BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF CULTIVABLE BACTERIAL DIVERSITY OF SURYAKUND HOT SPRING, YAMUNOTRI, INDIA

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ABSTRACT

The aim of this investigation was to identify the culturable bacterial population of the Surya kund hot spring site in the Garhwal region of the Central Himalaya. In this study, 21 morphologically distinct thermophilic bacteria were isolated and analyzed by biochemical and molecular methods. The phenotypic characters of the isolates were studied using standard methods and for the genotypic character, 16S rRNA gene was sequenced. Most of the colonies grown on tryptone soy agar plates at 55 °C following 24 hr incubation were circular. The isolates were able to grow in wide range of temperatures (35°C –90°C) and pH (5–10). The gram positive bacteria were in abundance. Standard biochemical tests were performed along with the exo-enzyme (protease, amylase, lipase and L-asparaginase) activity and carbohydrate fermentation test. Based on 16S rRNA gene sequence analysis, 16 of the isolates belong to the phylum Proteobacteria, four were Firmicutes (4) and one was related to the CFB group bacteria.

Keyword: *hot spring, 16S rRNA gene, thermophiles, bacterial diversity, phylogenetics.*

Introduction

The geothermal energy is naturally derived from the deep inside the earth's crust and if water comes to the contact with these geothermal energy areas it comes out to the earth's surface as volcanoes, fumaroles, geysers or hot springs. Geothermal springs are excellent source of thermophilic microorganism (Joshi et al., 2011). Bacterial communities can survive in very harsh habitats e.g. extreme temperature, pH, radiation, pressure, salinity, lack of water or oxygen is called extremophiles (Rampelotto, 2013). Bacteria grow on high temperature known as thermophile (50^0C -80^0C) and hyperthermophile (80^0C -120^0C) (Kristjansson, 1991). Bacterial cells have several adaptations to keep their cellular proteins and biocatalysts functional under such extreme conditions (Van Den Burg, 2003). The increasing demand of thermostable biocatalyst in biotechnological processes, research and industrial uses it required to explore more novel habitat for potent bacterial strains to fulfill the demands.

By applying the phenotypic and genotypic characterization methods to study the thermophilic bacteria has been done for many geothermal areas and hot springs found in different parts of the world e.g., North Island, New Zealand (Jones et al., 2000), Iceland (Alfredsson et al., 1998), Joshinetsu, National Park, Japan (Fukuyama et al., 2019), Geyser Valley, Kamchatka (Kiryukhin et al., 2020), El Tatio, Chile (Zhang et al., 2020), The Great Rift, Kenya (Avci et al., 2020), Tuscany, Italy (Nuvolone et al., 2020), Camiguin Island, Philippines (Martinez-Goss et al., 2020) and the bacterial communities have been studied and explored.

In India, there are many hydrothermal sites are well-known, found in different areas, Tattapani in Chhattisgarh (Mittal et al., 2017), Chhumathang and Puga in Ladakh (Gupta et al., 2017), Cambay Graben in Gujarat (Bhandari & Nailwal, 2020), Manikaran in Himachal Pradesh (Pathania et al., 2020), Surajkund in Jharkhand (Prabha & Nigam, 2020), Bakreshwar in West Bengal (Maji et al., 2020), Jakrem in Meghalaya (Panda et al., 2015), Reshi Hot Water Spring in Sikkim (Sherpa et al., 2013), Aravali hot water springs in Maharastra (Kumar et al., 2011), Dhuni Pani in Madhya Pradesh (Rao et al., 2018), and Gaurikund, Tapovan (Kumar & Sharma, 2020) are explored for bacterial diversity by applying various approaches.

Materials and Methods

Description of site

In the Garhwal region of Central Himalayas, the water samples were taken from the

Suryakund hot Spring, located at an altitude of 3,291 meters and geographic coordinates $31^0$1'0.12"N $78^0$27'0"E, Yamunotri Temple, District Uttarkashi, Uttarakhand, India. This temple opens in May and closes in the November. The water samples were collected just before the starting of pilgrimage to keep disturbance minimum at sampling site. The temperature and pH of hot spring water was noted on the spot.

