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Research article

Microbial diversity in relation to physico-chemical properties of hot water ponds located in the Yamunotri landscape of Garhwal Himalaya

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ABSTRACT

The current study includes the assessment of physico-chemical characteristics along with the microbial diversity of hot water samples collected from three important sacred hot water springs of the Uttarakhand Himalaya close to the world-famous Hindu shrine Yamunotri temple. Hot water samples were collected for two consecutive years 2015–2016 in two sampling operations each year. A total of fifteen physico-chemical attributes of hot water were recorded. Microbes of hot water ponds were characterized by morphological, biochemical, MALDI-TOF MS, and molecular approaches. A total of twenty-two microbial strains were identified from the Surya Kund with water temperature ranging between 86 °C and 89 °C; twenty-two microbial strains were identified from the Draupadi Kund with water temperature ranging between 65 °C and 69 °C and twenty-one microbial strains were identified from the Yamunotri Tapt Kund with water temperature ranging between 45 °C and 48 °C during the study period. The present study on the assessment of physico-chemical characteristics and thermophilic microbial diversity of all the three hot water springs can be a useful reference for further studies on similar aspects in other parts of the Himalaya. The available data will also help to understand the reasons behind the curative properties of these hot water springs. This baseline information will also be instrumental for the conservation and management of these hot water springs.

1. Introduction

Microbes are one of the most important components of any extreme environment. These microbes can take nutrition from these harsh environmental conditions. Thus, these extremophiles can survive and grow in harsh environmental conditions (Oarga, 2009). Among extremophiles, the thermophiles or heat-loving microbes can survive at extreme temperatures (Beg et al., 2000; Akmar et al., 2011). Hot water ponds have water temperatures higher than the atmospheric temperature in surroundings (Sen et al., 2010). Hot water ponds represent harsh environmental conditions available across the entire Himalayan region (Kumar et al., 2004). Hot water ponds are an important source of heat-loving or thermophilic microorganisms useful and effective even at the extremely high temperature ranges. These microorganisms are beneficial for various industries including the pharmaceutical sector (Tekere et al., 2015).

A considerable research has been undertaken on multiple aspects of hot water ponds. This includes the work of Baker et al. (2001) on the isolation and identification of bacterial diversity in the hot springs of Indonesia; Kumar et al. (2004) on identification of microbiological frequency of soil of two distinct hot water ponds in the Uttarakhand Himalaya; Kvist et al. (2007) on archaeal diversity in Icelandic hot water spring; Sharma et al. (2008) on isolation and characterization of *Geobacillus spp*; Akmar et al. (2011) on identification of a novel heat loving bacterial strain from hot water pond; Bhusare and Wakte (2011) on microbial diversity of Unkeshwar thermal spring; Huang et al. (2011) on diversity of archaea and bacteria in the hot springs of Tibetan Plateau; Sharma et al. (2012) on the identification of Manikaran hot spring microbial community; Pagaling et al., (2012) on diversity of bacteria and archaea in microbial mats obtained from two important thermal springs located in the geothermal region of Tengchong; Jiang et al., (2012) on diversity of actinomycetes in the thermal springs located in Central and Central-Eastern Tibet, an autonomous region of China; Wemheuer et al. (2013) on microorganims and their biochemical potential; while, Lopez et al. (2013) on hot spring metagenomics; Ghati et al. (2013) on Esterolytic heat loving bacterial strain from a thermal water pond in India; Bandyopadhyay et al. (2013) on a bacterium having a tolerant capacity against the high arsenic concentration that was isolated from a thermal

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spring in India; Meyer-Dombard and Amend (2014) on geochemistry and microbial ecology in a hot springs having alkaline pH located at Ambitle Island in Papua New Guinea; Pathak and Rathod (2014) on cultivable bacterial frequency of hot water pond located at Unkeshwar in Maharashtra; Rawat (2015) on bacterial enumeration from a thermal spring of Uttarakhand. Chan et al. (2015) on the enumeration of heat-loving microbial diversity in Malaysia through sequencing and metagenomic approaches; Tekere et al. (2015) on the diversity of bacteria dwelling from some African hot water ponds; Kumar and Sharma (2019a,b) on microbes and physico-chemical characteristics of two Garhwal Himalayan thermal springs; and Kumar and Sharma (2020) on the determination of microbes and physico-chemical characteristics of thermal springs located close to the Badrinath temple. However, no information is available in the public domain regarding the diversity of microbes and physico-chemical characteristics of the hot waters of Surya Kund, Draupadi Kund and Yamunotri Tapt Kund located in the vicinity of world-famous Hindu shrine Yamunotri in Uttarakhand Himalaya. Therefore, it was felt desirable to undertake the work on the diversity of microbes and physico-chemical features of all the three hot water bodies Surya Kund, Draupadi Kund and Yamunotri Tapt Kund hot water. Thus, the present obtained baseline data will have both academic and applied values.

