International Journal of Research in Advent Technology, Vol.7, No.4, April 2019
E-ISSN: 2321-9637
Available online at www.ijrat.org

Unexplored Bacterial Diversity of Rajapur-Unhale Hot Spring, Ratnagiri District, Maharastra, India- A Gold Standard and MALDI-TOF Based Approach

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Abstract: In our study sediment and water samples was collected from the Rajapur-Unhale hot spring having 42 ^{O}C temperature. This study aimed to explore the unexplored bacterial diversity within the Rajapur-Unhale hotsprings. Fifty five isolates were obtained from both sediment and water samples were subjected to MALDI-TOF identification, were able to identify 15 isolates and most of the isolates which was not identified by direct cell loading method but was identified using ethanol/ formic acid extraction method and others gave not reliable identification with both the methods. For identification of bacterial species and genera, the sequencing centered on 16S rRNA is most extensively used and accepted to categorize them phylogenetically. Whereas, 16S rRNA gene sequencing based identification could able to identify all the 55 isolates. This study shows the clear picture that the MALDI-TOF database needs to be updated with environmental isolates for effective and appropriate identification.

Keywords: Hot spring, 16S rRNA gene sequencing and MALDI-TOF

1. INTRODUCTION

The microbial biosphere is the major unexplored reservoir of biodiversity on the earth, and is an important forward-facing in ecology under exploration. Microbial diversity encompasses inquiry of microorganisms in relation to their biotic and abiotic environments. The enormous arrangement of microbial activities and their importance to the biosphere and to human economics provide strong rationale for understanding their diversity, conservation and exploitation. This is done with a variety of approaches and tools, including microscopy, culturing, biochemistry and molecular biology. The microorganisms are the living bodies which have ability to survive under extreme environmental conditions in which other organism are unable to survive. Microorganisms, in detail bacteria, continue to be revealed living under extreme ecological conditions such as low pH, high and low temperature, high salinity etc. Thermophilic bacteria, a group of extremophiles whose optimal temperature for the growth lies between 42-80°C, temperature goes beyond 80°C are called hyper-thermophiles. The bacteria can able to survive at very high temperature conditions such as hot springs, geysers, volcano etc. The ability of the bacterial enzymes and proteins which is produced from the extremophiles controls the specific metabolic and biological functions to be highly active at high temperature makes survival of these microorganisms in adverse conditions. In addition, cultivation of thermophiles at high temperature is technically and economically advantageous as it diminishes risk of contamination and viscosity which leads to high degree of substrate solubility.

Maharastra being rich in bio-diversity due to climatic differences owns a variety of hot-springs located in poles apart geographical locations and vary both in physio-chemical and microbial ecosystem parameters. In general, the temperature of rocks within the earth increases with depth. The rate of temperature increase with depth is known as the “geothermal gradient”. A spring of naturally hot water, typically heated by subterranean volcanic activity and the water issuing from a hot spring is heated by geothermal heat, essentially heat from the Earth's interior. Geothermal energy is the heat from the Earth. Resources of geothermal energy range from the shallow ground to hot water and hot rock found a few miles beneath the Earth's surface, and down even deeper to the extremely high temperatures of molten rock called magma.

In Maharastra, several hot springs distributed in different regions having temperature ranges between 42 and 71°C have been known by geologists for many years, geographical distribution, chemical and physical characteristics of these springs were described by many researchers whereas few hot springs from this region was not completely explored. In our study sediment and water samples of Rajapur-Unhale hot spring with the latitude 16^{0}38.7458''N and longitude 73^{0}31.8680’E was

collected from the hot spring having 42 ^{O}C temperature. This study aimed to explore the unexplored bacterial diversity within the Rajapur-Unhale hotsprings.

2. MATERIALS AND METHODS:

2.1. Study area and sample collection:

Sediment and water samples of hot springs of Rajapur-unhale were collected in April 2016. The temperature and pH of sediment and water was recorded at the site. The samples were collected in presterilized polypropylene bottles were placed in ice bags and transported in laboratory within 24 h (Table 1).