Sampling of water sample, cultivation and enumeration of bacteria

Water sample was collected in sterile plastic vials and kept in the thermos flasks to maintain the temperature of samples during the transportation. The bacterial cultures were obtained by the pour plate methods (Aneja, 2007). Sample was serially diluted with sterile water and 50-100 µl of sample was poured on the Tryptone Soy agar **(**Arya et al., 2015**)** plate with help of glass spreader. The inoculated plates were kept in the incubator 55 ^{0}C for 24-48 hr. The same sample was also observed for anaerobic growth in anaerobic medium. The anaerobic condition was created by using anaerobic chamber and kept in incubator at appropriate temperature for 24-48 hr. The colonies were counted with the help of colony counter and colony forming units were calculated. Each distinctive colony was subsequently streaked on plates to obtain pure culture. The pure culture of isolates was preserved by making glycerol stocks.

The overnight grown bacterial culture was used for the preparation of glycerol stocks to preserve the isolates. The 40% glycerol conc. was taken and preserved at -20^{0}C in deep freezer. The agar slants were prepared as working culture. Every experiment was conducted with fresh culture.

Morphological and Biochemical Characterization of Bacterial Isolates

The appearance and morphological feature of the colonies e.g. texture, color, margin, shape and forms were recorded. Himedia Gram Stain-Kit was used to perform Gram staining to distinguish strains as Gram positive and Gram negative bacteria. The physiological parameters (pH and temperature tolerance) and biochemical tests e.g. extracellular enzymatic activities (protease, amylase, lipase and L-asparaginase), Indole test, MR-VP test, Citrate Utilization test, Catalase test, H_2S Production, Oxidase test and Nitrate reduction test were performed by following the standard procedure described in (Cappucino and Sherman 2007).

Genomic DNA Extraction and PCR Amplification of 16S rRNA Gene

Overnight grown bacterial broth culture was taken to isolate the DNA. Total genomic DNA of each bacterial isolates was extracted by following CTAB method (Wilson, 2001) with some modifications. The quality of DNA was checked with agarose gel electrophoresis on 1% agarose and quantified by the spectrophotometer. PCR amplification of the 16S rRNA gene was done by using two universal primers, forward primer 16S 5'-GGA TGA GCCCGC GGC CTA -3' and reverse Primer 5'-CGG TGT GTACAA GGC CCG G-3' (Rajasekar et al., 2007). Primers were commercially synthesized by Biokart, India Pvt. Ltd. Bangalore. The final volume of PCR reaction mixture was set 100µl by adding the 50–100 ng template DNA, forward and reverse primers (100 ng each), dNTPs (200 µM each), Taq polymerase reaction buffer (10X) 10 µl and 1.0 U Taq polymerase. The PCR tubes containing reaction mixture were place inside the Thermocycler (BIO_RAD, T100) and conditions of PCR amplification steps were fellow: Initial denaturation for 5min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40s and extension at 72 °C for 1min and a final extension at 72 °C for 10min. PCR products were resolved by electrophoresis at 60 V for 1 h in 1.2 % agarose gel in 1X TAE buffer. Gels were then stained with ethidium bromide (10 mg/ml) and visualized on a gel documentation system (Alpha Imager, Alpha Innotech, Santa Clara, CA).

Sequencing of 16S rRNA Gene and Phylogenetic Analysis

The amplified DNA samples were sent to Biokart, Bangalore, India for the 16S rRNA gene sequencing. Instrument and chemistry details were provided by the facilitated company are given here. Sequencing Instrument and Chemistry Details Sequencing

Machine: ABI 3130 Genetic Analyzer Chemistry Cycle sequencing kit: Big Dye Terminator version 3.1". Polymer & Capillary Array: POP_7 pol Capillary Array. Analysis protocol: BDTv3-KB-Denovo_v 5.2

Data Analysis: Seq Scape_v 5.2. Software Reaction Plate: Applied Biosystem Micro Amp Optical 96-Well Reaction plate. The obtained sequences were confirmed by BLAST (Basic Local Alignment Search Tool) (https://www.ncbi.nlm.nih.gov) searching tool and phylogenetic tree was made by using MEGA version10. The sequence results were submitted to the Genebank, NCBI.

