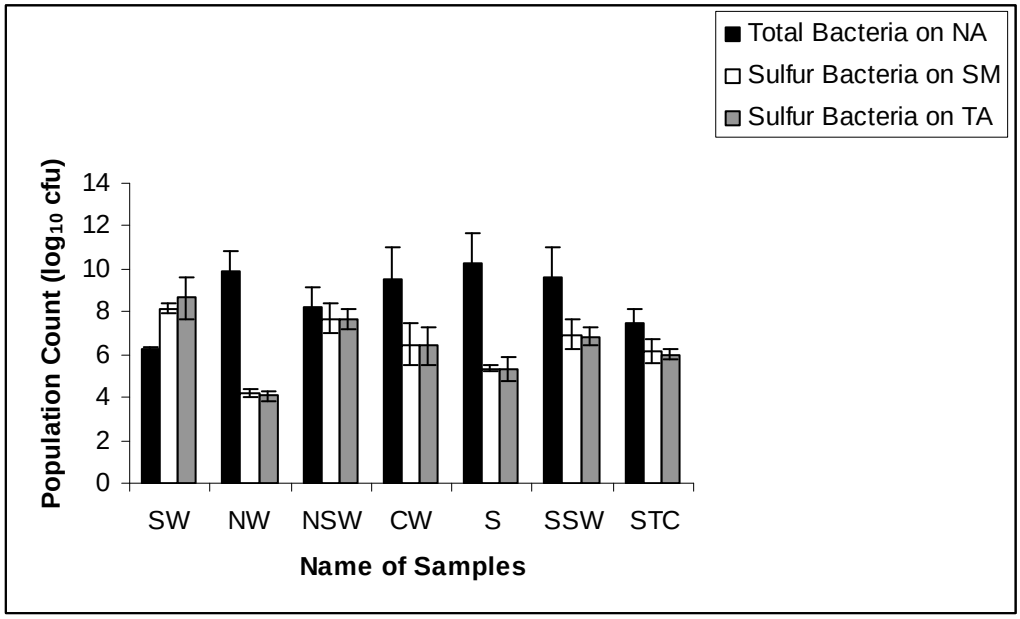
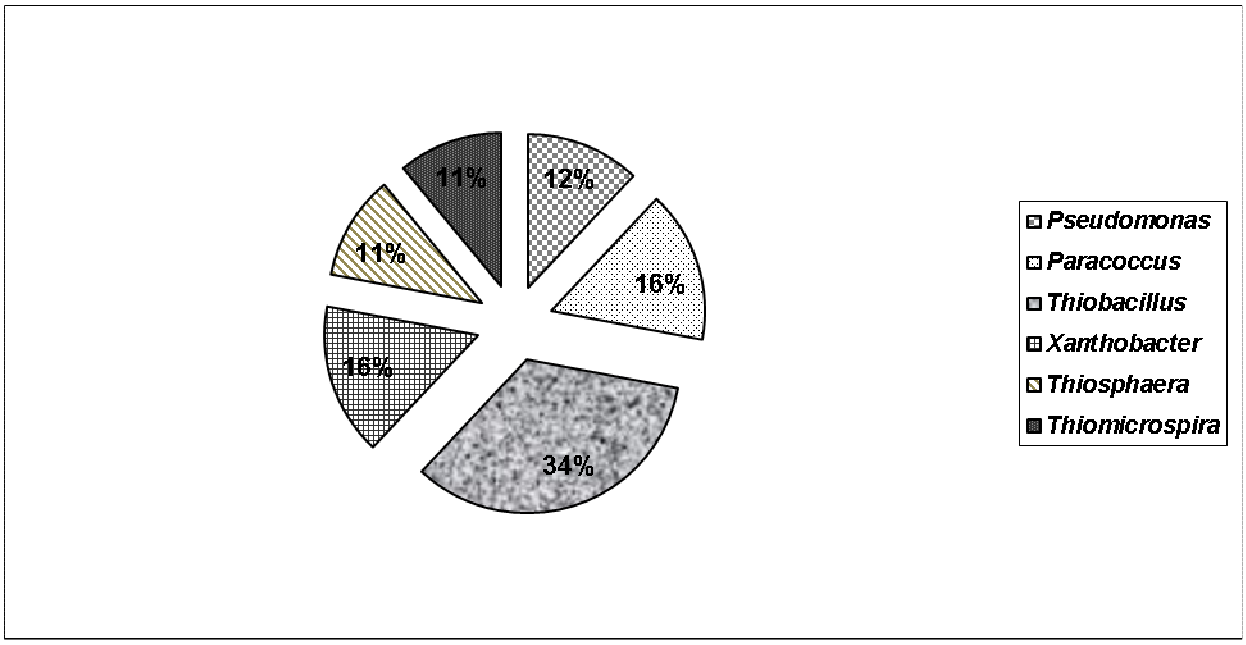
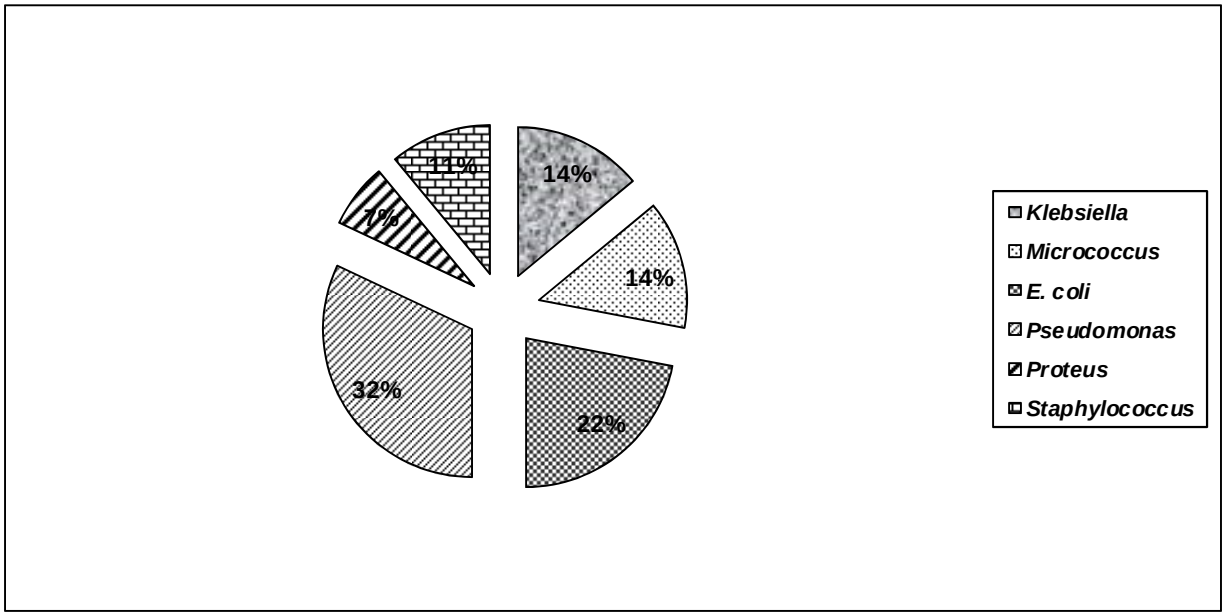