Table 1: Sampling location and Physical parameter of the hot spring

Sub-district	Hot spring	Coordinates		pH	Sampling maximum depth in feet measurements	Temperature (^{0}C)	
		Latitude	Longitude			Observed	Reported
Rajapur	Unhale (RS)	16^{0} 38.7458 'N	73^{0} 31.8680 'E	7.5	1.8	42	42

2.2. Media composition, serial dilution and plating:

A total of 8 different medium were used out of which seven were purchased from Himedia, India and the one were prepared to mimic the natural niche composition. In the process of preparation of the media hot spring water was taken and filtered with 0.4 micron filter paper to remove the bacterial contamination and then 2% of agar was mixed with it and given for autoclave. Both concentrated and diluted medium was used to isolate maximum bacterial colonies from the given consortium. The list of the medium used with their components and dilutions are provided herewith (Table 2)

Table 2: List of media used for isolating microbes

S.No	Medium	Components	gm/L
1	Minimal medium	$(NH_4)_2SO_4$	3
		K_2HPO_4	0.5
		$MgSO_4.7H_2O$	0.5
		KCl	0.1
		$Ca(NO_3)_2.4H_2O$	0.01
		Trace elements	**1ml**
		$FeCl_3.6H_2O$	1.1
		$CuSO_4.\ 5H_2O$	0.05
		H_3BO_3	0.2
		$MnSO_4.H_2O$	0.2
		$Na_2MoO_4.2H_2O$	0.08
		$CoCl_2.6H_2O$	0.06
		$ZnSO_4.7H_2O$	0.09
2	Reasoner's 2A agar (R2A) Without dilution and 1:10 dilutions	Casein acid hydrolysate	0.5
		Yeast extract	0.5
		Proteose peptone	0.5
		Dextrose	0.5
		Starch, soluble	0.5
		Dipotassium phosphate	0.3
		Magnesium sulphate	0.024
		Sodium pyruvate	0.3
		Agar	15
3	Tryptic Soy Agar (TSA) Without dilution and 1:10 dilutions	Pancreatic digest of casein	15
		Papaic digest of soyabean meal	5
		Sodium chloride	5
		Agar	15
4	Luria-Bertani (LB) Agar Without dilution and 1:100 dilutions	Casein enzymic hydrolysate	10
		Yeast extract	5
			10

		Sodium chloride Agar	15
5	Nutrient Agar (NA) Without dilution and 1:100 dilutions	Peptic digest of animal tissue Sodium chloride Beef extract Yeast extract Agar	5 5 1 1 15
6	Actinomyces Isolation agar (AIA) Without dilution and 1:100 dilutions	Sodium caseinate L-Asparagine Sodium propionate Dipotassium phosphate Magnesium sulphate Ferrous sulphate Agar	2 0.1 4 0.5 0.1 0.001 15
7	Tryptone Yeast Extract Agar	Casein enzymic hydrolysate Yeast extract powder Agar	6 3 15
8	Hot-water spring agar	Hot spring water (From each niche) Agar Filter the hot-spring water, add agar and then autoclave it	1 L 15

Both sediment and water were suspended in normal saline (0.85%) and serially diluted. The serially diluted samples were plated on the respective agar mediums listed above followed by incubation at the natural niche temperature. CFU was recorded for each samples against each media composition used.

2.3. DNA isolation

The isolates were subjected to DNA isolation (Mayilraj et al, 2006) using ZymoResearch kit based method in which it contains lysis buffer to lyse the bacterial cells by adding 500µl and then vortexes for 5 mins to disrupt the bacterial cells, centrifuge for 2 mins at 10000rpm. Collect the supernatant and add 12000µl of binding buffer to bind the reagents with bacterial DNA and separates it. Add 600µl of the solution into the spin column and centrifuge at 10000rpm for 2 mins. Discard the flow through and save the column, add 500µl of DNA pre wash buffer into the column and centrifuge at 10000 rpm for 2 mins. Discard the flow through and add DNA wash buffer 500µl, centrifuge at 10000rpm for 2 mins and discard the flow through. Empty spin the column to remove the extra buffer which hinders or interferes the DNA. Add elution buffer directly into the column with the filter that can elute the DNA. The eluted DNA can be stored in to a fresh tube for further experiments and checked for their quality by gel electrophoresis (0.8% agarose) at 100 V for 10-15 min.