Results and Discussion

Temperature and pH of the Site

The temperature at source of spring was recorded 86.5±0.2^{0}C and the pH of water at source was recorded slightly alkaline 7.5±0.2. The same observation of alkaline nature of this hot spring was reported by Ranawat & Rawat, 2017. An another study of two hot springs from Himachal, India, by Kumar et al., 2014, was also reported alkaline with high temperature (85^{0}C -95^{0}C). The temperature of hot spring is remain same throughout the year and not affected by the seasonal variation of the surroundings.

Cultivation and enumeration of bacterial isolates

The bacterial population in terms of CFU, was calculated 4.23 (CFU/ml 10^3) for water sample. Total 21 isolates were finally selected by performing the morphological, physiological and biochemical tests. Some other studies on bacterial population of Suryakund hot spring have been conducted, in one of them 34 isolates were reported but isolates were only characterized by biochemical methods (Ranawat & Rawat, 2017) while in another study molecular data was published but they reported only 12 bacterial isolates (Kumar & Sharma, 2020). No bacterial growth or colony forms were observed in anaerobic condition. Although there were not so much morphological variances seen among the colonies but each slightly distinctive colony was subsequently streaked on plates to obtain pure culture.

Morphological and biochemical features of isolates

All bacterial strains isolated from the water samples were carefully examined to study their morphological and biochemical characteristics. The morphological characteristics and Gram's reaction results of the isolates are summarized in Table 1.

Most of the colonies were small in size with slimy consistency. Most of the colonies were circular with diverse elevation like convex, unmbonate and flat but umbonate feature was more frequent. Colonies with different type of margin like undulate, serrate, and entire were observed. The pigmented colonies had yellowish appearance while non pigmented colonies were white or cream in color with dull appearance. Only 7 isolates (YII-1, YII-14, YII-20, Y1, Y2, Y10, Y13 and Y16) were Gram-negative and rests of the bacterial isolates were Gram-positive. Both types of Gram's positive and negative bacteria was also reported in a Himalayan hot spring, Soldhar India by the Arya et al., 2015 and the bacterial population of Gram's positive was dominating. Isolates were found to grow over a wide pH range 5-10 whereas, temperature range varied in between 35^{0}C-90^{0}C.

Among the 21 isolates, 4 isolate were found able to produce protease enzyme, 8 isolate were capable to produce amylase enzyme and only 2 isolates were lipase enzyme producing. Isolate YII-3 was able to produce protease and lipase and isolate Y7 and Y19 were able to produce protease and amylase enzyme. No isolate were showing L-asparaginase enzyme activity. All other results of biochemical tests are summarized in Table 2. None of the isolates respond to MR-test, H_2S and mobility tests while YII-15, YII-20, Y9-II, Y16 and Y13 were positive for VP-test, all the isolates were unable to utilize citrate as sole source of energy. Most of the isolates were catalase positive except YII-3, YII-20, Y10 and Y8-I. The bacterial isolates from the soils of Himalayan hot springs, Soldhar and Ringigad, India were also found incapable to reduce H_2S (Kumar et al., 2004)