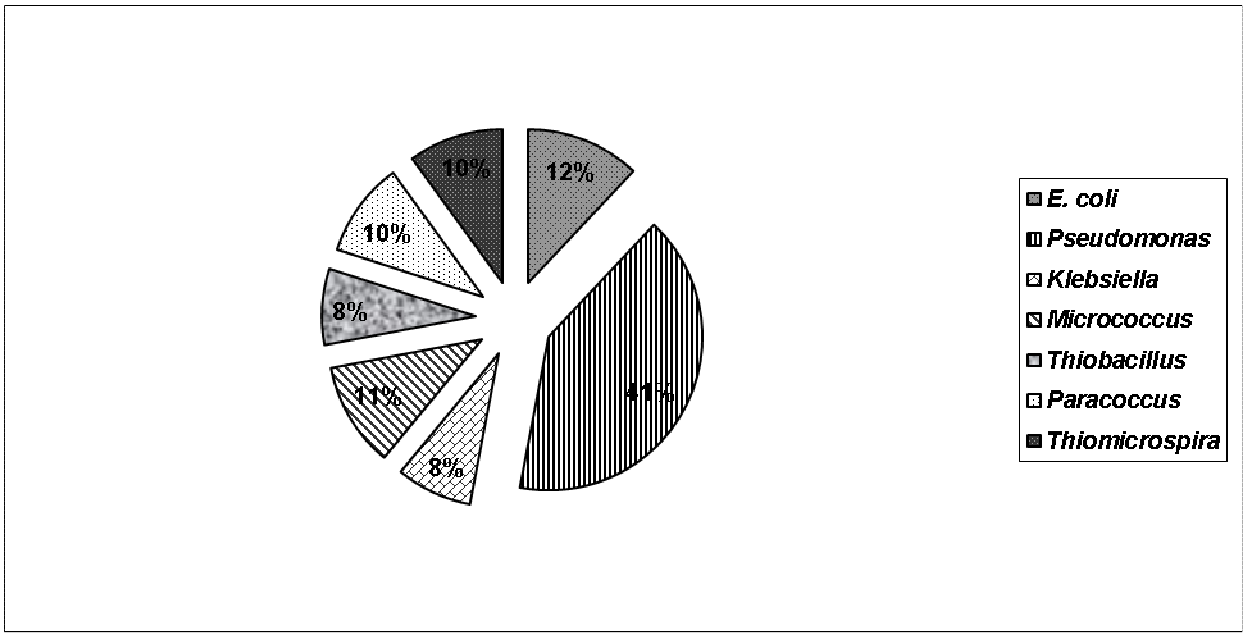
Fig.1: Population count of bacteria recovered from sulphur spring



