International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **8(2),** 6-14, February **(2019)** *Int. Res. J. Biological Sci.*

Seasonal variation in bacterial diversity of Tuva Timba thermal springs of Gujarat, India

Disha N. Patel[1,2*], Shailesh R. Dave[2], Vincent J. Braganza[2] and Hasmukh A. Modi[1]
[1]Department of Life Sciences, Gujarat University, Ahmedabad, India
[2]Xavier Research Foundation, Loyola Centre for Research & Development, Ahmedabad, India
dishu.patel85@gmail.com

Available online at: www.isca.in, www.isca.me
Received 9th October 2018, revised 15th January 2019, accepted 5th February 2019

Abstract

The seasonal bacterial diversity study of thermophilic isolated from TuvaTimba hot springs in terms of morphological, physicochemical, biochemical tests and molecular characterization. A total of 48 isolates were cultivated using various media. Out of these isolates 25 were Gram-positive, 19 Gram-negative and 4 were Gram variable. Based on the metabolic activity of these isolates, 29 could produce catalase, 45, 47 and 24 utilized casein, starch, and citrate respectively. 22 produced H_2S by sulphate reduction. Sugar utilization varied in all the isolates. Diversity indices such as Simpson, Shanon, Menhinick and Margalef index were also studied based on their metabolic activity. 25 well isolated purified colonies were selected for molecular analysis. Based on this study the identified isolates belonged to genera Bacillus, Brevibacillus, Geobacillus, Anoxybacillus and Brevibacterium. All these isolates may have potential biotechnological and industrial application.

Keywords: Thermophilic bacteria, Hot springs, Physicochemical, Metabolic activity, Diversity indices, 16S rRNA sequencing.

Introduction

Microorganisms can survive under extreme environments in different ecosystems. Hot springs are a major source of thermotolerant and thermophilic bacteria. Their optimum growth temperatures are between 45°C to 75°C[1,2]. Survival, as well as growth rate of microorganisms, depends on the temperature and based on this they are divided into different categories like psychrophiles, mesophiles, thermophiles and hyperthermophiles[3,4]. The main purpose of the study of hot springs resources is to find out diversity of the microorganism and also the molecular substantiality of the active components of the ecosystem[4-6]. The phenotypic and genotypic characterization studies have been done on thermophilic bacteria from hot springs in the world at large as well as in India[7-9]. Thermophilic bacteria have the capacity to produce thermostable and thermo-active enzymes. These enzymes have various applications and they have been used in many industries like paper, textiles, foods, fermentation, pharmaceuticals and detergents[10-12]. Conventional microbiological methods have been used for classification of microorganisms and depend for their characterization based on morphology, physiology and metabolic activity[13]. Molecular-based techniques have been used for the phylogenetic relationship between the different types of bacteria from various sources[14-16].

Several valuable reports have been available based on study on Yellowstone National Park in the USA[17,18], Greece[19], Italy[20], Iceland[21], Bulgaria[22,23], China[24], India[7] and Turkey[25] and these studies have attracted the attention of researchers[3]. Studies on Tuva Timba, Lasundra, Tulsishyam and Unai have been reported from the geothermal region of Gujarat. In this context thermophilic bacteria were isolated in different season from TuvaTimba hot spring and characterized. The study was carried out by conventional isolation based methods and molecular identifications.

Materials and methods

Sample collection: Tuva Timba location hot location spring was selected from Gujarat. The water as well as soil samples were collected in sterile glass container. Samples were transported in a thermocol container to our laboratory and processed immediately for experiments and then stored at room temperature. The samples were collected in three different seasons: monsoon, winter and summer.

Physicochemical analysis: pH and temperature were recorded at the time of sampling. Also conductivity, TDS and salt of the collected samples were measured.

Isolation of bacteria: Serial dilution were prepared for collected water and soil samples and they were inoculated in Nutrient agar medium (gm/litre) (Peptone 5.0, Beef extract 3.0, NaCl 5.0, Agar 20.0), Luria-Bertani medium (Tryptone 10.0, NaCl 10.0, Yeast extract 5.0 , Agar 20.0), ATCC Thermus medium (NaCl 5.0, Peptone 5.0, Beef extract 4.0, Yeast extract 2.0, Agar 20.0), Starkey`s sulphate reducing medium (Himedia

M1981), Sulphate reducing medium (Himedia M803) and Medium77 medium (K_2HPO_4 5.0 , NH_4Cl 10.0, $CaCl_2.2H_2O$ 1.0, $MgSO_4.7H_2O$ 1.0, Sodium lactate 50.0, Yeast extract 10.0, $FeSO_4.7H_2O$ 50.0, Sodium thioglycolate 10.0, Ascorbic acid 10.0, Agar 20.0). All the plates were incubated at 45±2°C and 50 ± 2°C. After the growth, morphologically distinct colonies were selected and sub-cultured on respective media to get pure isolates. Then from these well isolated purified colonies, 20% glycerol stocks of bacterial cultures were prepared for preservation and further study on the isolates.

The conventional method for identification and characterization of the isolates: The selected isolates were studied for morphological and growth characteristics. The bacterial isolates were characterized by Gram staining and observed in light microscopy. The thermophilic isolates were identified by conventional method, physiological tests and characteristics based on biochemical tests. These tests are based on Gram nature, shape, temperature, pH, catalase production test, casein utilization test, starch utilization test, citrate utilization test, Sugars profile like D-Glucose, D-Fructose, Maltose, Mannose, Sucrose, Mannitol, Lactose, Xylose, Galactose, H_2S production test and Sulphate reduction test as per the Bergy's manual.

Diversity indices: A range of diversity indices profile, cluster analysis and similarity in diversity were generated using statistical software Paleontological Statistics (PAST) considering the results of biochemical tests[26].