TABLE 1: Colony morphology of the isolates from the water samples of Suryakund, Yamunotri

s/n	Isolates	Size	Form	Elevation	Margin	Pigmentation	consistency	Gram's reaction	Optimum temperature(^{0}C)	pH
1	YII-1	Small	Circular	Convex	Undulate	Yellow	Slimy	Negative	35-85	6-9
2	YII-2	Large	Circular	Umbonate	Serrate	White	Slimy	Positive	35-85	5-9
3	YII-3	Large	Circular	Umbonate	Serrate	Yellow	Slimy	Positive	35-90	6-9
4	YII-14	Small	Circular	Flat	Entire	yellow	Slimy	Negative	35-85	6-9
5	YII-10	Large	Irregular	Convex	Entire	Yellow	Slimy	Positive	30-75	6-10
6	YII-12	Small	Circular	Flat	Entire	White	Slimy	Positive	40-85	5-9
7	YII-16	Small	Circular	Flat	Entire	White	Slimy	Positive	30-85	5-9
8	YII-19	Small	Irregular	Flat	Undulate	Cream	Slimy	Positive	30-85	6-9
9	YII-20	Small	Circular	Convex	Entire	White	Slimy	Negative	35-90	6-9
10	Y1	Small	Circular	Umbonate	Undulate	Cream	Slimy	Negative	35-80	6-10
11	Y2	Small	Irregular	Umbonate	Entire	Yellow	Slimy	Negative	30-80	6-9
12	Y7	Small	Circular	Umbonate	Entire	Yellow	Slimy	Positive	35-85	6-10
13	Y8-I	Large	Circular	Umbonate	Entire	Cream	Slimy	Positive	35-80	6-10
14	Y9-II	Small	Circular	Umbonate	Serrate	White	Slimy	Positive	30-80	5-9
15	Y10	Large	Circular	Umbonate	Entire	Yellow	Slimy	Negative	30-85	6-10
16	Y12-I	Small	Circular	Umbonate	Undulate	White	Slimy	Positive	40-80	6-10
17	Y12-II	Small	Circular	Umbonate	Lobate	Cream	Slimy	Positive	40-80	6-9
18	Y13	Large	Circular	Umbonate	Entire	White	Slimy	Negative	30-85	5-9
19	Y14-I	Small	Circular	Flat	Undulate	Yellow	Slimy	Positive	35-80	6-9
20	Y16	Small	Irregular	Umbonate	Undulate	White	Slimy	Negative	40-80	6-9
21	Y19	Small	Irregular	Flat	Entire	Cream	Slimy	Positive	40-80	6-9

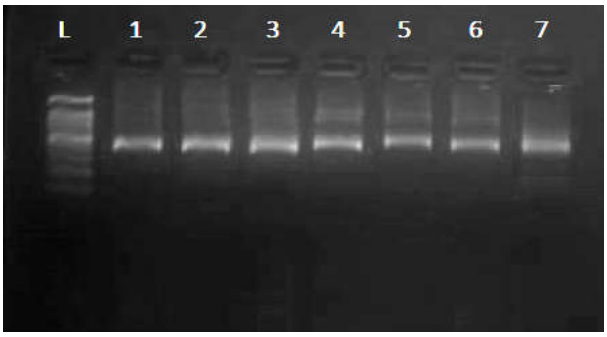
Table 2: Results of biochemical and carbohydrate utilization tests

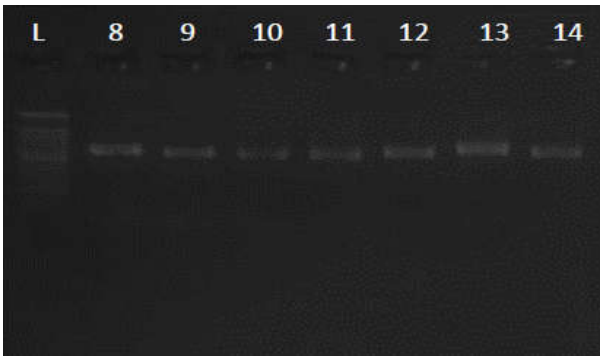
s/n	**Biochemical test**		**YII-3**	**YII-19**	**YII-2**	**YII-10**	**YII-20**	**Y12-II**	**YII-14**	**YII-1**	**YII-16**	**Y9-II**	**Y7**	**Y14-I**	**Y12-II**	**Y10**	**Y8-I**	**Y12-I**	**Y2**	**Y16**	**Y13**	**Y1**	**Y19**
1	Casein hydrolysis		+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+
2	Starch hydrolysis		-	-	+	-	-	+	-	+	-	-	+	+	-	+	-	-	-	+	+	-	+
3	Lipid hydrolysis test		+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	L-asparaginase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Indole production test		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	Methyl red test		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	Voges Proskauer test		-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-
8	Citrate utilization test		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	Catalase test		-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
10	H_2S production test		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	Mobility test		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	**Carbohydrate utilization**	Lactose	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
		Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
		Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Mannose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Fructose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