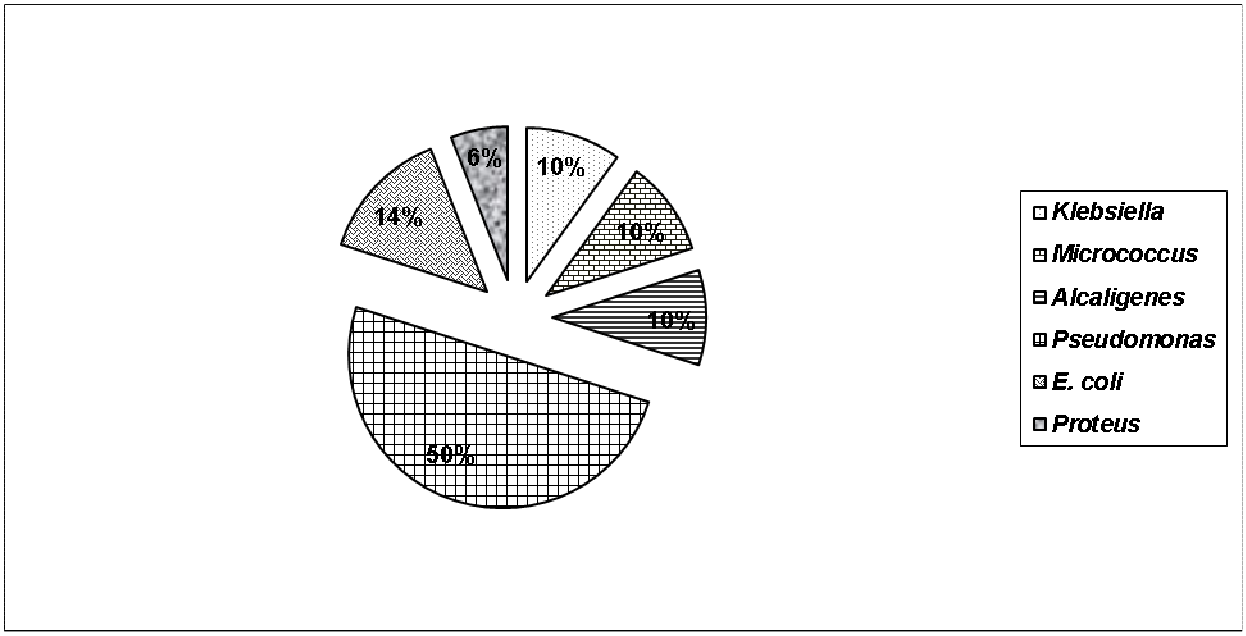
(a) Sulphur water (SW)