2. Materials and methods

2.1. Study area

All the hot water ponds, Surya Kund, Draupadi Kund and Yamunotri Tapt Kund are located just near to the World famous Hindu's shrine Yamunotri Temple in the Uttarakhand Himalaya (Figure 1). Surya Kund or Suraj Kund is located at a height of 3,291 m above m.s.l. at latitude 31°1′0.12″ N and longitude 78°27′0″ E. Draupadi Kund is situated next to Surya Kund at a height of 3,270 m above m.s.l. This hot water of Draupadi Kund is known for its curative and medicinal properties. The maximum *in-situ* water temperature of Draupadi Kund is 67 °C. However, Yamunotri Tapt Kund is also situated in the vicinity of Surya Kund at a height of 3,262 m above m.s.l. The maximum *in-situ* water temperature of Yamunotri Tapt Kund is 45 °C.

2.2. Water sampling

Water samples were collected from all the three hot water bodies (Surya Kund, Draupadi Kund and Yamunotri Tapt Kund) in two sampling operations (June and August) for the period of two years (2015; 2016). The hot water ponds were only accessible during the period from June to October every year due to their locations in the high altitude Himalayan range. Water temperatures, pH, free CO2, the concentration of dissolved oxygen were recorded at the collection sites. For rest of the parameters, water samples were collected in autoclaved thermosteel flask and transported to the Laboratory of Environmental Microbiology and Biotechnology (L.E.M.B.), Department of Environmental Sciences, H.N.B. Garhwal University, Srinagar Garhwal, Uttarakhand. The physico-chemical parameters and microbial analyses were made within 24 h of the sample collection following the standard methodology outlined in APHA (2012); Harley and Prescott (2002) and Morello et al. (2003). The samples of water collected from the sampling sites were inoculated in the desired media and incubated under the similar environmental conditions available at the sampling sites like temperature and pH. The inoculated plates were incubated at the same temperature observed at the sampling site for 48–72 h in case of bacteria and 3–5 days in the case of fungi and actinomycetes. All the unique microbial colonies were cultured in similar growth media to obtain a pure culture for further growth and identification (Davis et al., 2005; Huq et al., 2012; Anand and Aoyagi, 2019).

2.3. Physical and chemical characteristics of hot water

Samples of water from all the three hot water ponds were collected for the analysis of a predefined set of fifteen physico-chemical parameters. Water temperature was observed by submerging the digital Centigrade Thermometer having a temperature range of -50 to +300 °C up to 10 cm in water. The pH was recorded on-site by using portable pH meter (Electronics India Model No. 7011) and off-site by using a benchtop pH meter (Toshcon, Model No. TPC-17). The concentration of DO was estimated on-site following the Modified Winkler's Method. Free CO_2 was also recorded at the sites. Electronic conductivity, the concentration of salinity and TDS were recorded with the help of Multiparameter Analyzer (Toshcon, Model No. TPC-17). However, the concentration of total alkalinity, chlorides, total hardness, Calcium and Magnesium were recorded at the Laboratory using the standard methodology available in APHA (2012). The concentrations of nitrates, sulfates and phosphates were analyzed with the help of a UV-VIS Spectrophotometer (Systronic, Model No. 117) at the Laboratory.

2.4. Microbial isolation and enumeration

Bacterial colonies were isolated by using the Nutrient Agar Media (HiMedia, India, Bangaluru) commonly known as NA Media. The fungal strains were isolated by using the Sabaroud Dextrose Agar (SDA) Media (HiMedia, India, Bangaluru). To avoid any type of bacterial

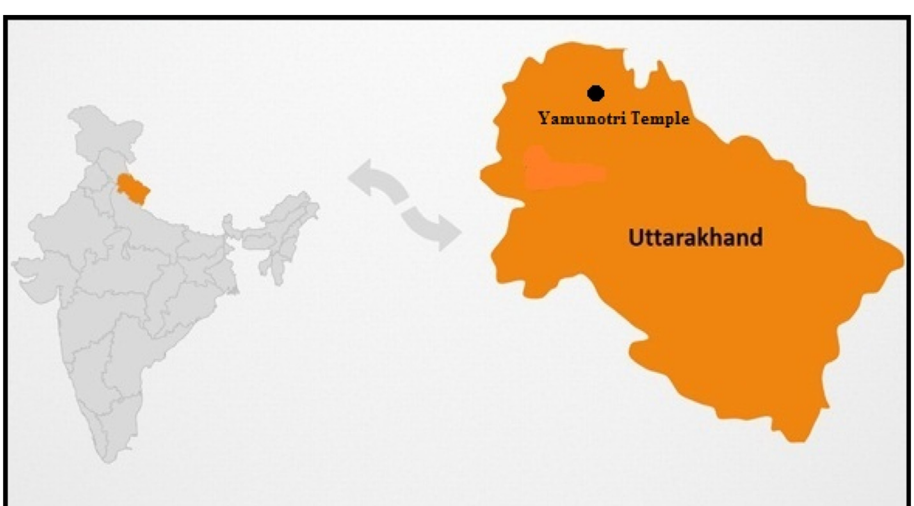

Figure 1. The Study area (Surya Kund; Draupadi Kund and Yamunotri Tapt Kund located near Yamunotri temple).

contamination during the isolation of fungal strains, the SDA media was enriched with 50 mg l^{-1} of each Ampicillin (HiMedia, India, Bangaluru) and Streptomycin (HiMedia, India, Bangaluru). For isolation of Actinomycetes, the Actinomycetes Isolation Agar (AIA) media (HiMedia, India, Bangaluru) was used. The pH of each culture medium was adjusted matching to the pH recorded at the sampling site. After the enumeration of microorganisms, the isolated colonies were streaked on separate suitable media plates for getting the pure cultures of each microbe (Clesceri et al., 1998).

