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CHARACTERIZATION OF MICROORGANISMS FROM SITAKUNDA HOT SPRING

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ABSTRACT

Water, leaf, stone and sand samples from Sitakunda hot spring were studied and the microorganisms isolated from these samples were thoroughly characterized. The pH of the collected samples was found to range from 8.5 to 8.9 and the recorded temperature varied from 26.1 to 29.8 ºC during winter season. BOD of the collected water samples ranged from 10 to 92 mg/L, while COD was almost zero. The total alkalinity and chlorine content ranged from 12 to 20 mg/L and 13.9 to 18.3 gm/L respectively. Quantitative enumeration of bacterial population showed the range of 8.0×10^2 to 3.8×10^5 cfu/ml or gm sample. All the 9 selected isolates were identified as *Bacillus coagulans* (S_1B_4), *Bacillus laterosporus* (SB_{18}), *Bacillus megaterium* (S_2B_{11}), *Bacillus popilliae* (S_4P_1), *Bacillus firmus* (S_5B_1), *Citrobacter intermedius* (S_4B_{17}), *Listeria monocytogenes* (S_1B_1), *Listeria denitrificans* (S_2B) and *Pseudomonas mendocina* (S_4P_3). *Bacillus laterosporus*, *Bacillus megaterium* and *Listeria denitrificans* showed better growth in presence of methane gas in nutrient broth. By direct microscopic observation 7 genera of cyanobacteria (*Arthrospria*, *Microcoleus*, *Oscillatoria*, *Schyzothrix*, *Scytonema*, *Nostoc* and *Gloeocapsa*) and 4 genera of diatoms (*Gomphonema*, *Melosira*, *Navicula* and *Pinnularia*) were detected in the water sample.

Key words: Characterization, Microbe, Hot spring.

INTRODUCTION

Little was known about how microbes live their lives in hot spring. Mainly thermophiles are found in hot spring and among them Archaea and Cyanobacteria are common (Encyclopedia of Environmental Microbiology 2002. vol.3). Brock (1966) made the remarkable discovery that microorganisms were growing in the boiling hot springs of Yellowstone National Park Since Brock's discovery, thermopiles have been discovered in geothermal features all over the world including areas in Iceland, Kamchatka, New Zealand, Italy, Mt. Lassen, and other locations. Microorganisms other than *Sulfolobus* are reported for the first time in

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the low pH high temperature springs of Waiotapu, North Island, New Zealand (Ellis *et al.* 2005*)*. The bacterial diversity of a hot spring in Bakreshwar, India, was investigated by a culture-independent approach (Ghosh *et al.* 2003). A halophilhic, thermotolerant *Bacillus* strain (B3-15) was isolated from water of a shallow, marine hot spring at Vulcano Island (Eolian Islands, Italy). From 16S rDNA analysis, strain B3-15 was related to *B. licheniformis* (Teresa *et al.* 2002). A thermophilic bacterium *Bacillus* sp. strain TB-1 was isolated in association with the yeast *Debaryomyces vanriji* from hot springs at 46°C (Eugene *et al.* 1999). A polyphasic taxonomic study was performed on a novel facultatively anaerobic, hydrogen- or sulfur/thiosulfate-oxidizing, thermophilic chemolithoautotroph recently isolated from subsurface hot aquifer water in a Japanese gold mine (Takai *et al.* 2003).Laminatd mats of unique character in siliceous alkaline hot springs of Yellowstone Park are formed predominantly by two organisms, a unicellular blue green alga, *Synechococcus lividus*, and a filamentous,gliding,photosynthetic bacterium, *Chloroflexus aurantiacus* (Doemel and Brock 1977)

Sitakunda Hot Spring is the only hot spring in Bangladesh. It is located at Barobkunda, Sitakunda, Chittagong. Still no work has been done on this hot spring. This spring mainly consists of two rectangular wells. In one well, methane gas and water are continuously discharging from the ground to the surface. Fire can be burnt on the surface of water and it would be continued for long time, even never switched off spontaneously, because of the continuous flow of methane gas. From this well water flows to another well that receives no flow of methane gas. The present work was undertaken to study the micro flora associated with this hot spring.