Isolation of Genomic DNA: Genomic DNA was extracted using the miniprep method described by Wilson with some modifications[27,28]. Bacterial cultures (2-5ml) were grown at 45±2°C and 50±2°C for 24 hours. Overnight cultures (2.0ml) were centrifuged at 10,000–15,000Xg for 15 minutes. The supernatant was decanted. The pellet was collected and re-suspended in 1 ml of TE buffer. Then 20µl lysozyme was added and mixed thoroughly by inverting the Eppendorf tubes several times. These treated samples were incubated at 37°C for 30 minutes. To this, 60µl of 10% SDS and 5µl Proteinase K (10mg/ml) were added. Samples were mixed thoroughly and incubated at 65°C for 10 minutes. Then, 170µl of 5M NaCl and 85µl CTAB solution were added and mixed thoroughly and again incubated at 65°C for 10 minutes. In this 1 ml solution of Phenol:Chloroform:isoamyl alcohol in the ratio of 25:24:1 was added. Then it was centrifuged (10,000Xg, 10 minute). Again this step was repeated once. Upper phase was separated into 2.0 ml Eppendorf tube and extracted with 1 volume of Chloroform:isoamyl alcohol (24:1) solution. Then it was centrifuged (10,000Xg, 10minute). The nucleic acids containing upper phase was again transferred to a 2.0ml Eppendorf tube. 700 µl of upper phase was taken and 70µl Sodium acetate was added and double volume of Alcohol was incubated at -20°C overnight. Samples were centrifuged at 10,000Xg for 10 minutes. Upper phase was discarded and 70% of 200µl of ethanol was added and it was centrifuged at 10,000Xg for 10 minutes. Again upper phase was discarded. Pellets were air dried. Pellets were re-suspended into 50µl of elution buffer and kept for 2 minutes in a dry bath. DNA was re-suspended completely at 37°C. The DNA was quantified by NanoDrop spectrophotometer by taking absorbance at 260 and 280 nm (Nucleic acid software). The DNA size was measured by agarose gel (0.5% W/V) electrophoresis by Gel.Doc.System. (Gene snap software).

Results and discussion

Some studies have been done on Tuva Timba, Tulsi – Shyam, Unai and Lasundra hot springs[29-32]. Tuva Timba was selected for the present study, for seasonal variation with respect to thermotolerant organism isolates from this ecosystem.

Physicochemical characteristics: Physicochemical characteristics of water and soil samples of Tuva Timba hot spring are listed in Table-1. The soil sample collected from Tuva Timba was blackish in colour which may be due to the presence of Sulphur (S) or Sulphate (SO_4) coming from the continuous flow of hot water from the central spring. The temperature of the soil was about 55°C which was equivalent to the hot spring. In spite of 55°C temperature of the central springs, the inner wall of spring showed a luxurious growth of green algae. The temperature of the water was 60°C to 65°C in Tuva Timba. The TDS (Total Dissolved Salt) value varied from 2.16 to 2.25 ppt (parts per thousand) in Tuva Timba. Observed pH value was 7.25 to 7.9 in Tuva Timba at sampling point. Conductivity was 3.05 to 3.29mS (milli-Siemens) Salt concentration range observed was 1.62 to 1.69 ppt (parts per thousand). Significantly differences were not found in seasonal physicochemical variation in Tuva Timba. Overall difference was not observed in conductivity, TDS and salt concentration. Some studies have reported that due to high – temperature in geothermal region, seasonal variation in microbial community is less observed as compared to any other environments. Also, the physicochemical parameters can vary due to seasonal effects in some hot springs, while in other studies they have observed that physicochemical characteristics can be stable with no significant changes in seasons[33-36].

Table-1: Season-wise physicochemical characteristics observed in samples from TuvaTimba.

TuvaTimba	Summer	Monsoon	Winter
Temperature	65°C	60°C	60°C
pH	7.9	7.5	7.25
Conductivity	3.29ms	3.05ms	3.29ms
TDS	2.24 ppt	2.16 ppt	2.25 ppt
Salt	1.69 ppt	1.62 ppt	1.67 ppt

Microbial analysis: At total of 48 isolates were cultivated and purified using Nutrient agar medium, Luria-Bertani medium, ATCC Thermus medium, Starky`s medium, Sulphate reducing medium and Medium77 medium, from TuvaTimba hot spring sample collected during summer, monsoon and winter season. The colonies found were round, irregular shaped, pigmented and non-pigmented with uniform margin. The texture of the colonies was smooth, dry and sticky. Most of them were opaque, shiny or translucent. The different shapes were observed like short, long rods, thread, curved and chain forms. Out of these isolates; 25 isolates were Gram-positive, 19 were Gram-negative and the remaining 4 were Gram variable. A wide range of pigmented colonies were observed. Pigmented colonies of 7 different colours were observed, among these there were13 cream coloured, 16 brown, 6 black, 5 yellow, 4 white, 1 red and 1 grey coloured. Geographically distinct microbial communities were observed in the studied isolates. Recently geographical hot springs reports of culture-based study of thermophilic bacteria, have explored their potential applications in various biotechnology fields[37-44].

Metabolic activity based on biochemical tests: A variety of metabolic activities were performed to have a broad view of the characterization of bacteria and also to identify them. Out of 48 isolates tested, catalase production, was utilized by 29 isolates. 45, 47 and 24 isolates were able to utilize casein, starch and citrate respectively. 22 isolates indicated positive results of H_2S production and sulphate reduction. Sugar derivatives activity varied in all the isolates.