16S rRNA Gene Sequencing and Phylogenetic Analysis

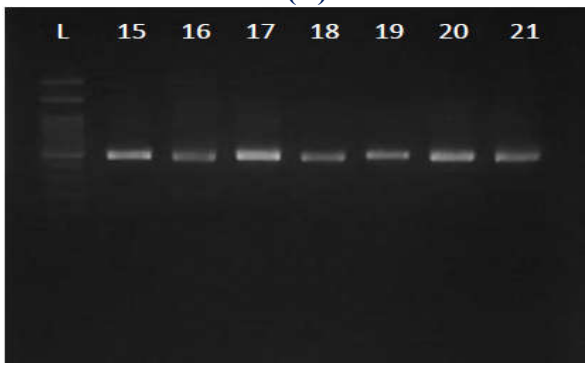
Total genomic DNA was successfully extracted from all the isolates and amplified with the thermo-cycler. The partial 16S rRNA gene sequencing of approximately 1200 bp size was provided from Biokart, Banglore, India Pvt. Ltd. for all 21 isolates. The BLAST results revealed that 16 of the isolates belong to the phylum *Proteobacteria, 4* were *Firmicutes* (4) *and* 1 was related to the CFB group bacteria. At phylum level *Proteobacteria* was most abundant but at genus level *Pseudomonas* and *Acinetobacter* were found dominating. The evolutionary history was inferred using the neighbor-joining method. The 5 isolates, namely, YII-16, Y2, Y9-II, Y12-II and Y16 shared homology (100-97.29%) with genus *Pseudomonas, 2* isolates (YII-20 and Y1) shared homology (98.36-93.31%) with genus *Stenotrophomonas, 5 isolates (*YII-1, YII-3, YII-19, Y7, Y13*)* shared homology (97.99-93.94%) *with* genus *Acinetobacter,* 2 isolates (Y12-I and Y19) shared homology (99.72-99.21%) with genus *Enterobacter*, Y8-I shared homology (96.45%) with genus *Parageobacillus,* Y10 shared homology (99.24%) with genus *Paenibacillus,* YII-14 shared homology (99.57) with genus *Lysinibacillus,* Y14-I shared homology (99.93%) with *Burkholderia,* YII-10 shared homology (99.86%) with *Klebsiella* and YII-12 shared homology(99.81%) with genus *Sphingobacterium.* The isolates Y1, Y7 and Y8-I shows nearest neighbor similarity score of <97% and can be a new species as it is suggested by many sudies (Janda and Sharon, 2007).

(A)





(B)



(C)

Fig1: Representative pictures (A,B&C) of Amplified 16S rRNA gene of isolates (1-21) (Y9II, Y12I, Y12II, Y14II, Y16, Y19, YII1, YII16, YII20, Y1, Y81, Y7, Y13, Y2, YII10, YII12, YII2, YII3, YII10, YII14, YII19). M=500bp (DNA ladder).

Bacterial isolates belongs to genus *Pseudomonas and Burkholderia* were also reported from Manikaran hot water spring of Himachal Pradesh (Sharma et al. 2012) and *Paenibacillus and Lysinibacillus* and in Yamunotri Tapt Kund hot springs (Rahul & Sharma 2020). The members of phylum *Firmicutes* (YII-2, YII-14, Y8-I & Y10) were most dominant after genus *Pseudomonas* among the identified bacteria isolated from thermal springs. The *Firmicutes* in extreme habitats have been previously reported in the Manikaran and Yumthang hot springs (Sahay et al., 2017). Bacteria from the genus *Klebseilla* (YII-10) and *Enterobacter* (Y12-I & Y19) surviving in hot springs were also reported by the Sen & Maiti, (2014) in Odisha, India. *Stenotrophomonas* was reported in Manikaran hot springs by Sharma et al., 2018.