MATERIALS AND METHODS

Samples were collected from different parts of the hot springs in cleaned, dried and surface sterilized (with 70% alcohol) plastic bottles and polyethylene bags. The solid samples were taken in the polyethylene bags while liquid samples were taken in plastic bottles. At each time of collection, hands were sterilized with 70% alcohol. Temperature in the field was measured with a maximum recording mercury thermometer. pH of each of the samples was determined by the electric pH meter (pH Hanna Instrument Ltd. & 3310, pH meter Jenway, UK).

After collection, samples were brought to the laboratory and carefully preserved in the refrigerator at 4°C before and after the microbial analysis. BOD,

COD, total alkalinity and Chlorine content of the collected samples were determined.

For the enumeration and isolation of bacteria, serial dilution was carried out up to 10^6 dilutions. Plating in triplicate plates was made for each dilute sample either by Pour Plate Technique or by Spread Plate Technique using Nutrient agar as the medium. The plates were incubated at 37°C in an incubator for 24 to 48 hours. After incubation, well-spaced plates were placed on a colony counter (Stuart scientific, UK) and the colonies were counted. . Characters of the colonies were recorded as colour, form, elevation, margin, surface etc. (Eklund and Lankford 1967, Bryan 1950). The marked and observed colonies were transferred on nutrient agar slant for purification. Morphological, physiological and biochemical characteristics of the isolates were recorded.

In order to study the utility of methane gas by the selected isolates, methane gas was aseptically introduced into Nutrient broth and Inorganic Salt media and was inoculated with 72 hour old cultures.

Collected samples were observed directly under microscope for Cyanobacteria and diatoms using 10x, 40x, 60x and 100x objectives.

RESULTS AND DISCUSSION

Table-1 presents the features of the samples collected from different parts of the spring and of the microbial populations of each sample.

The temperature of the spring ranged from 26.1 to 29.8^0C during winter season and pH from 8.5 to 8.9. BOD of the collected samples from both wells of the hot spring was also determined within one hour of the collection. The BOD of the methane containing well was found 10 mg/L while BOD of other well was found 92 mg/L. The reason of higher BOD in the second well was the growth of different types of algae, cyanobacteria, arthropods etc., which were not found in the first well. COD of the collected samples was found almost zero or very poor. The reasons of this result may be the unusual conditions of the hot spring.

TABLE-1: FEATURES OF THE SAMPLES COLLECTED FROM DIFFERENT PARTS OF THE SPRING.

Collected samples	Colour	Temp. (^{0}C)	pH	Total alkalinity (mg/L)	Chlorine content (gm/L)	Total bacterial count *
Water from the well with methane and water discharging	Colourless	29.8	8.8	12	13.9	8.0×10^2
Water from the well without methane and water discharging	Colourless	26.7	8.9	19	18.3	1.3×10^3
Water from the well without methane and water discharging	Light green	26.1	8.5	20	17.4	6.2×10^4
Leaves, stones, sands etc. from the well without methane and water discharging	Black, green	26.1	8.8	17	18.1	3.8×10^5
Leaves, stones, sands etc. from the well with methane and water discharging	Black, white	29.8	8.7	14	15.2	2.5×10^3

*cfu/ml in case of water sample and cfu/gm incase of solid sample.

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Different cultural (Table- 2), morphological and staining features of finally selected 9 isolates (Table- 3) were recorded. Among the colony types, circular type colony was found to be dominant type.

TABLE- 2: COLONY CHARACTERISTICS OF THE SELECTED ISOLATES ON NUTRIENT AGAR .

Isolate	Color	Form	Elevation	Margin	Surface	Slant character
S_1B_1	Translucent	Circular	Flat	Entire	Smooth	Filiform
S_1B_4	Translucent	Punctiform	Flat	Entire	Smooth	Filiform
S_1B_{18}	Milk white	Punctiform	Flat	Entire	Smooth	Filiform
S_2B_5	Cream	Circular	Raised	Entire	Smooth	Echinulate
S_2B_{11}	White	Circular	Flat	Erose	Contoured	Echinulate
S_4P_1	Cream	Circular	Flat	Entire	Smooth	Filiform
S_4P_3	Light Yellow	Circular	Flat	Entire	Smooth	Filiform
S_4B_{17}	Milk white	Circular	Raised	Entire	Smooth	Filiform
S_5B_1	White	Filamentous	Flat	Filamentous	Smooth	Arborescent

Of 9 isolates, 8 were short rods and only 1 isolate (S_1B_{18}) was long rod. No cocci were found in the selected isolates. The isolates were also different in their arrangement of cells. Grams staining of the selected isolates revealed that majority of them were gram positive while only 3 of them were gram negative. Acid fast staining of the isolates showed that all of them were non-acid fast. Among 9 isolates, 5 were spore formers and 4 were non-spore formers (Table- 3). Spores were round to cylindrical and central.