The overall results are (shown in the graph) among the bacterial isolates and biochemical parameters. The results of the Tuva Timba water samples are shown in Figure-1. Utilization of catalase was overall dominant in all the three seasons. The highest number of casein utilizing isolates was recorded during monsoon as compared to summer and winter. During monsoon all isolates utilized starch but in winter and monsoon, it was indicated in some of the isolates. On the other hand, it was observed that citrate utilization was significantly less in winter isolates as compared to summer and monsoon seasons isolates. Fructose utilization (Sugar utilization profile) indicates an equal distribution of positive results in all three seasons isolates. Xylose was present in only one isolate of the summer season while in the monsoon season it was present in many isolates as compared to winter isolates. H_2S production and sulphate reduction tests were dominant in all the three seasons.

The results of TuvaTimba soil samples are shown in Figure-2. During the monsoon and winter seasons isolates catalase utilization were dominant, while in summer season it was observed significantly less. Casein and starch utilization were observed positive in all three seasons isolates. Citrate utilization was positively observed in 50% isolates of winter and summer seasons, while it was less observed in monsoon isolates. Sugar profile (eg: Galactose, Fructose, Maltose and Glucose) were indicated positive in all isolates of the summer season. Similarly Galactose, Fructose and Maltose sugar derivatives showed positive results in the winter season isolates. When total isolates of all the three seasons were considered almost 50% isolates showed positive results.

Cluster analysis and diversity indices based on metabolic activity: Diversity study of various isolates was carried out in terms of 15 biochemical tests on the basis of their results Dendograms are prepared, and are shown in Figure-3(a) and Figure-3(b). The blue colour label indicates summer season, green colour indicates the monsoon season and pink colour winter season. All the isolates obtained from water samples collected from TuvaTimba were divided in 2 major group as A and B. (Figure-3(a)). A group was divided into 2 clusters A1 and A2. B group represented sulphate reducing isolates in all three seasons and showed significant sulphate reduction as per their metabolic activity results. All the isolates obtained from soil samples collected from TuvaTimba were divided in 2 major group as A and B. (Figure-3(b)) but only, A group showed 2 clusters A1 and A2. Again A1 showed 2 tight clusters A1.1 and A1.2. Similarly, A2 showed 2 groups A2.1 and A2.2. The A2.1 group showed clear-cut clusters of sulphate reducing isolates in all three seasons based on their biochemical activity.

Morphologically distinct 48 isolates from TuvaTimba thermal ecosystem were studied to determine their similarity. Irrespective of the seasonal collection distinct isolates range from 6 to 9 in each sample. (Data not shown). Among the different isolates studied the maximum similarity between any 2 isolates was 86 to 87 %. Except TS 26 – TS 28 they showed 100% similarity and they were identified as a *Bacillus subtillis.*

Diversity indices data in terms of Simpson, Shanon, Menhinick and Margalef were also studied for all the 6 samples collected in the different seasons from TuvaTimba hot spring ecosystem (Table-2). There are earlier reports about Simpson index, salt concentration and seasonal variations affecting the microbial diversity in studies done in Southern Brazil[45,46].

Molecular identification of selected organisms: From the 48 isolates 29 colonies were selected and identified based on molecular method and results are shown in Table-3. Among the 25 identified cultures 15 belong to *Bacillus* genus, 3 to *Brevibacillus,* 2 to *Geobacillus*, 2 to *Anoxybacillus*, 1 to *Brevibacterium* and 2 show similarity with uncultured organisms. Details of the NCBI identification numbers are given in Table-3.

Earlier reported *Bacillus* species produced metabolites like extracellular enzymes, biosurfactants, biopesticides, biopolymers and also a variety of eco-friendly products[47]. Mackenzie et al. studied seasonal variation of bacterial diversity in three geothermal springs of Patagonia (Argentina). Among the three hot springs only one hot spring was observed with variation in temperature and bacterial diversity[34]. Biosurfactant producing Bacilli spp. were also reported from Lasundra and

Tuva Timba hot water spring[29]. Lipase producing *Bacillus subtillis* and *Bacillus licheniformis* was found in Lasundra, which is also supported by this study[32]. *Bacillus licheniformis* was found in Unai hot spring which have importance of organic solvent-tolerance, thermostability, alkali tolerance and detergent stability[31]. Species of *Anoxybacillus, Geobacillus* and *Bacillus* are present in Tulsi Shyam hot springs and also Himalayan hot spring and are also useful for potential biotechnological application using their biochemical properties[46,44]. After the identification and description of *Brevibacillus*, members of the genus have been found widely distributed throughout the biosphere such as hot springs[48], Soil[49], Sediments[50]. *Brevibacillus halotolerans* organisms have the ability to tolerate high level of salt concentration[51]. Aanniz et al., in 2015, first time reported that the presence of *Bacillus tequilensis* as a thermophilic organisms based on their study of Moroccan hot springs[52]. Thermophilic *Brevibacillus brostelensis* is reported for its capable of degrading polyethelene at 50-60°C[53]. An earlier study showed that Enterobacter sp, Pseudomonas sp. and Bacillus sp. were used in Biodesulfurization[54,55]. Bacillus licheniformis also showed H_2S production[31].