Table 4: GenBank accession numbers and the BLAST analysis for the nearest neighbor search results for bacterial isolates from the hot spring water samples

s/n	Isolate	Accession No.	Nearest phylogenetic neighbor	16SrRNA similarity (%)	Phylum
1.	**YII-1**	MZ330689	*Acinetobacter sp.* strain LSN1-10	99.36	Proteobacteria
2.	**YII-2**	MW946987	*Caryophanon sp.* TP-Snow-C57	90.06	Firmicutes
3.	**YII-3**	MZ047313	*Acinetobacter vivianii* strain BC3402	97.99	Proteobacteria
4.	**YII-14**	MZ054237	*Lysinibacillus macroides* strain IAE195	99.57	Firmicutes
5.	**YII-10**	MZ052095	*Klebsiella pneumoniae* strain E16KP0102	99.86	Proteobacteria
6.	**YII-12**	MW946986	*Sphingobacterium thalpophilum* strain S8SF4	99.81	CFB group bacteria
7.	**YII-16**	MZ330857	*Pseudomonas aeruginosa* strain MLTBM2	100	Proteobacteria
8.	**YII-19**	MZ092845	*Acinetobacter sp.* strain L161	97.79	Proteobacteria
9.	**YII-20**	MZ330858	*Stenotrophomonas maltophilia* strain PEG-390	98.36%	Proteobacteria
10.	**Y1**	MZ156961	*Stenotrophomonas sp.* BAB-5640	93.31	Proteobacteria
11.	**Y2**	MW899049	*Pseudomonas aeruginosa* strain MLTBM2	99.68%	Proteobacteria
12.	**Y7**	MZ156962	*Acinetobacter sp.* strain S2-L	93.74	Proteobacteria
13.	**Y8-I**	MZ164963	*Parageobacillus toebii* NBRC 107807 strain DSM 14590	96.45	Firmicutes
14.	**Y9-II**	MZ164970	*Pseudomonas punonensis* strain LMT03	97.29	Proteobacteria
15.	**Y10**	MW917243	*Paenibacillus thiaminolyticus* strain NBRC 15656	99.24	Firmicutes
16.	**Y12-I**	MZ164971	*Enterobacter bugandensis* strain RH-13	99.21	Proteobacteria
17.	**Y12-II**	MZ164972	*Pseudomonas alloputida* strain BG-3	99.44	Proteobacteria
18.	**Y13**	MZ164967	*Acinetobacter vivianii* strain BC3402	97.80	Proteobacteria
19.	**Y14-I**	MZ318714	*Burkholderia tropica* strain CATux-138	99.93	Proteobacteria
20.	**Y16**	MZ319379	*Pseudomonas aeruginosa* strain PP	99.78	Proteobacteria
21.	**Y19**	MZ330682	*Enterobacter hormaechei* strain A26	99.72	Proteobacteria