The morphological, physiological and biochemical features are shown inTable-3 & 4. All the 9 isolates were found to belong to 4 genera (*Bacillus, Citrobacter, Listeria,* and *Pseudomonas*). Among these genera, *Bacillus* and *Pseudomonas* genera were reported from different hot spring by different workers (Belly and Brock 2008). The reason of finding of *Listeria* in study may be due to

the presence of leaf litter in the spring (Buchanan and Gibbons 1974). The isolates were provisionally identified as *Bacillus coagulans* (S_1B_4), *Bacillus laterosporus* (SB_{18}), *Bacillus megaterium* (S_2B_{11}), *Bacillus popilliae* (S_4P_1), *Bacillus firmus*, (S_5B_1), *Citrobacter intermedius* (S_4B_{17}), *Listeria monocytogenes* (S_1B_1), *Listeria denitrificans* (S_2B_5) and *Pseudomonas mendocina* (S_4P_3), by comparing with the standard description of "Bergey's Manual of Determinative Bacteriology" 8th ed. (Buchanan and Gibbons 1974).

TABLE-3: MICROSCOPIC FEATURES OF THE SELECTED ISOLATES

Isolates	**Form**	Arrangement	Gram reaction	Acid fast staining	Spore staining
S_1B_1	Short rod	Single, pair and cluster	Gram positive	Non acid fast	Non spore former
S_1B_4	Short rod	Single, pair and cluster	Gram positive	Non acid fast	Spore former
S_1B_{18}	Long rod	Single and pair	Gram positive	Non acid fast	Spore former
S_2B_5	Short rod	Single and short chain	Gram positive	Non acid fast	Non spore former
S_2B_{11}	Short rod	Single, pair and chain	Gram positive	Non acid fast	Spore former
S_4P_1	Short rod	Single, pair and chain	Gram positive	Non acid fast	Spore former
S_4P_3	Short rod	Single and pair	Gram negative	Non acid fast	Non spore former
S_4B_{17}	Short rod	Single and pair	Gram negative	Non acid fast	Non spore former
S_5B_1	Short rod	Single, pair and chain	Gram positive	Non acid fast	Spore former

Natural methane gas was aseptically diffused in the Nutrient broth and in the Inorganic salt media of screw-capped test tubes. Control without methane gas was also maintained in every case. Selected isolates were inoculated separately in the media and incubated at 37^0C. The isolate $S_1B_{18,}$ S_2B_5 and S_2B_{11} showed enhanced growth in Nutrient broth. Again, isolate S_1B_{18} and S_2B_{11} showed trace growth in natural gas containing Inorganic salt medium, which showed no growth in this medium in the absence of natural gas (Table- 5).

In the present study, samples were also directly observed under microscope. The samples collected from outer well was found to have cyanobacteria and diatoms. The observed genera were compared with the standard descriptions illustrated in different books (Sharma 1986, Vashishta 1999, Bold and Wynne 1985). Seven genera of cyanobacteria and 4 genera of Bacillariophyceae were identified. The identified genera of cyanobacteria were *Gloeocapsa, Arthrospira, Oscillatoria, Microcoleus*, *Nostoc*, *Scytonema* and *Scyzothrix.* Among these genera, *Arthrospira, Oscillatoria* and *Scytonema* were very common (Table- 6). Similar genera were reported by various workers from different hot springs [*Oscillatoria* (Castenholz 1967), *Gloeocapsa* (Bonny and Jones 2003), *Arthrospira* (Walter *et al*, 2006) and *Scytonema* (Rezanka *et al* 2003)]. The identified genera of Bacillariophyceae were *Gomphonema*, *Melosira, Navicula* and *Pinnularia* (Table- 7).

TABLE 4: BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE SELECTED ISOLATES.