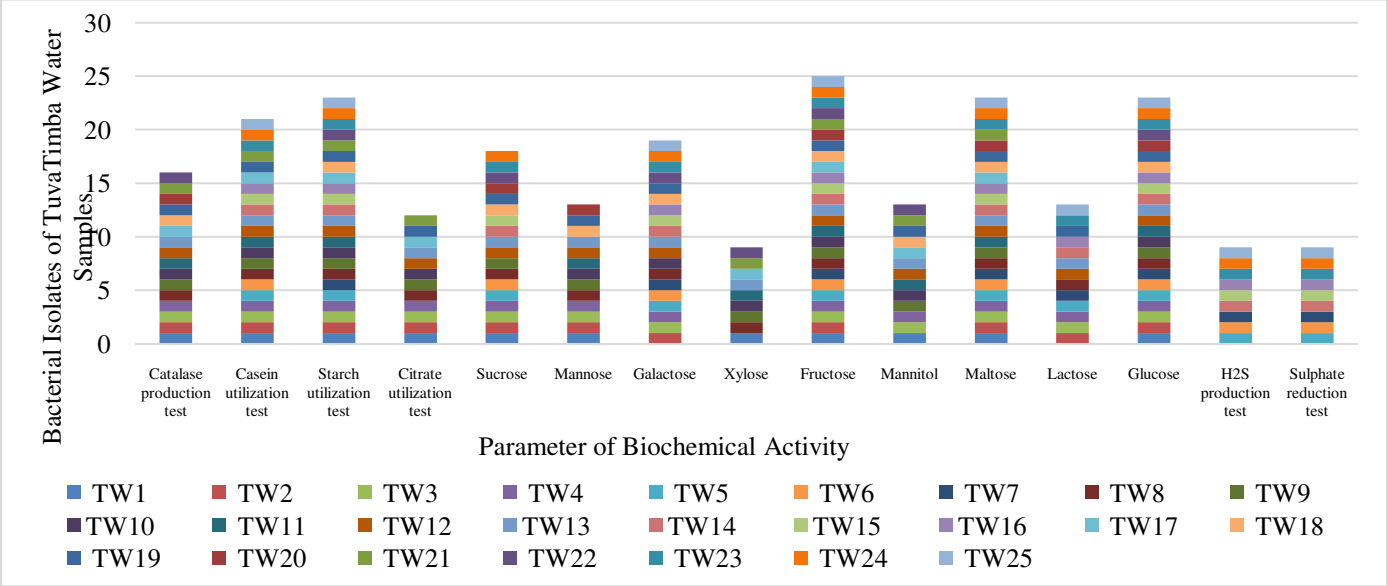


Figure-1: Comparison of metabolic activities of the isolated bacteria from Tuva Timba hot spring water samples [collected during summer (TW1- TW7), winter (TW8-TW16) and monsoon (TW17-TW25) season]. TW1 to TW25 are the bacterial isolates.

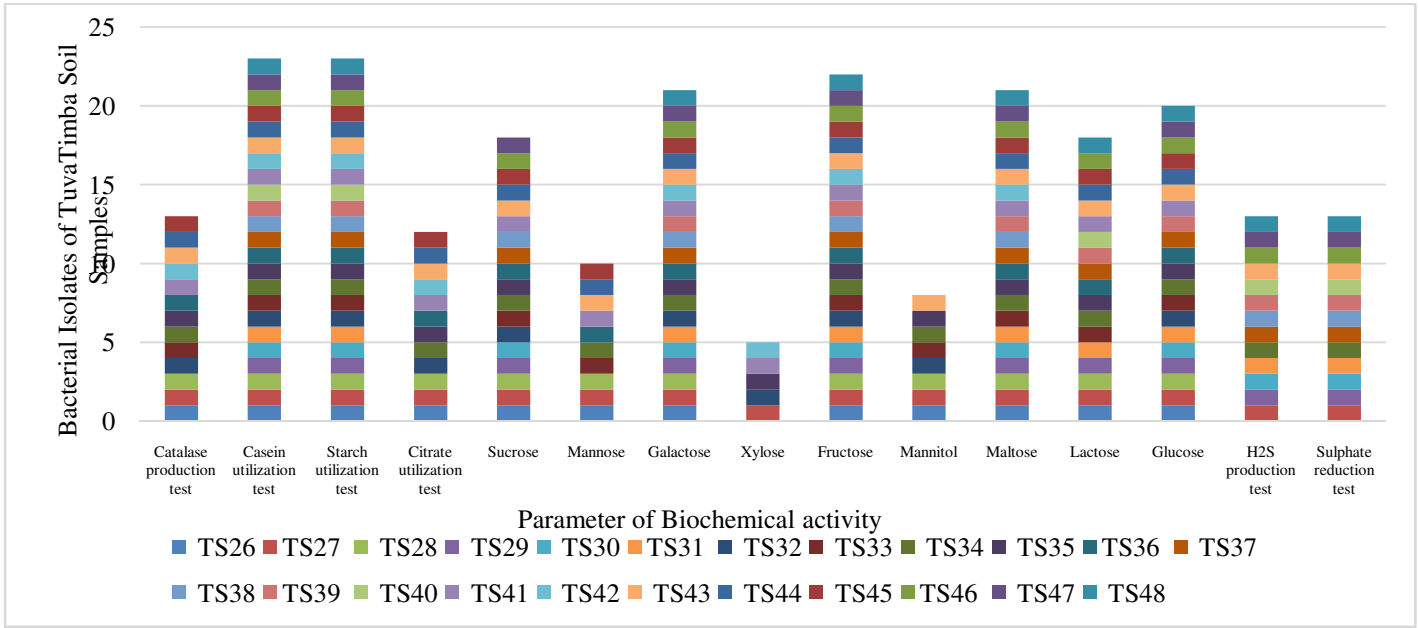
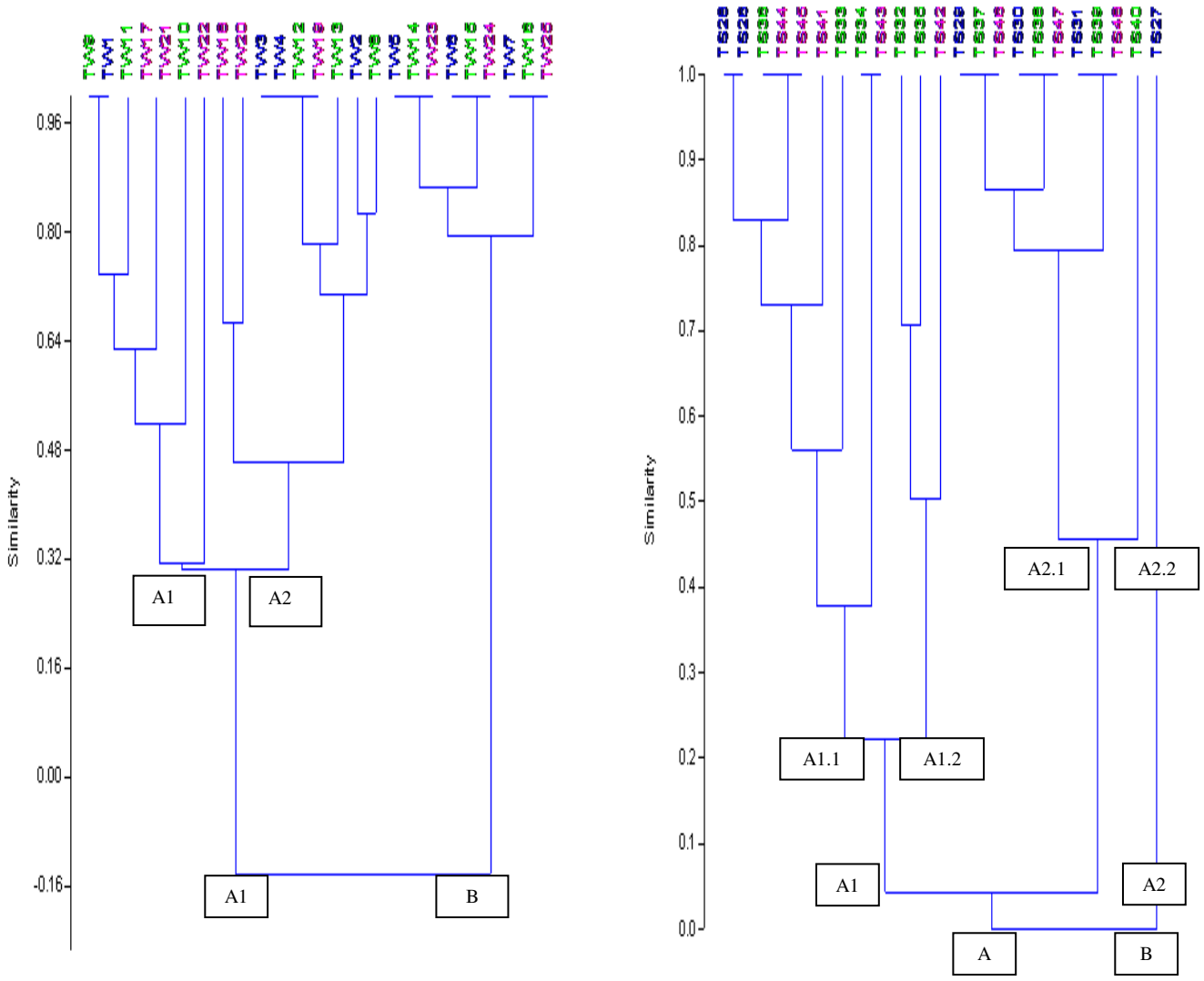