2.4. PCR amplification and sequencing of the 16S rDNA

The 16S rRNA gene was amplified by the method in which the primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'AGAAAGGAGGTGATCCAGGC-3') were used [9]. The PCR program was set up as follows: initial incubation at 94^{o}C for 5 min followed by 35 cycles (94^{o}C for 1 min, 52.5^{o}C for 1 min and 72^{o}C for 2 min) and final extension at 72^{o}C for 10 min using thermal cycler. The amplified DNA fragment was separated on 1 % agarose gel eluted from the gel and purified using Macherey-Nagel PCR/Gelpurification kit (Macherey-Nagel, Germany). The purified PCR product was sequenced with forward and reverse primers namely 8-27f (5'AGAGTTTGATCCTGGCTCAG-3'), 357f (5'-CTCCTACGGGAGGCAGCAG-3') and518r (5'-ATTACCGCGGCTGCTGG-3'), 1100r (5'-GGGTTGCGCTCGTTG-3'), 1492r (5'-GGTTACCTTGTTACGACTT-3'), respectively (*Escherichia coli* numbering system) (Kumar et al, 2015) (table 3).

Table 3. PCR set up for16S rRNA gene amplication	
Thermo pol buffer (10X)	5 µl

Genomic DNA	100- 200 ng
Forward primer	2 µM
Reverse primer	2 µM
dNTPs	800 µM
Taq Polymerase	1 U/ 50 µl

2.5. PEG- Nacl (polyethylene glycol) purification of PCR amplified products

Polyethylene glycol (PEG), used for precipitation of DNA. In this process a solution containing salt and PEG were added to an aqueous nucleic acid sample, which was then incubated. This results in precipitation of nucleic acids, which were then precipitated by centrifugation and then washed twice in 70% ethanol.

PEG- Nacl purification was done to precipitate the PCR product, removing the primer dimer and other reaction mixture. The steps followed were as stated below; 0.6 volumes of 20% PEG-Nacl were added to the final volume of the PCR product. It was followed by incubation at 37^0C for 20 minutes. Centrifuge at 12000rpm for 30 minutes, Decant/if plate Invert spin up to 400 rpm. 50 µl of the 70% ethanol was added to wash the pellet. Centrifugation was done at 12,000 (tube)/3800 (plate) rpm for 30 minutes.Supernatant was decanted/Invert spin at 400rpm for 5-10seconds. Air dry and then 10-20 µl of the distilled water was added to the tubes and the purified products were checked on 1% agarose gel electrophoresis.

2.6. Sequencing of 16S rDNA

Sequencing of the purified PCR product (~200 ng/reaction) was carried out using 2 pmoles of a given sequencing primer and 4 µl of ready reaction The rDNA sequence was determined by the dideoxy chain-termination method using the Big - Dye terminator kit using ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) mix from the Big Dye Terminator sequencing kit (ABI) in a total volume of 10µl. Cycle sequencing was carried out in a PCR machine (Eppendorf-Gradient) for 30 cycles. Initial incubation at 96^oC for 1 min followed by 25 cycles of sequencing and each cycle consisted of a denaturation step at 96^oC for 10 s, an annealing step at 50^oC for 10 s and an extension step at 60^oC for 4 min. After the PCR, the products were precipitated using 1 µl of 3 M sodium acetate (pH 4.6) and 50 µl of ethanol and incubated on ice for 15 min. The pellet was recovered by centrifugation at 15000 rpm for 20 min at 4^oC, washed with 70% ethanol, dried under vacuum and dissolved in 10 µl l of loading buffer [formamide:25 mM EDTA (4:1)]. About 2 µl of the sample was used to analyze the sequence and the rDNA sequence was determined by the dideoxy chain-termination method using the Big - Dye terminator kit using ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) (table 4). The purified DNA was used for sequencing of either partial or full 16S rDNA.

Table 4. PCR reaction mixture for sequencing	
DNA	50 ng
Sequencing buffer (5X)	1.5 µl
Primer (forward or reverse)	2 picomoles
Terminator ready raction (TRR) mix	1 µl

2.7. 16S rRNA gene sequence analysis

The identification of phylogenetic neighbors and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al, 2012). The 16S rRNA gene sequence of hot spring sediment and water sample isolates were aligned using the MEGA software version 6.0 (Tamura et al, 2013). Phylogenetic trees were constructed using the Neighbor-joining algorithm (Saitou & Nei 1987). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1981; Felsenstein,1985).