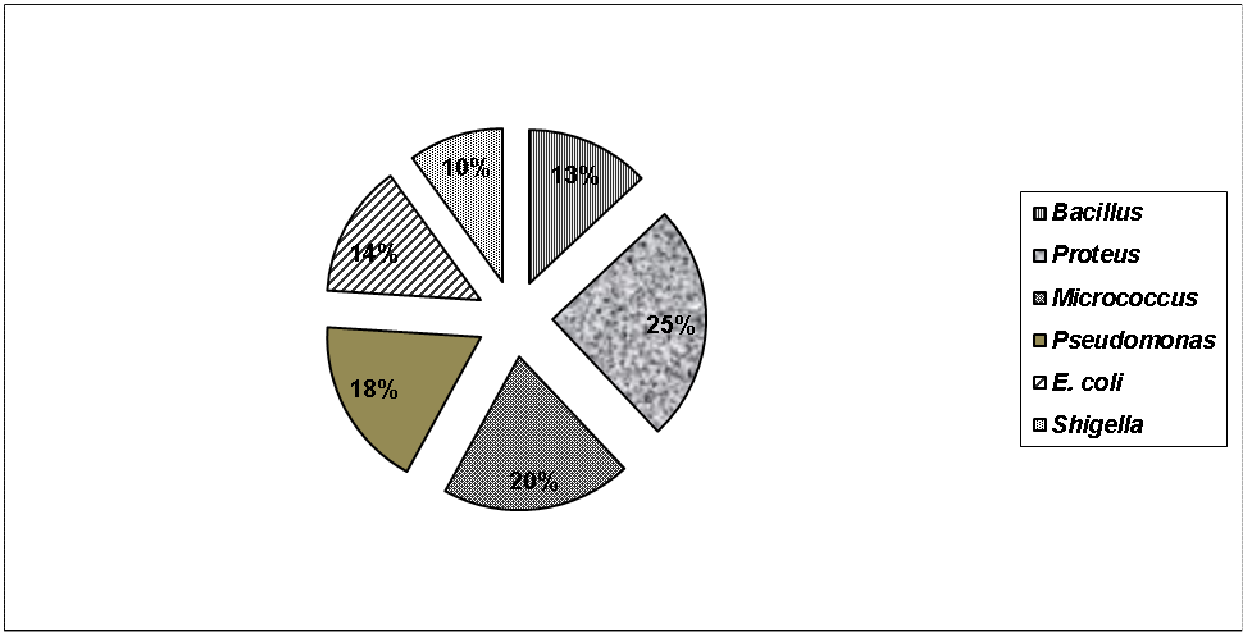
(b) Normal water (NW)



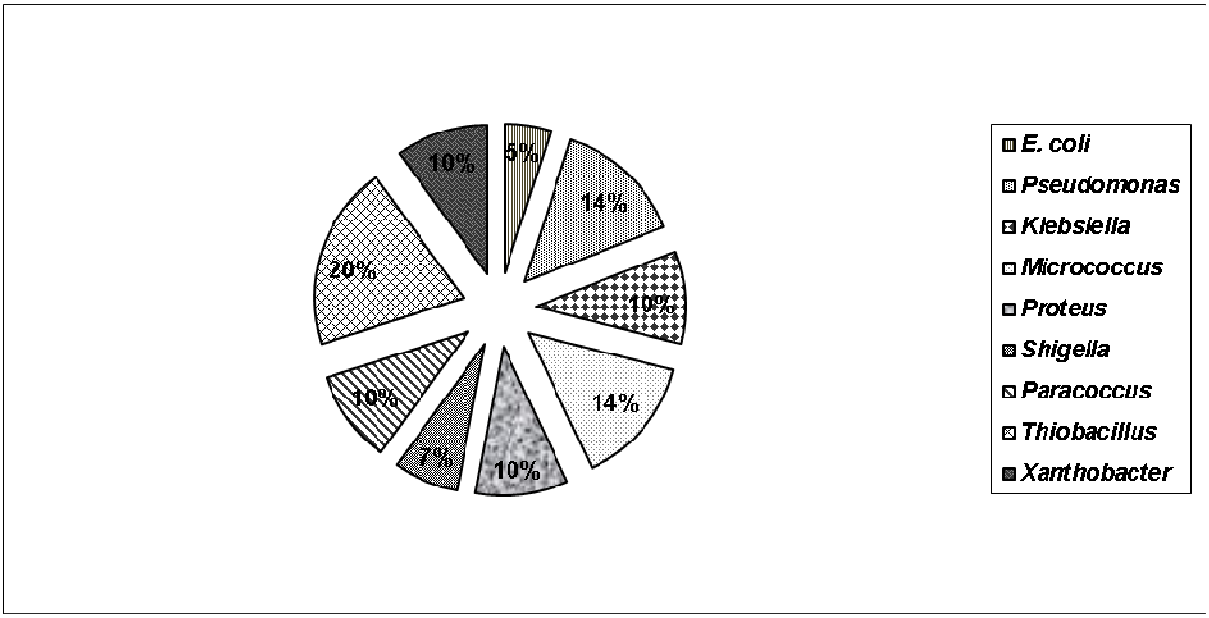
(c) Normal water mixed with sulphur water (NSW)