2.5. Morphological and biochemical characteristics of microbes

A total of twenty-seven morphological and biochemical characteristics were analyzed during the study period. During analysis, pure cultures of microorganisms were carefully recognized by unaided eyes and under the Phase Contrast Microscope (Nikon Eclipse TS100) for studying the morphological structures and arrangements of the bacterial strains. The morphological characteristics of microorganisms included shape, size, margin, elevation, color, cell shape, motility, spore formation, flagellation, and gram straining. However, various biochemical tests were performed to identify a microbial strain up to the lowest possible taxon (Rohomania et al., 2015; Ayitso and Onyango, 2016). Biochemical tests included the assessment of catalase, citrate, urease, fructose, MR-VP, indole test, and maltose, etc.

2.6. Identification of microbial isolates by using MALDI-TOF MS

Pure culture of each isolate was sent to the National Centre for Microbial Resources (NCMR), Pune for final confirmation using the MALDI-TOF mass spectrometry technique. Mass spectrometry is an analytical technique in which samples are ionized into charged molecules and the ratios of their mass-to-charge (m/z) can be measured. In MALDI-TOF mass spectrometry, the ion source is matrix-assisted laser desorption/ionization (MALDI), and the mass analyzer is time-of-flight (TOF) analyzer. MALDI-TOF MS is an emerging tool used for high-throughput and rapid microbial identification. The principle behind microbial identification using MALDI-TOF MS is based on the fact that every microorganism has a specific protein composition, which gives its characteristic and unique mass spectra (Risch et al., 2010; Marko et al., 2012). The procedure of identification of microorganisms by using MALDI-TOF MS had two sections: the first section involves the preparation of the HCCA (α-Cyano-4-hydroxycinnamic acid) matrix solution; and the second section involves the direct transfer method, in which the single microbial colony is transferred to the MALDI target plate and later using the HCCA matrix solution and MALDI-TOF MS instrument for the identification of microbial colonies based on the mass spectra.

2.7. DNA extraction and gene amplification

Almost all the microbial isolates were identified by using the technique of MALDI-TOF MS. However, few microbial isolates were identified through molecular or sequencing approaches, as their identification was not successfully made through the MALDI-TOF-MS technique. The DNA was extracted from these selected microbial isolates using HiPurA Bacterial Genomic DNA Extraction Kit (HTBM008; HiMedia, India, Bangaluru) for bacteria and GSure Fungal DNA Extraction Kit (G45331; GCC Biotech, India, New Delhi) for fungal isolates. Genes of 16S rRNA were amplified by PCR using specific universal primers, forward primer 8F (AGAGTTTGATCCTGGCTCAG) and reverse primer 1492R (TACGGYTACCTTGTTACGACTT) (GCC Biotech, India, New Delhi) (Takahashi et al., 2014); whereas, the genes of 18S rRNA were amplified by PCR using specific universal ITS primers, forward primer ITS 1F (TCCGTAGGTGAACCTGCGG) and ITS 4R (TCCTCCGCTTATTGATATGC) (GCC Biotech, India, New Delhi) (Pryce et al., 2003; Raja et al., 2017; Usyk et al., 2017).

2.8. Identification of microbial isolates by using rRNA sequencing approach

Few of the microbial isolates, whose identifications could not be possible through MALDI-TOF MS, were sent for 16S rRNA and 18S rRNA sequencing at the same facilitation center. The facilitation center provided an accession number only to those microbial strains whose identification was done through the gene sequencing method. The accession numbers of such microbial strains are given in Table 1.

2.9. Statistical treatment of data

MS Office 2013 was used for the statistical treatment (minimum; maximum; mean; standard deviation) of the data related with the physico-chemical characteristics of hot water samples of all the hot water ponds.

3. Results and discussion

Results of all the physico-chemical parameters of hot waters of Surya Kund, Draupadi Kund and Yamunotri Tapt Kund have been presented in Table 2. Detailed biochemical and morphological characteristics of few isolates selected randomly from all the examined strains are given in Table 3. The majority of the isolated and identified bacteria were found to be gram-positive and such bacteria are known to be comparatively stress-resistant and long-range migrants, especially the Actinobacteria (Cerritos et al., 2011; Kumar et al., 2014).