2.8. Sample preparation for MALDI analysis

Sample preparation was done with some modifications to the manufacturer's method which are as mentioned below:

2.8.1. Direct Transfer Method

Bacterial culture was homogenously smeared as a thin film directly onto a cleaned MALDI target plate or grids. Each sample position was overlaid with 1 µl matrix solution. The sample was allowed to dry at room temperature. The sample was inserted into the MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) for analysis.

2.8.2. Protein Extraction Method

At first 300 µl of ultra-pure water was pipetted into a clean Eppendorf tube. 5-10 mg of biological material

was transferred into the tube and vortexed to form a homogenous suspension. 900 µl of pure ethanol was added into the tube and vortexed for at least 1 minute. The tube was centrifuged for 2 minutes at 13,000 rpm and the supernatant was discarded. 50 µl of 70% formic acid was added into the Eppendorf tube and was vortexed. An equal volume of 100% acetonitrile was added to the tube and mixed carefully. 1 µl of cell suspension was plated on the target overlaid with 1 µl of matrix. The sample was allowed to dry at room temperature and inserted into the MALDI-TOF mass spectrometer. The cell suspension was centrifuged for 2 minutes at 13,000 rpm. 1 µl of extract supernatant was pipetted onto a cleaned MALDI target. The sample was allowed to dry at room temperature. The sample was overlaid with 1 µl matrix, dried and inserted into MALDI-TOF mass spectrometer for analysis.

2.9. MALDI-TOF MS analysis

For the analysation of MALDI-TOF mass spectra obtained from Ultraflex III instrument operated in linear positive mode under FlexControl 3.1 software. External calibration of the mass spectra was done using *Escherichia coli* DH5 alpha standard peaks (4346.3, 5095.8, 5380.4, 6254.4, 7273.5 and 10,299.1 Da). Laser power was set to 120% of the threshold laser power was used for the sample. Five independent spectra comprising 240 laser shots were acquired from each spot. Within an individual spot, the laser was manually directed when required in addition to a pre-defined lattice raster. Mass spectra were processed using Flex Analysis (version 3.1; Bruker Daltonik) and BioTyper software (version 3.1; Bruker Daltonik).

3. RESULTS AND DISCUSSION

3.1. Serial Dilution, Plating And Purification Of The Isolates

The serially diluted sediment and water samples were plated (Table 5) in a respective medium and incubated at natural niche temperature at 42^{O}C for 2 weeks (Fig.1). Colonies were observed after 5 days of incubation. Among the sediment and water samples, the sediment sample showed more isolates than water samples. The isolates obtained from the spread plating were then patch streaked and incubated at respective temperature (Fig.2). The samples comprising both pigmented and non-pigmented colonies were observed and patch streaked. The patch streaked isolates were purified and preserved in -80 ^{O}C for further experiments (Fig.3). In Rajapur-Unhale sediment, 40 isolates were obtained and 15 isolates were obtained from Rajapur-Unhale water sample.

Table 5: Serial dilution of hot spring sediment and water samples

1	Rajapur-Unhale hot spring sediment	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
2	Rajapur-Unhale hot spring water	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}