Figure-2: Comparison of metabolic activities of the isolated bacteria from Tuva Timba hot spring soil samples [collected during summer (TS26-TS31), winter (TS32-TS40) and monsoon (TS41-TS48) season]. TS26 to TS48 are bacterial isolates.



(a) TuvaTimba water seasonal cluster analysis. (b) TuvaTimba soil seasonal cluster analysis.

Figure-3: Cluster analysis of all seasonal samples. [Water samples collected during summer (TW1- TW7), winter (TW8-TW16) and monsoon (TW17-TW25) season]. [collected during summer (TS26-TS31), winter (TS32-TS40) and monsoon (TS41-TS48) season]. [Seasons colour indicated (Summer – Blue colour, Monsoon – Green colour, Winter – Pink colour)

Table-2: Comparison of selected diversity indices of isolates from both ecosystem during sampling season.

Index	Tuva Timba					
	Water			Soil		
	Summer	Monsoon	Winter	Summer	Monsoon	Winter
Simpson	0.8– 0.91	0.92	0.92	0.92 - 0.93	0.92	0.92
Shannon	2.1 – 2.4	2.6	2.6	2.6 – 2.7	2.6	2.6
Menhinick	3.0 – 3.4	3.2 – 3.5	3.0 – 3.5	3.2 – 3.8	3.0 – 3.7	3.1- 3.7
Margalef	3.6 – 4.4	4.5 – 4.8	4.4 – 4.8	4.5 – 5.1	3.0 – 3.7	3.1 – 3.7

Table-3: Detail of NCBI identification.