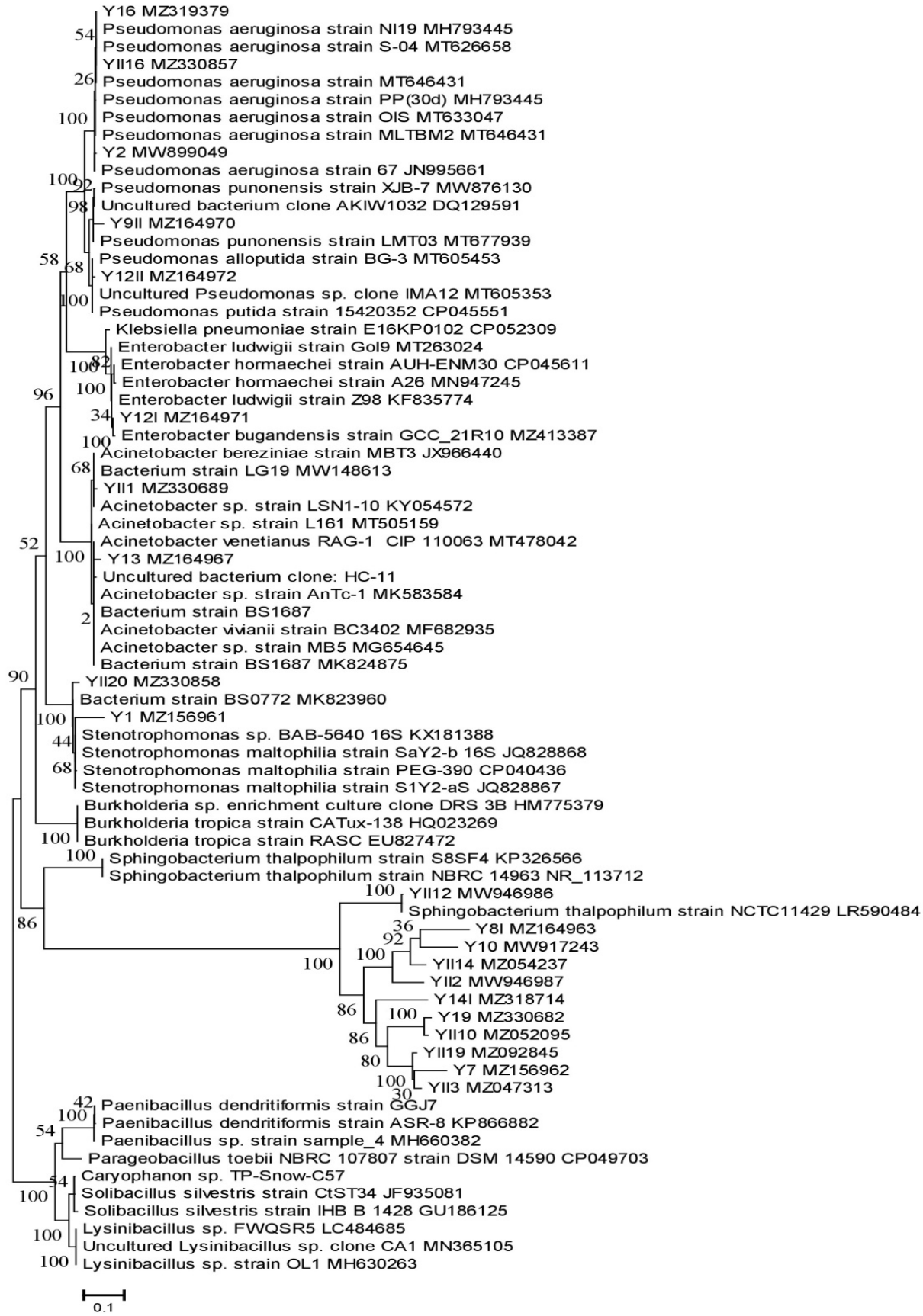


Figure 2. Unrooted phylogenetic trees based on comparison of 16S rDNA sequences of 211 isolates along with their closest phylogenetic relatives by the using Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992).

The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 72 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1988 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al, 2013).

Conclusion

In the present study, total 21 different bacterial isolates were identified and characterized from the alkaline hot spring water of Suryakund based on biochemical and molecular analysis. The isolates belonged to the phylum of *Proteobacteria*, *Firmicutes* and *CFB* group and genus *Pseudomonas* and *Acinetobacter* were most abundant. This hot spring is a rich source of thermophilic bacteria, majority of them were gram positive. Through the BLAST results it was found that isolates Y8-I, Y7 *and* Y1 were showing homology score <97% and they could be new strains. Although the protease and lipase enzyme producing isolates were few but several were capable to produce amylase enzyme. These enzyme producing bacteria can be further studied and can be useful to fulfill the increasing demands of thermophilic enzymes. Such type of unique habitats should be conserved because of peculiar bacterial population that might serve novel activities and applications.

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