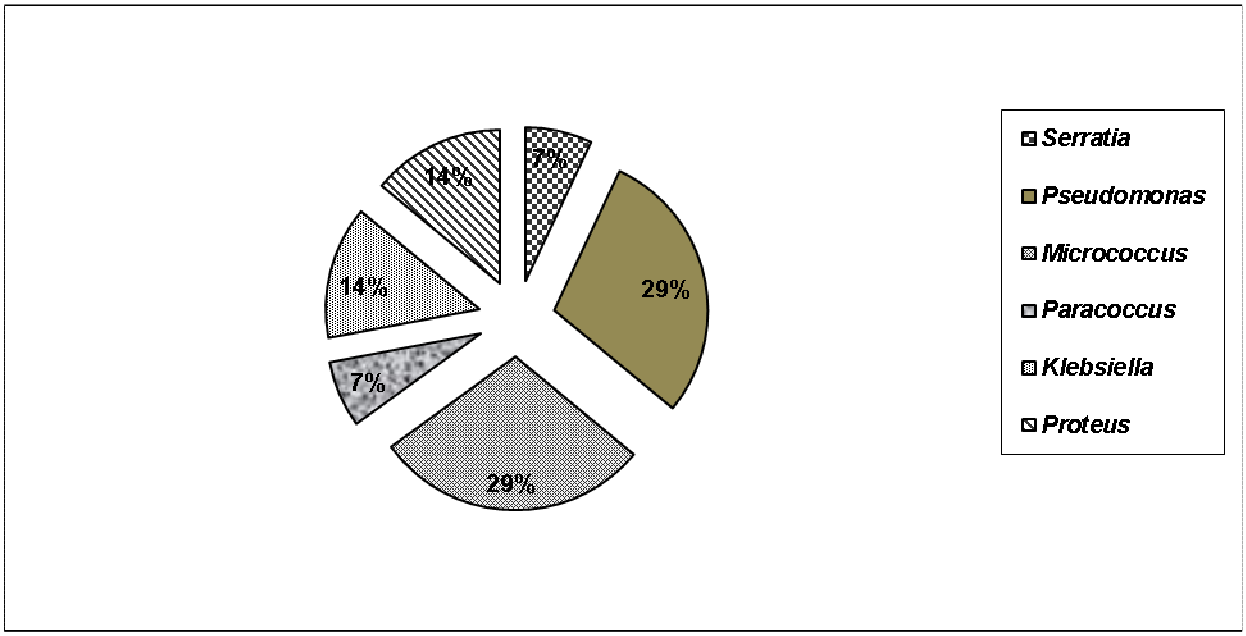
(d) Cave water (CW)



(e) Soil



(f) Soil mixed with sulphur water (SSW)



(g) Stalactite (STC)

Fig. 2: Distribution profile of bacteria in different samples of sulphur spring

3.2 Functional diversity

The functional divergence was observed amongst the recovered microflora. Amongst protease producers maximum diversity was observed in SSW (H'=2.01; E1=0.64), maximum richness in NW (R1=4.32) while amongst xylanase producers, maximum diversity in NSW (H'=0.95; E1=0.90) and maximum richness in CW (R1=3.36). Cellulolytic microflora was most diverse in STC (H'=0.98; E1=0.93) and more rich in NW, CW and S (R1=2.88). In sulfur water amylolytic microflora was most diverse (H'=0.96; E1=0.88); while maximum richness in NW (R1=4.32). Amongst pectinolytic microflora, maximum diversity in NSW (H'=0.64; E1=1.80) and maximum richness in NW (R1=2.88). P solubilizers were most diverse in SW (H'=0.66; E1=0.96); more rich in NW (R1=4.32. Maximum diversity amongst siderophore producers was observed in CW (H'=0.72; E1=0.63) while maximum richness in STC (R1=3.41).