Sequence number	Isolates name	Accession number	Isolates number
XRF01	*Bacillus mojavensis*	MH220879	TW1
XRF02	*Bacillus halotolerans*	MH220880	TW10
XRF03	*Bacillus paralicheniformis*	MH220881	TS33
XRF04	*Brevibacillus brostelensis*	MH220882	TW8
XRF05	*Uncultured oceanibaculum*	MH220883	TS40
XRF06	*Bacillus subtillis*	MH426307	TS28
XRF07	*Bacillus licheniformis*	MH426308	TW2
XRF08	*Bacillus sonorensis*	MH426309	TW9
XRF09	*Geobacillus thermoleovorans*	MH426310	TW11
XRF10	*Anoxybacillus gonensis*	MH426311	TW22
XRF11	*Bacillus tequilensis*	MH426312	TW13
XRF12	*Bacillus subtillis subsp spizizenii*	MH426313	TS26
XRF13	*Anoxybacillus salavatliensis*	MH426314	TW18
XRF15	*Uncultured staphylococcaceae bacterium*	MH426316	TW20
XRF16	*Brevibacterium species*	MH426317	TS42
XRF17	*Bacillus atrophaeus*	MH426318	TS32
XRF19	*Brevibacillus thermoruber*	MH426320	TW21
XRF20	*Geobacillus stearothermophilus*	MH426321	TS35
XRF22	*Brevibacillus brostelensis*	MH426323	TS41
XRF25	*Bacillus licheniformis*	MH426326	TS44
XRF26	*Bacillus subtillis*	MH614328	TW19
XRF27	*Bacillus subtillis subsp spizizenii*	MH614329	TW4
XRF28	*Bacillus subtillis*	MH614330	TS28
XRF29	*Bacillus licheniformis*	MH614331	TS36
XRF30	*Bacillus licheniformis*	MH614332	TS45

Conclusion

The focus of this preliminary study is comparison of seasonal bacterial diversity of Tuva Timba hot spring, as it is not reported with respect to soil samples of Gujarat hot springs. Our investigation therefore focused on the elucidation of seasonal diversity to get insights into novel species or metabolic diversity. Based on the bacterial identification study, almost

negligible difference was found in the diversity due to seasons. But when water and soil sample cluster analysis was performed the number of groups found in soil samples as compared to water was more. Based on morphological and biochemical studies, both in water and soil samples the isolates obtained formed two major groups, as sulphate reducer and sulphate non-reducer. However in case of soil samples A2.2 and B group isolate formed separate groups. Sulphate reducer isolates were studied further. They form a separate group.

Acknowledgement

Authors are thankful to "Xavier Research Foundation" for supporting this research through an internal grant.

References

1. Adiguzel A., Ozkan H., Baris O., Inan K., Gulluce M. and Sahin F. (2009). Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *Journal of microbiological methods*, 79(3), 321-328. http://dx.doi.org/10.1016/j.mimet.2009.09.026.

2. Khuntia H.K., Suryakanta S., Sahoo R.K., Kar S.K., Pal B.B. and Bihari K.M. (2010). Isolation and characterization of thermophilic bacteria from hot spring in Orissa, India. *Biosciences, Biotechnology Research Asia*, 7(1), 449-451.

3. Krıstjonsson J.K. and Stetter K.O. (1991). Thermophilic Bacteria Thermophilic Bacteria (Ed: Kristjonsson JK) CRC Pres. Inc. London, 1-13.

4. Baltaci M.O., Genc B., Arslan S., Adiguzel G. and Adiguzel A. (2017). Isolation and characterization of thermophilic bacteria from geothermal areas in Turkey and preliminary research on biotechnologically important enzyme production. *Geomicrobiology journal*, 34(1), 53-62. DOI: 10.1080/01490451.1137662.2015.

5. Adiguzel A., Inan K., Sahin F., Ozbek T., Gulluce M., Belduz A.O. and Baris O. (2011). Molecular Diversity of Thermophilic Bacteria Isolated From Erzurum City Pasinler Hot Spring (Erzurum/Turkey). *Turkish Journal of Biology,* 35(3), 267-274.

6. Genc B., Nadaroglu H., Adiguzel A. and Baltaci O. (2015). Purification and characterization of an extracellular Cellulase from Anoxybacillus gonensis O9 isolated from geothermal area in Turkey. *Journal of Environmental Biology,* 36(6), 1319-1324.

7. Sharma A., Pandey A., Shouche Y.S., Kumar B. and Kulkarni G. (2009). Characterization and identification of Geobacillus spp. isolated from Soldhar hot spring site of Garhwal Himalaya, India. *Journal of basic microbiology*, 49(2), 187-194.

8. Rath C.C. and Subramanyam V.R.J. (1996). *Microbes.* 86, 157-161.

9. Panda S.K., Jyoti V., Bhadra B., Nayak K.C., Shivaji S., Rainey F.A. and Das S.K. (2009). Thiomonas bhubaneswarensis sp. nov., an obligately mixotrophic, moderately thermophilic, thiosulfate-oxidizing bacterium. *International journal of systematic and evolutionary microbiology*, 59(9), 2171-2175.

10. Fooladi J. and Sajjadian A. (2009). Screening the thermophilc and hyperthermophilic bacterial population of three Iranian hot springs to detect thermostable amylase producing bacteria. *Iranian Journal of Microbiology*, 2(1), 40-53.

11. Mageswari A., Subramanian P., Chandrasekaran S., Sivashanmugam K., Babu S. and Gothandam K. M. (2012). Optimization and immobilization of amylase obtained from halotolerant bacteria isolated from solar salterns. *Journal of Genetic Engineering and Biotechnology*, 10(2), 201-208. http://dx.doi.org/10.1016/j.jgeb.2012.09.001.

12. Indriati G. and Megahati R.R.P. (2018). Isolation of Thermophilic Bacteria and Optimizing the Medium Growth Conditions. *Int. J. Curr. Microbiol. App. Sci*, 7(1), 1457-1464.

13. Scow K.M., Schwartz E., Johnson M.J. and Macalady J.L. (2001). Microbial biodiversity, measurement of. Levin. SA, Editor, 177-190.

14. Cai H., Archambault M. and Prescott J.F. (2003). 16S Ribosomal RNA Sequence-Based Identification of Veterinary Clinical Bacteria. *Journal of Veterinary Diagnostic Investigation,* 15(5), 465-469.