3.1. Physico-chemical characteristics and microbial diversity of Surya Kund

The water temperature of Surya Kund ranged from 86 °C to 89 °C with a mean value of 87.8 °C. High temperature is important for the growth of thermophiles. Those microorganisms which can grow at a temperature of more than 80 °C are known as hyperthermophiles. The pH of the hot water was recorded within a range from 8.4 to 8.8, indicating the slightly alkaline nature of water of Surya Kund. An acidic nature of one hot water spring of Kullu district, Himachal Pradesh was recorded by Kumar et al. (2013). The same observation (pH 6.9–9.5) of acidic nature of hot water was reported by Singh et al. (2015) **o**n the thermal springs of Jharkhand and West Bengal. A very low concentration of DO (1.8 mg l^{-1}) was recorded in the hot water of Surya Kund during all sampling operations. Kumar et al. (2013) also observed low concentration of DO (2.52 mg l^{-1}) in hot water springs of Himachal Pradesh. The water temperature is inversely proportional to the concentration of DO. As the water temperature increases, the amount of DO decreases (Rana et al., 2018). Fazlzadeh et al. (2016) observed 3.25 mg l^{-1} to 3.57 mg l^{-1} concentration of dissolved oxygen in Iran. Electrical conductivity (EC) ranged between 12.0 mS/cm to 12.4 mS/cm with 12.2 mS/cm as mean value in the hot water of Surya Kund. Haki and Gezmu (2012) reported almost an identical range of electrical conductivity in Ethiopian hot spring. The salinity of water of Surya Kund varied from 6.6^{SAL} to 6.8^{SAL} during the study period. High salinity of thermal springs was observed by Hamzah et al. (2013) from Malaysia. TDS in the hot water of Surya Kund was recorded between 6.5 mg l^{-1} to 6.8 mg l^{-1}. However, a high concentration of TDS was observed by Hamzah et al. (2013) in Malaysia. Free CO_2 was recorded between 22.0 mg l^{-1} to 26.4 mg l^{-1} during the study period. Total hardness of hot water of Surya Kund ranged between 164 mg l^{-1} to 168 mg l^{-1}. Concentration of calcium ranged from 21.60 mg l^{-1} to 24.05 mg l^{-1} in the hot water of Surya Kund. Magnesium concentration was recorded between 26.34 mg l^{-1} to 27.34 mg l^{-1} Singh et al. (2015) recorded the concentration of Magnesium in the range of 12.60 mg l^{-1} to 15.62 mg l^{-1} in the thermal springs of Jharkhand and West Bengal. Chlorides concentration was recorded between 157.6 mg l^{-1} to 166.1 mg l^{-1} with an average of 161.9 mg l^{-1}. Total alkalinity of hot water ranged from 130 mg l^{-1} to 150 mg l^{-1} in the water of Surya Kund

Table 1. Summary of the closest neighbour for the samples sent for identification to the sequencing facility centre.

Isolate No.	Closest Neighbour	Accession No.	% Similarity
1.	*Bacillus licheniformis* ATCC 14580(T)	AE017333	100.00
2.	*Bacillus tequilensis* KCTC 13622(T)	AYTO01000043	100.00
3.	*Bacillus paralicheniformis* KJ-16(T)	LBMN01000156	99.84
4.	*Aspergillus costaricaensis* CBS 115574	NR_103604.1	99.21
5.	*Bacillus tequilensis* KCTC 13622(T)	AYTO01000043	99.76
6	*Bacillus paralicheniformis* KJ-16(T)	LBMN01000156	99.33

Table 2. Physicochemical properties of hot water ponds located in the Yamunotri landscape of Garhwal Himalaya.

Physico-chemical Parameters	Surya Kund			Draupadi Kund			Yamunotri Tapt Kund		
	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD
Water Temperature (°C)	86.0	89.0	87.8 ± 1.5	65.0	69.0	67.0 ± 1.63	45.0	48.0	46.8 ± 1.26
Dissolved Oxygen (mg.l^{-1})	1.8	1.8	1.8 ± 0.0	3.4	3.8	3.7 ± 0.19	5.8	6.2	6.0 ± 0.16
pH	8.4	8.8	8.5 ± 0.19	9.2	9.7	9.4 ± 0.22	7.3	7.8	7.5 ± 0.24
Conductivity (mS/cm)	12.0	12.4	12.2 ± 0.16	14.0	14.5	14.3 ± 0.21	8.19	8.28	8.24 ± 0.04
Free CO_2 (mg.l^{-1})	22.0	26.4	24.2 ± 2.54	74.8	79.2	77.0 ± 2.54	92.4	96.8	95.7 ± 2.2
Salinity (^{SAL})	6.6	6.8	6.7 ± 0.08	7.5	7.8	7.6 ± 0.13	4.1	4.5	4.3 ± 0.19
TDS (mg.l^{-1})	6.5	6.8	6.6 ± 0.15	7.4	7.6	7.6 ± 0.1	4.51	4.59	4.55 ± 0.04
Chlorides (mg.l^{-1})	157.6	166.1	161.9 ± 3.65	170.4	177.5	174.7 ± 3.48	92.3	96.56	94.43 ± 2.46
Total Alkalinity (mg.l^{-1})	130.0	150.0	141.3 ± 10.31	80.0	100.0	90.0 ± 9.13	95.0	120.0	107.5 ± 11.90
Nitrates (mg.l^{-1})	0.080	0.082	0.081 ± 0.00	0.137	0.142	0.140 ± 0.00	0.092	0.098	0.095 ± 0.00
Sulfates (mg.l^{-1})	0.789	0.804	0.797 ± 0.00	1.201	1.213	1.207 ± 0.00	0.241	0.244	0.243 ± 0.00
Phosphates (mg.l^{-1})	0.071	0.075	0.073 ± 0.00	0.022	0.028	0.025 ± 0.00	0.081	0.090	0.085 ± 0.00
Total Hardness (mg.l^{-1})	164.0	168.0	166.5 ± 1.91	114.0	122.0	118.5 ± 3.42	128.0	132.0	130.0 ± 1.63
Calcium (mg.l^{-1})	21.60	24.05	23.03 ± 1.23	29.6	33.6	32.0 ± 1.73	27.2	31.2	29.2 ± 1.66
Magnesium (mg.l^{-1})	26.34	27.34	26.60 ± 0.50	8.81	9.78	9.41 ± 0.46	12.71	15.64	13.92 ± 1.23