Table 2: Functional diversity indices of sulphur spring

Indices	Name of Samples						
	SW	NW	NSW	CW	SSW	S	STC
Protease							
H'	0.33	0.00	0.69	0.00	2.01	0.50	0.65
R1	1.38	4.32	1.24	1.44	1.64	1.44	2.18
E1	0.25	0.00	0.53	0.00	0.64	0.48	0.53
Xylanase							
H'	0.91	0.35	0.95	0.66	0.58	0.72	0.65
R1	1.37	2.88	1.24	3.36	3.29	3.24	2.28
E1	0.86	0.00	0.90	0.51	0.48	0.54	0.50
Cellulase							
H'	0.61	0.35	0.32	0.48	0.82	0.89	0.98
R1	0.46	2.88	1.24	2.88	2.62	2.88	2.66
E1	0.33	0.00	0.00	0.12	0.42	0.46	0.93
Amylase							
H'	0.83	0.00	0.32	0.48	0.61	0.96	0.61
R1	0.46	4.32	1.24	0.48	1.32	2.52	1.52
E1	0.48	0.00	0.00	0.00	0.50	0.88	0.70
Pectinase							
H'	0.25	0.35	0.64	0.55	0.00	0.34	0.33
R1	0.92	2.88	0.62	2.40	0.93	0.36	1.52
E1	0.00	0.00	1.80	0.50	0.00	0.00	0.30
P solubilization							
H'	0.66	0.00	0.32	0.61	0.28	0.52	0.64
R1	1.38	4.32	1.24	0.96	1.32	1.44	1.90
E1	0.60	0.00	0.00	0.50	0.25	0.25	0.56
Siderophore							
H'	0.60	0.35	0.00	0.72	0.62	0.68	0.32
R1	1.20	2.88	1.86	2.88	2.30	2.88	3.41
E1	0.53	0.00	0.00	0.63	0.46	0.50	0.15

H'= Shanon's diversity index; R1=Richness index; E1= Evenness index

DISCUSSION

Sulfur springs constitute an important ecosystem which is of great relevance from microbial ecological point of view as it represents a complete spectrum of microbial diversity.

Microbial community succession occur at a very fast pace with the change in eco-physiological conditions of spring [4]. The present study was focused on structure and function of microbial communities in Sahastradhara.

The enrichment culture technique revealed many interesting observations. Bacteria surviving in sulfur springs with neutral pH often form mats whose appearance seems to be governed by prevailing physical and chemical conditions [7]. In this study yellow, black, brown and white colour depositions were observed. The yellow colour depositions were probably sulfur granules as many sulfur oxidizers excrete internal or external sulfur in the form of hydrophilic spherical globules [18] that can serve as an energy reserve when environmental concentrations of hydrogen sulphide substantiallydecrease. Brown depositions were indicative of presence of ferrihydrite while a blak deposition observed was because of formation of iron sulfide. Yellow alongwith black colour depositions were observed in soil samples which could be due to concomitant accumulation of sulfate with iron sulfide. Similar observations were reported by [19] in marine sediments. Brock [20] reported that the thick bacterial mats could be spectacularly white or brown yellow from precipitated sulfur are formed in neutral sulfur springs. The changes in colour, depositions and pH in broth with time period were consequence of the microbial growth and activity.

The population profile varied significantly in different samples. Maximum bacterial diversity was observed in SSW which harboured an assemblage of soil and sulfur water microflora. *Thiobacillus* was dominant microflora of sulphur water (34%) and soil mixed with sulphur water (20%). *Pseudomonas* was documented to be the dominant microflora of normal water (41%), normal water mixed with sulphur water (41%), cave water (50%) and stalactite (29%). *Proteus* was predominant in soil sample (25%). *Pseudomonas* was recovered from all water and soil samples. The dominance of *Pseudomonas* amongst heterotrophic population of springs had been reported by [4].

The functional diversity was studied by employing various diversity indices viz., Shannon index [15], evenness index [17] and richness index [16] to ascertain the role played by various morphotypes in their indigenous environment. Though the use of various diversity indices created discrepancies among some observations but the real picture of diversity was obtained since it permitted removal of bias on account of use of nutritional media,

physicochemical environment, the likely element of human experimental error. Higher the Shannon's index and evenness index, higher would be the diversity. The abundance of a particular functional type in samples were indicative of the role played by that microflora in their niche. Normal water was rich in proteolytic, cellulolytic, amylolytic microflora and p solubilizers. Cave water was observed to be rich in xylanolytic and cellulolytic microflora. Soil was rich in cellulolytic microflora. Siderophore producers population was most rich in stalactite.

The biasedness shown by cultivation approach due to intrinsic selectivity of each component of the cultivation technique nearly always resulted either in enhancement or decrease or even inhibition of growth of some specific members and therefore cultivation-dependent methods does not give complete diversity picture of a habitat. To tide over these difficulties, cultivation-independent approaches should be employed to have a complete picture.

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