15. Clarridge J.E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews*, 17(4), 840-862.

16. Mignard S. and Flandrois J.P. (2006). 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. *Journal of microbiological methods*, 67(3), 574-581.

17. Miller S.R., Purugganan M.D. and Curtis S.E. (2006). Molecular population genetics and phenotypic diversification of two populations of the thermophilic cyanobacterium Mastigocladus laminosus. *Applied and environmental microbiology*, 72(4), 2793-2800.

18. Boomer S.M., Noll K.L., Geesey G.G. and Dutton B.E. (2009). Formation of multilayered photosynthetic biofilms in an alkaline thermal spring in Yellowstone National Park, Wyoming. *Applied and environmental microbiology*, 75(8), 2464-2475.

19. Sievert S.M., Ziebis W., Kuever J. and Sahm K. (2000). Relative abundance of Archaea and Bacteria along a thermal gradient of a shallow-water hydrothermal vent quantified by rRNA slot-blot hybridization. *Microbiology*, 146(6), 1287-1293.

20. Maugeri T.L., Gugliandolo C., Caccamo D. and Stackebrandt E. (2001). A polyphasic taxonomic study of thermophilic bacilli from shallow, marine vents. *Systematic and Applied Microbiology*. 24(4), 572-587.

21. Takacs C.D., Ehringer M., Favre R., Cermola M., Eggertsson G., Palsdottir A. and Reysenbach A.L. (2001). Phylogenetic characterization of the blue filamentous bacterial community from an Icelandic geothermal spring. *FEMS Microbiology Ecology,* 35(2), 123-128.

22. Atanassova M., Derekova A., Mandeva R., Sjøholm C. and Kambourova M. (2008). Anoxybacillus bogrovensis sp. nov., a novel thermophilic bacterium isolated from a hot spring in Dolni Bogrov, Bulgaria. *International journal of systematic and evolutionary microbiology*, 58(10), 2359-2362.

23. Derekova A., Mandeva R. and Kambourova M. (2008). Phylogenetic diversity of thermophilic carbohydrate degrading bacilli from Bulgarian hot springs. *World Journal of Microbiology and Biotechnology*, 24(9), 1697-1702.

24. Lau M.C., Aitchison J.C. and Pointing S.B. (2009). Bacterial community composition in thermophilic microbial mats from five hot springs in central Tibet. *Extremophiles,* 13(1), 139-149.

25. Akkaya S.E. and Kivanc M. (2009). Thermophilic Bacteria; Methods of isolation and identification of Gram-negative Bacilli in hot springs. *Electronic Microbiology Journal,* 07(1), 01-23.

26. Hammer R., Harper D. and Ryan P. (2001). PAST : Paleontological Statistics Software Packages for Education and Data Analysis. *Palaeontologia Electronica,* 4, 9.

27. Moore Edward and Angelika Arnscheidt (2004). Simplified Protocols for the Preparation of Genomic DNA from Bacterial Cultures Simplified Protocols for the Preparation of Genomic DNA from Bacterial Cultures. *Molecular Microbial Ecology Manual*, Second Edition, 1.01, 3-18.

28. Wilson K. (1987). Preparation of genomic DNA from bacteria. 2.4. 1–2.4. 5 in FM Ausubel, R. Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K. Struhl, eds. Current protocols in molecular biology. John Wiley & Sons, New York, NY.

29. Joshi S.J., Suthar H., Yadav A.K., Hingurao K. and Nerurkar A. (2012). Occurrence of biosurfactant producing Bacillus spp. in diverse habitats. *ISRN biotechnology*, 2013, 6. http://dx.doi.org/10.5402/2013/ 652340.

30. Gohel H.R., Ghosh S.K. and Bragazna V.J. (2013). Production, Purification and Immobilization of Extracellular Lipases Xrf11 and Bacillus Licheniformis Xrf12 for Production of Alkyl Esters. *International Journal of Life Sciences Biotechnology and Pharma Research*., 2(3), 278-286.

31. Dudhagara P., Bhavasar S., Ghelani A. and Bhatt S. (2014). Isolation, characterization and investing the industrial applications of thermostable and solvent tolerant serine protease from hot spring isolated thermophililic Bacillus licheniformis U1. *International Journal of Applied Sciences and Biotechnology*, 2(1), 75-82.

32. Kikani B.A. and Singh S.P. (2011). Single step purification and characterization of a thermostable and calcium independent -amylase from Bacillus amyloliquifaciens TSWK1-1 isolated from Tulsi Shyam hot spring reservoir, Gujarat (India). *International Journal of Biological Macromolecules,* 48(4), 676-681. http://dx.doi.org/10.1016/j.ijbiomac.2011.02.010.

33. Ferris M.J. and Ward D.M. (1997). Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Applied and environmental microbiology*, 63(4), 1375-1381.

34. Mackenzie R., Pedrós-Alió C. and Díez B. (2013). Bacterial composition of microbial mats in hot springs in Northern Patagonia: variations with seasons and temperature. *Extremophiles,* 17, 123-136.

35. Brandon R. Briggs, Eoin L. Brodie, Lauren M. Tom, Hailiang Dong, Hongchen Jiang, Qiuyuan Huang, Shang Wang, Weiguo Hou, Geng Wu, Liuquin Huang, Brian P. Hedlund, Chuanlun Zhang, Paul Dijkstra and Bruce A. Hungate. (2014). Seasonal Patterns in Microbial Communities Inhabiting the Hot Springs of Tengchong, Yunnan Province, China. *Environmental Microbiology,* 16(6), 1579-1591.