during the period of study with an average of 141.3 mg l^{-1}. High concentration of total alkalinity (196 mg l^{-1}) was also observed in hot water ponds located in Himachal Pradesh by Kumar et al. (2013). Nitrates concentration in the hot water of Surya Kund varied from 0.080 mg l^{-1} to 0.082 mg l^{-1}. However, the sulfates concentration ranged from 0.789 mg l^{-1} to 0.804 mg l^{-1} Sherpa et al. (2013) recorded a high concentration of sulfates and nitrates in the hot water ponds of Sikkim. Phosphates concentration in the water samples of Surya Kund was recorded between 0.071 mg l^{-1} to 0.075 mg l^{-1}.

An overall, twelve species of bacteria, four species of actinomycetes and six species of fungi were isolated and identified from the hot water of Surya Kund. A total of twenty-two microbial species were isolated and identified during the present study on Surya Kund (Table 4). ***Bacillus subtilis***, ***Bacillus megaterium*** and ***Paenibacillus*** were also reported by Sharma et al. (2013) from the thermal springs of Himalayan region. *Bacillus thermoamylovorans, Bacillus simplex* and *Bacillus megaterium* were also reported by Baker et al. (2001) from Indonesian hot springs. Khalil et al. (2003) reported the isolation of *Streptococcus thermophilus* from Zerka-Maeen thermal spring of Africa. The presence of thermophilic *Actinobacillus spp*. was also reported from cattle compost (Saggu and Shrivastava, 2013).

3.2. Physico-chemical characteristics and microbial diversity of Draupadi Kund

The water temperature of Draupadi Kund ranged from 65 °C to 69 °C with a mean value of 67 °C during the period of study. The pH of Draupadi Kund ranged from 9.2 to 9.7, indicating the highly alkaline nature of water. Similar observations were recorded by Kumar and Sharma (2019a,b) for the hot water springs of Uttarakhand. The similar observations (pH: 7.76 to 9.98) were recorded by Guzman et al. (2004) for thermal springs of Mexico. The presence of cyanobacteria or Blue-Green Algae (BGA) or other microscopic algae may be the reason for high pH, as these algae use carbonates and bicarbonates present in hot water and contribute towards the high alkalinity. The presence of algal mat of cyanobacteria in the Draupadi Kund has been recorded around the hot water source. The concentration of dissolved oxygen (DO) in hot water of Draupadi Kund was recorded between 3.4 mg l^{-1} to 3.8 mg l^{-1} during the study period. Fazlzadeh et al. (2016) also recorded amount of dissolved oxygen between 3.25 mg l^{-1} to 3.57 mg l^{-1} in Iran. The concentration of electrical conductivity (EC) of hot water of Draupadi Kund was recorded between 14.0 mS/cm to 14.5 mS/cm. Haki and Gezmu (2012) also reported the electrical conductivity within the same range in the hot water spring located in Ethiopia. The salinity of water samples obtained from Draupadi Kund varied from 7.5^{SAL} to 7.8^{SAL} during two-year of the study period. High salinity was observed by Hamzah et al. (2013) from the hot water springs located in Malaysia. TDS ranged from 7.4 mg l^{-1} to 7.6 mg l^{-1} in the water of Draupadi Kund. A high range of TDS concentration was recorded by Hamzah et al. (2013) in hot water springs of Malaysia. Free CO_2 concentration in Draupadi Kund was recorded between 74.8 mg l^{-1} to 79.2 mg l^{-1} during the study period with an average of 77.0 mg l^{-1}. Total hardness of hot water of Draupadi Kund ranged from 114 mg l^{-1} to 122 mg l^{-1} with an average value of 118.5 mg l^{-1}. Calcium concentration ranged from 29.6 mg l^{-1} to 33.6 mg l^{-1} with an average value of 32.0 mg l^{-1}. Magnesium concentration in hot water of Draupadi Kund was recorded from 8.81 mg l^{-1} to 9.78 mg l^{-1} Singh et al. (2015) reported 12.60 mg l^{-1} to 15.62 mg l^{-1} concentration of magnesium in hot water ponds of Jharkhand and West Bengal. Chlorides concentration in Draupadi Kund ranged from 170.4 mg l^{-1} to 177.5 mg l^{-1} with an average value of 174.7 mg l^{-1}. The total alkalinity of hot water of Draupadi Kund ranged from 80 mg l^{-1} to 100 mg l^{-1} with an average value of 90.0 mg l^{-1}. High alkalinity (196 mg l^{-1}) was reported from hot water ponds located in the Kullu district of Himachal Pradesh by Kumar et al. (2013). Nitrates concentration in hot water of

Table 3. Morphological and Biochemical characterization of bacterial isolates from hot water ponds located in the Yamunotri landscape.