Characteristic	S_1B_1	S_1B_4	S_1B_{18}	S_2B_5	S_2B_{11}	S_4P_1	S_4P_3	S_4B_{17}	S_5B_1
Catalase test	+	+	+	+	+	+	+	+	+
Casein hydrolysis	-	+	-	+	+	-	-	+	-
Starch hydrolysis	-	-	-	+	+	-	-	+	-
Gelatin hydrolysis	-	+	+	+	+	-	-	+	+
Nitrate reduction	-	-	±	±	+	-	+	+	+
Deep glucose agar test	A	F	A/F	A/F	A/F	A	A/F	A/F	A/F
Proteolysis	-	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	±	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	+	-	-
Indole test	-	-	-	-	-	-	-	+	-
Methyle Red test	-	-	-	-	-	-	-	-	+
Voges Proskauer test	-	-	-	-	-	-	-	-	-
Citrate test	+	+	+	+	±	-	+	+	±
Urease test	+	+	+	-	±	+	+	+	-
Motility	±	+	±	+	+	±	+	+	+
Growth at pH 4.5	+	++	±	+	±	+	±	+	++
Growth at pH 8.5	+++	+++	++	+++	+++	+++	+++	+++	+++
Heat tolerance at 60^0C	+	+	+++	+++	+++	+++	+	+	+++
Maximum salt tolerance	3%	3%	3%	12%	4%	6%	2%	2%	2%
Glucose fertmentation	Alk-ali	Acid	Alk-ali	Alk-ali	Acid	Acid	Acid	Acid	Acid
Lactose fermentation	Alk-ali	Alk-ali	Alk-ali	Alk-ali	Alk-ali	Alk-ali	Alk-ali	Alk-ali	Acid

Note: A= Aerobic, F= Facultative, + = Positive, - = Negative, ± = Variable

TABLE-5: DIFFERENCES BETWEEN THE GROWTH IN THE MEDIA WITH OR WITHOUT METHANE GAS.

Isolates	Nutrient Broth		Inorganic Salt	
	without CH_4	With CH_4	without CH_4	with CH_4
S_1B_1	+ + +	+ + +	-	-
S_1B_4	+ + +	+ + +	-	-
S_1B_{18}	+ +	+ + +	-	Trace
S_2B_5	+ +	+ + +	-	-
S_2B_{11}	+	+ + +	-	Trace
S_4P_1	+ +	+ +	-	-
S_4P_3	+ +	+ +	-	-
S_4B_{17}	+ + +	+ + +	-	-
S_5B_1	+ +	+ + +	-	-

Note: + = Positive (+ = Scanty, ++ = Moderate, +++ = Heavy), - = Negative.

TABLE-6: FEATURES OF THE IDENTIFIED GENERA OF CYANOBACTERIA.

Name of the family	Name of the genera	Description
Chroococcaceae	*Gloeocapsa*	Subaerial, palmelloid, colonies were in gelatinous masses, cells were more or less spherical.
Oscillatoriaceae	*Arthrospira*	Filamentous. closely spiral, trichomes are enclosed with mucilaginous sheath, brown to blue green colour.
	Oscillatoria	Long filamentous, filaments were naked; trichomes were cylindrical and unbranched, dark blue green colour.
	Microcoleus	Filamentous, more than one trichomes, trichomes are enclosed with mucilaginous sheath, blue green colour.
	Scyzothrix	Filamentous, single trichome was observed, yellowish green in colour.
Scytonemataceae	*Scytonema*	Filamentous, trichomes were enclosed with mucilaginous seath like bundles, single filament was also enclosed with sheath, false branching occurred, blue green colour.
Nostocaceae	*Nostoc*	Germinating hormogonia was observed, trichomes were enclosed with gelatinous matrix, blue green colour.

TABLE-7: FEATURES OF THE IDENTIFIED GENERA OF BACILLARIOPHYCEAE.

.	Genus	Description
	Gomphonema	Cell was golden to brown, one end was narrow and another end was broad and blunt, one polar nodule was present in each end.
	Melosira	Three distinct cells were present; cells were rectangular, long and sometimes coiled filamentouss, green colour.
	Navicula	Two ends of the cells were tapping, attenuated with round apics; raphe was axile, distinct, straight with small polar and central nodule; golden brown cell wall.
	Pinnularia	Two ends of the cells were equally thick, not attenuated, contain nodule in each end.

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