36. Prieto-Barajas C.M., Alfaro-Cuevas R., Valencia-Cantero E. and Santoyo G. (2017). Effect of seasonality and physicochemical parameters on bacterial communities in two hot spring microbial mats from Araró, Mexico. *Revista Mexicana de Biodiversidad*, 88(3), 616-624. http://dx.doi.org/10.1016/j.rmb.2017.07.010.

37. Archna S., Priyank V., Nath Y.A. and Kumar S.A. (2015). Bioprospecting for extracellular hydrolytic enzymes from culturable thermotolerant bacteria isolated from Manikaran thermal springs. *Research Journal of Biotechnology*, 10(4), 33-42.

38. Huang Q., Dong C.Z., Dong R.M., Jiang H., Wang S., Wang G., Bin Fang, Xiaoxue Ding, Lu Niu, Xin Li, Chuanlun Zhang and Hailiang Dong (2011). Archaeal and Bacterial Diversity in Hot Springs on the Tibetan Plateau, China. *Extremophiles,* 15(5), 549-563.

39. Khiyami Mohammad A., Ehab A. Serour, Maher M. Shehata and Ali H. Bahklia (2012). Thermo-Aerobic Bacteria from Geothermal Springs in Saudi Arabia. *African Journal of Biotechnology,* 11(17), 4053-4062. http://www.academicjournals.org/AJb/abstracts/abs2012/28 Feb/Khiyami et al.htm.

40. Sahoo R.K., Subudhi E. and Kumar M. (2015). Investigation of bacterial diversity of hot springs of Odisha, India. *Genomics Data*, 6, 188-190.

41. Sen R. and Maiti N.K. (2014). Genomic and functional diversity of bacteria isolated from hot springs in Odisha, India. *Geomicrobiology Journal*, 31(7), 541-550.

42. Stefanova K., Tomova I., Tomova A., Radchenkova N., Atanassov I. and Kambourova M. (2016). Archaeal and bacterial diversity in two hot springs from geothermal regions in Bulgaria as demostrated by 16S rRNA and GH-57 genes. *International Microbiology*, 18(4), 217-223.

43. Yadav A.N., Verma P., Kumar M., Pal K.K., Dey R., Gupta A, Jasdeep Chatrath Padaria, Govind T. Gujar, Sudheer Kumar, Archna Suman, Radha Prasanna and Anil K. Saxena (2015). Diversity and Phylogenetic Profiling of Niche-Specific Bacilli from Extreme Environments of India. *Annals of Microbiology,* 65(2), 611-629.

44. Sahay H., Yadav A.N., Singh A.K., Singh S., Kaushik R. and Saxena A.K. (2017). Hot Springs of Indian Himalayas : Potential Sources of Microbial Diversity and Thermostable Hydrolytic Enzymes. *3 Biotech*., 7(2), 1-11.

45. Kiel G. and Gaylarde C.C. (2007). Diversity of salt-tolerant culturable aerobic microorganisms on historic buildings in Southern Brazil. *World Journal of Microbiology and Biotechnology*, 23(3), 363-366.

46. Kikani B.A., Sharma A.K. and Singh S.P. (2015). Culture dependent diversity and phylogeny of thermophilic bacilli from a natural hot spring reservoir in the Gir Forest, Gujarat (India). *Microbiology*, 84(5), 687-700.

47. Schallmey M., Singh A. and Ward O.P. (2004). Developments in the use of Bacillus species for industrial production. *Canadian Journal Microbiology,* 50(1), 1-17. PMid:15052317. http:// dx.doi.org/10.1139/w03-076.

48. Inan K., Canakci S., Belduz A.O. and Sahin F. (2012). Brevibacillus aydinogluensis sp. nov., a moderately thermophilic bacterium isolated from Karakoc hot spring. *International Journal of Systematic and Evolutionary Microbioogy,* 62, 849-855.

49. Baek S.H., Im W.T., Oh H.W., Lee J.S., Oh H.M. and Lee S.T. (2006). Brevibacillus ginsengisoli sp. nov., a denitrifying bacterium isolated from soil of a ginseng field. *International journal of systematic and evolutionary microbiology*, 56(11), 2665-2669. http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.64382-0.

50. Choi M.J., Bae J.Y., Kim K.Y., Kang H. and Cha C.J. (2010). Brevibacillus fluminis sp. nov., isolated from sediment of estuarine wetland. *International journal of systematic and evolutionary microbiology*, 60(7), 1595-1599.

51. Song Jinlong, Wang Yanwei, Song Yi, Zhao Bingqiang, Wang Huimin, Zhou Shan, Kong Delong, Guo Xiang, Li Yanting, He Mingxiong, Ma Kedong, Ruan Zhiyong and Yan Yanchun (2017). Brevibacillus Halotolerans Sp. Nov., Isolated from Saline Soil of a Paddy Field. *International Journal of Systematic and Evolutionary Microbiology,* 67(4), 772-777.

52. Aanniz T., Ouadghiri M., Melloul M., Swings J., Elfahime E., Ibijbijen J. and Amar M. (2015). Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. *Brazilian Journal of Microbiology*, 46(2), 443-453.

53. Hadad D., Geresh S. and Sivan A. (2005). Biodegradation of polyethylene by the thermophilic bacterium Brevibacillus borstelensis. *Journal of applied microbiology*, 98(5), 1093-1100.

54. Rath K., Mishra B. and Vuppu S. (2012). Biodegrading ability of organo-sulphur compound of a newly isolated microbe Bacillus sp. KS1 from the oil contaminated soil. *Archives of Applied Science Research*, 4(1), 465-471.

55. Babu G.P., Subramanyam P., Sreenivasulu B. and Paramageetham C. (2014). Isolation and identification of sulfate reducing bacterial strains indigenous to sulphur rich barite mines. *International Journal of Current Microbiology and Applied Sciences*, 3(7), 788-793.