Characteristics	Bacterial Isolates							
	Bacillus licheniformis	*Bacillus megaterium*	*Streptococcus thermophilus*	*Bacillus subtilis*	*Actinobacillus hominis*	*Paenibacillus alvei*	*Streptococcus pyogenes*	*Geobacillus stearothermophilus*
Shape	Round	Round	Round	Round	Round	Circular	Spherical	Circular
Size	4 mm	3 mm	2 mm	2–3 mm	1 mm	1 mm	1 mm	2–3 mm
Margin	Undulate	Undulate	Entire	Lobate	Regular	Regular	Smooth	Entire
Elevation	Semi-raised	Elevated	Convex	Flat	Flat	Semi-raised	Convex	Convex
Color	White	Pale-white	Cream	White	White	Pale-white	Translucent	Cream
Cell shape	Straight rods	Straight rods	Cocci in chain	Rod	Pleomorphic	Rod	Cocci	Rod
Spore formation	+	+	-	-	-	+	-	+
Motility	Motile	Motile	Non-motile	Motile	Non-motile	Motile	Non-motile	Motile
Grams staining	+	+	+	+	-	+	+	+
Flagella	Peritrichous	Peritrichous	Atrichous	Peritrichous	Atrichous	Peritrichous	Atrichous	Monotrichous
Catalase	+	+	+	+	+	+	-	-
Citrate	-	+	+	+	-	-	+	v
Urease	+	+	+	-	+	+	-	-
Methyl Red (MR)	-	-	-	-	-	-	-	-
Voges Proskauer (VP)	-	-	-	+	-	-	-	-
Fructose	-	+	+	+	-	+	+	-
Indole Test	-	-	-	-	-	-	-	-
Raffinose	-	+	-	+	+	-	-	-
Ribose	+	+	-	+	+	-	-	+
Sorbitol	+	-	-	+	-	+	-	-
Sucrose	+	-	+	+	+	-	+	-
Xylose	-	-	-	+	+	+	-	+
Trehalose	+	-	-	+	-	-	+	-
Mannose	+	-	+	+	-	-	+	-
Mannitol	+	+	-	+	+	-	-	+
Lactose	+	+	+	v	-	-	+	-
Maltose	+	-	-	+	-	-	+	-

Characteristics	Bacterial Isolates							
	Bacillus mycoides	*Bacillus thermoamylovorans*	*Streptomyces* rangoon	*Streptomyces clavifer*	*Lysinibacillus sphaericus*	*Actinobacillus seminis*	*Brevibacillus thermoruber*	*Bacillus simplex*
Shape	Round	Round	Round	Round	Round	Round	Circular	Round
Size	3 mm	2–3 mm	1–3 mm	1–3 mm	3 mm	2–4 mm	2 mm	3 mm
Margin	Rhizoid	Smooth	Entire	Entire	Regular	Entire	Undulate	Regular
Elevation	Raised	Flat	Centrally raised	Centrally raised	Raised	Convex	Convex	Semi-raised
Color	Cream	White	Cream	Sandy yellow	Pale-white	White	Cream	Cream
Cell shape	Rod	Rod	Rod	Rod	Rod	Coco-bacillary	Rod	Rod
Spore formation	+	-	+	+	+	-	+	+
Motility	Non-motile	Motile	Motile	Motile	Motile	Non-motile	Non-motile	Motile
Grams staining	+	+	+	+	+	-	+	+
Flagella	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Atrichous	Peritrichous	Peritrichous
Catalase	+	+	-	-	+	+	+	+
Citrate	v	-	+	+	+	-	-	-
Urease	v	-	-	-	-	-	-	+
Methyl Red	+	-	-	-	-	-	-	-
Voges Proskauer (VP)	+	+	+	+	-	-	+	-
Fructose	v	+	+	+	-	-	+	-
Indole Test	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	+	-
Ribose	v	-	-	-	-	-	-	+
Sorbitol	-	-	+	+	-	-	-	-
Sucrose	v	+	+	+	-	-	+	+
Xylose	-	+	-	-	-	-	-	+
Trehalose	+	+	-	-	-	-	+	-

(*continued on next page*)

Table 3 (*continued*)

Characteristics	Bacterial Isolates							
	Bacillus mycoides	*Bacillus thermoamylovorans*	*Streptomyces* rangoon	*Streptomyces clavifer*	*Lysinibacillus sphaericus*	*Actinobacillus seminis*	*Brevibacillus thermoruber*	*Bacillus simplex*
Mannose	-	+	-	-	-	-	-	-
Mannitol	-	-	+	-	+	-	-	-
Lactose	-	+	-	-	-	-	-	-
Maltose	-	+	-	-	-	-	-	+

Abbreviations: +: positive; −: negative; v: variable.

Table 4. Microbial diversity present in hot water ponds located in the Yamunotri landscape of Garhwal Himalaya.

S. No.	Microorganisms	Surya Kund	Draupadi Kund	Yamunotri Tapt Kund
A. Bacteria				
1	*Bacillus subtilis*	+	+	+
2	*Brevibacillus borstelensis*	-	+	-
3	*Bacillus licheniformis* (AE017333)	+	+	+
4	*Streptococcus thermophilus*	-	-	+
5	*Actinobacillus seminis*	-	-	+
6	*Bacillus mycoides*	+	-	+
7	*Paenibacillus dendritiformis*	+	-	-
8	*Paenibacillus ehimensis*	+	-	-
9	*Bacillus simplex*	+	-	-
10	*Bacillus thermoamylovorans*	+	+	-
11	*Geobacillus stearothermophilus*	+	+	-
12	*Bacillus megaterium*	+	+	-
13	*Paenibacillus alvei*	+	+	-
14	*Actinobacillus hominis*	+	+	+
15	*Bacillus tequilensis* (AYTO01000043)	+	+	-
16	*Brevibacillus thermoruber*	-	+	-
17	*Brevibacillus choshinensis*	-	+	+
18	*Streptococcus pyogenes*	-	+	5
19	*Thiobacillus denitrificans*	-	-	+
20	*Paenibacillus thiominolyticus*	-	-	+
21	*Lysinibacillus sphaericus*	-	-	+
22	*Bacillus paralicheniformis* (LBMN01000156)	-	+	-
B. Actinomycetes				
1	*Streptomyces ruber*	+	+	+
2	*Nocardiopsis prasina*	+	+	+
3	*Streptomyces thermoviolaceus*	+	-	-
4	*Thermobifida fusca*	+	+	+
5	*Streptomyces erumpens*	-	+	+
6	*Streptomyces clavifer*	-	-	+
7	*Streptomyces rangoon*	-	-	+
C. Fungi				
1	*Aspergillus nidulans*	+	-	+
2	*Thermoascus crustaceus*	+	+	-
3	*Thermoascus viride*	+	-	-
4	*Cladosporium cladosprioides*	+	-	-
5	*Cladosporium herbarum*	+	-	-
6	*Penicillium soppii*	+	-	-
7	*Canariomyces thermophila*	-	+	-
8	*Chaetomium thermophile*	-	+	-
9	*Talaromyces thermophilus*	-	+	-
10	*Sporotrichum thermophile*	-	+	-
11	*Aspergillus niger*	-	-	+
12	*Aspergillus flavus*	-	-	+
13	*Aspergillus costaricaensis* (NR_103604.1)	-	-	+
14	*Thermomyces lanuginosus*	-	-	+
Total		**22**	**22**	**21**

Abbreviations: +: present; -: absent.

Draupadi Kund ranged from 0.137 mg.l-1 to 0.142 mg l^{-1}. Sulfates concentration of hot water of Draupadi Kund ranged from 1.201 mg l^{-1} to 1.213 mg l^{-1}. The high concentration of sulfates and nitrates in hot water ponds of Sikkim was reported by Sherpa et al. (2013). Phosphates concentration in hot water of Draupadi Kund was recorded between 0.022 mg l^{-1} to 0.028 mg l^{-1}.

A total of twenty-two species of microorganisms with thirteen species of bacteria, four species of actinomycetes and five species of fungi were isolated and identified from the hot water of Draupadi Kund (Table 4). *Geobacillus stearothermophilus* was also reported by Ghati et al. (2013) from Indian hot spring. Reysenbach et al. (2000) also reported the identification of *Bacillus subtilis* in the Yellowstone National Park.

3.3. Physico-chemical characteristics and microbial diversity of Yamunotri Tapt Kund

The water temperature of Yamunotri Tapt Kund ranged from 45 °C to 48 °C with a mean value of 46.8 °C. The pH of Yamunotri Tapt Kund water ranged from 7.3 to 7.8, showing slightly alkaline nature of water. Singh et al. (2015) reported wide variation (6.9–9.5) in pH of hot water springs located in West Bengal and Jharkhand. Considerable concentration of DO (5.8 mg l^{-1} to 6.2 mg l^{-1}) as recorded in Yamunotri Tapt Kund. Fazlzadeh et al. (2016) reported low concentration (3.25 mg l^{-1} to 3.57 mg l^{-1}) of dissolved oxygen from hot water springs located in Iran; Kumar et al. (2013) also reported low concentration of DO (2.52 mg l^{-1}) from thermal springs of Himachal Pradesh. Electrical conductivity of water of Yamunotri Tapt Kund ranged from 8.19 mS/cm to 8.28 mS/cm. Homma and Tsukahara (2008) also reported the same range of electrical conductivity from the thermal springs of the Itoigawa Shizuoka Tectonic Line. Salinity of water of Yamunotri Tapt Kund ranged from 4.1^{SAL} to 4.5^{SAL}. Total Dissolved Solids (TDS) ranged from 4.51 mg l^{-1} to 4.59 mg l^{-1} in hot water of Yamunotri Tapt Kund. A high concentration of TDS was recorded by Hamzah et al. (2013) in Malaysia. Free CO_2 concentration in Yamunotri Tapt Kund was recorded within a range of 92.4 mg l^{-1} to 96.8 mg l^{-1}. Total hardness of hot water of Yamunotri Tapt Kund ranged from 128 mg l^{-1} to 132 mg l^{-1}. Calcium concentration ranged from 27.2 mg l^{-1} to 31.2 mg l^{-1} with an average of 29.2 mg l^{-1}. Magnesium concentration fluctuated between 12.71 mg l^{-1} to 15.64 mg l^{-1} Singh et al. (2015) reported magnesium concentration from 12.60 mg l^{-1} to 15.62 mg l^{-1} for hot water springs of Jharkhand and West Bengal, India. Chlorides concentration was recorded between 92.3 mg l^{-1} to 96.56 mg l^{-1} in water samples of Yamunotri Tapt Kund. Total alkalinity ranged from 95 mg l^{-1} to 120 mg l^{-1} during the period of study. Nitrates concentration was found within the range of 0.092 mg l^{-1} to 0.098 mg l^{-1}. Sulfates concentration ranged from 0.241 mg l^{-1} to 0.244 mg l^{-1}. Phosphates concentration in hot water of Yamunotri Tapt Kund was recorded from 0.081 mg l^{-1} to 0.090 mg l^{-1}.

An overall, ten species of bacteria, six species of actinomycetes and five species of fungi were isolated and identified from the Yamunotri Tapt Kund. The α-diversity of microbes in hot water of Yamunotri Tapt Kund was recorded to be twenty-one during the study period (Table 4). *Bacillus licheniformis* was also reported by Sharma et al. (2012) in Manikaran hot water spring of Himachal Pradesh. Three species of bacteria (*Bacillus subtilis*, *Bacillus licheniformis*, and *Actinobacillus hominis*) and three species of actinomycetes (*Streptomyces ruber*, *Nocardiopsis prasina*, and *Thermobifida fusca*) were found common from the hot water of all the three hot water springs.

Temperature is considered, as one of the major environmental factors that control the activities and survival of microbes (Kumar and Sharma, 2020; Kumar et al. 2013; Takacs-Vesbach et al. 2008; Abou-Shanab, 2007). Most of the thermophiles belong to the domain of Archaea. *Methanopyrus kandleri* is a thermophilic microorganism that can grow and reproduce at a temperature in excess of 250°F (Vieille and Zeikus, 2001). The other thermophilic microorganisms are *Thermus aquaticus, Thermococcus litoralis*, *Bacillus stearothermophilus*, *etc*. A broad range of thermophilic microorganisms has been isolated from thermal springs, mud pots, geysers, hot spring algal mat, active volcanoes of the world. These thermophilic habitats are the home of a wide range of thermophilic and thermotolerant microbes. Protein stability, DNA stability, Lipid stability are the three main mechanisms that are responsible for the growth and development of thermophiles at such a high temperature.

4. Importance to the society

4.1. Importance of thermal springs

Natural hot water contains Na^+, K^+, Ca^{2+}, S, SO_4^{2-} and Cl^- which are good for balneotherapy. The chemical composition of the hot water of these natural water bodies contributes to the well being and health of the people. It generally increase metabolism, accelerate healing, soothe muscles, improve blood circulation and detoxify the body's lymphatic system (Javed et al., 2009; Hamzah et al., 2013). The collected data from the thermal springs shows that water of these springs is suitable for bathing purposes but not for human consumption. The major benefits of bathing in these hot water bodies are: (i) It enhances the circulation of blood; (ii) it reduces stress and promotes sleep; (iii) gives relief from pain or body ache naturally; (iv) it cures skin ailments like rough and dry skin, *etc*; (v) helps in removal of toxic compounds or elements out of the body; (vii) anti-aging; and (viii) other therapeutic effects such as positive mindset, etc. These possible benefits have been discussed by Joshi (2015).

4.2. Importance of thermophilic microorganisms

Enzymes obtained from heat-loving microorganisms are of paramount importance as they act as biocatalysts for various industrial applications. Such enzymes can work effectively even at high temperatures (Lopez et al., 2013). The bacterial species, *Bacillus subtilis* has been isolated from Surya Kund, Draupadi Kund and Yamunotri Tapt Kund act as an antidiarrhoeal microorganism, which has an ability to treat diarrhea. This species is also used to produce fermented soybeans, yogurt, ice cream, milk and cheese (Earl et al., 2008; Shahcheraghi et al., 2015; Ulrich et al., 2018). The bacterial species *Streptococcus thermophilus* has been isolated from Yamunotri Tapt Kund has a property to boost the immunity of a human body and can also be used in the fermentation of cheese. *Brevibacillus borstelensis* can be used for the degradation of polyethylene and long-chain hydrocarbons. This has also been reported by Hadad et al. (2005) and Khalil et al. (2018). *Geobacillus stearothermophilus* showed significant esterase producing ability, hence it can be used or the production of thermostable esterase (Ghati et al., 2013). *B. licheniformis, B. megaterium, A. hominis, L. sphaericus, P. alvei, B. simplex, A. seminis, P. fragii* and *S. cohnii* belong to class Firmicutes and Gamma proteobacteria and showed the production of amylase, gelatinase, lipase (Pathak and Rathod, 2014).

5. Conclusion

The present study on the physico-chemical characteristics of hot water of Surya Kund, Draupadi Kund and Yamunotri Tapt Kund revealed that the water of all the three hot water ponds is highly alkaline. All the three hot water ponds are the rich sources of thermophilic microbial diversity (bacteria, actinomycetes and fungi). *Bacillus subtilis, Bacillus licheniformis, Actinobacillus hominis, Streptomyces ruber, Nocardiopsis prasina, Thermobifida fusca* are the common microorganisms present in all the three hot water bodies. Almost all bacterial species were gram-positive. The present study can provide baseline data for further studies on similar aspects and also useful in providing characteristics of indigenous thermophilic microbial isolates that produce thermostable enzymes. Keeping in view the importance of these natural hot water bodies and their rich microbial diversity, an attempt should be made by the Government of Uttarakhand for the conservation and management of these high altitude natural hot water bodies.

Declarations

Author contribution statement

Rahul Kumar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ramesh C. Sharma: Conceived and designed the experiments; Analyzed and interpreted the data.

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