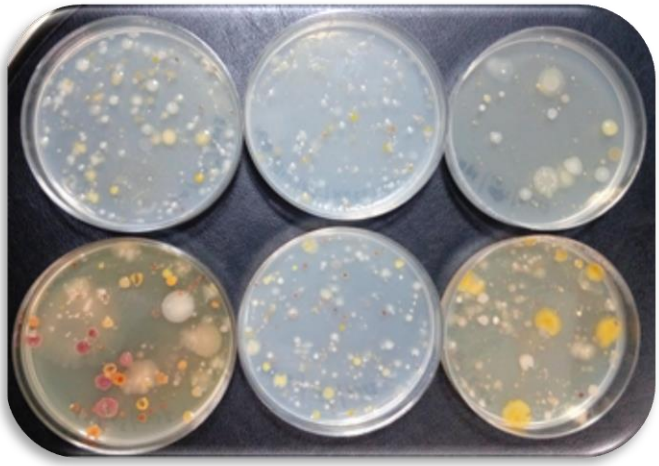

Fig.1. Spread plating of hot spring sediment and water

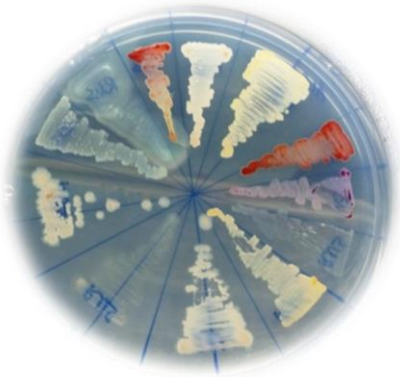

Fig.2. Patch streaking of the individual isolates

Fig.3. Purification of isolates

3.2. MALDI-TOF Identification

One sixty six isolates were subjected to MALDI-TOF based identification. MALDI-TOF mass spectra were obtained from an ultraflex III instrument operated in linear positive mode under flex-Control 3.1 software. External calibration of the mass spectra was done using standard peaks from *E. coli* DH5α (4346.3, 5095.8, 5380.4, 6254.4, 7273.5 and 10 299.1 da). Laser power was set to 120 % of the threshold laser power. This software generates classification models from large numbers of spectra and detects small differences among different clusters, based on mass, signal-to-noise, intensity, peak heights and peak areas. Among 55 isolates obtained only 15 isolates were identified as *Kocuria palustris, Staphylococcus hominis, Bacillus cereus, Pseudomonas alcaligenes, Enterobacter kobei, Aeromonas veronii, Escherichia coli, Staphylococcus capitis, Micrococcus luteus, Pseudomonas mendocina* and *Enterobacter ludwigii* were taken into consideration which showed above 2% cut-off value by MALDI-TOF and rest of the isolates failed in identification through MALDI-TOF based approach

Few isolates from Rajapur water and sediments samples were identified in MALDI-TOF identification. Two methods of MALDI identification of 55 isolates were done using 1) Ethanol/ formic acid extract and 2) direct cell loading. In Direct Method of identification the isolates RS9, RS17, RS19, RS41, RS53, RW8, RW12, RW17 and RW18 were identified. Whereas, in ethanol/ formic acid extraction method the isolates RS2, RS9, RS17, RS19, RS22, RS23, RW5, RW7, RW8, RW12, RW17 and RW18 were identified. Both the methods were able to identify 15 isolates and most of the isolates which was not identified by direct cell loading method (table 6) but was identified using ethanol/ formic acid extraction method (table 7) and others gave not reliable identification with both the methods. This shows the clear picture that the necessity of MALDI-TOF database to be updated with the large number of environmental bacterial isolates. Further the isolates were subjected to DNA isolation for the 16S rRNA gene sequence based identification to know the exact/ proper identification of the hot spring isolates.

Table 6. MALDI-TOF based identification by Direct smear method

Strain ID	**Organism identified**	**Cut off value**	**Organism identified**	**Cut off value**
MKAS - RS2	**no peaks found**	< 0	**no peaks found**	< 0
MKAS - RS9	Enterobacter kobei	2.067	Enterobacter cloacae	1.862
MKAS - RS17	Escherichia coli	2.297	Escherichia coli	2.194
MKAS - RS19	Staphylococcus capitis	1.715	**not reliable identification**	1.655
MKAS - RS22	**not reliable identification**	1.528	**not reliable identification**	1.505
MKAS - RS23	**not reliable identification**	1.524	**not reliable identification**	1.475
MKAS - RS41	Pseudomonas alcaligenes	1.849	**not reliable identification**	1.645
MKAS - RS53	Bacillus subtilis	1.825	Bacillus subtilis	1.803
MKAS - RW5	**not reliable identification**	1.583	**not reliable identification**	1.553

MKAS - RW7	**no peaks found**	< 0	**no peaks found**	< 0
MKAS - RW8	Staphylococcus hominis	2.233	Staphylococcus hominis	2.011
MKAS - RW9	**not reliable identification**	1.373	**not reliable identification**	1.305
MKAS - RW12	Bacillus sp.	1.897	Bacillus subtilis	1.83
MKAS - RW17	Enterobacter hormaechei	2.134	Enterobacter cloacae	2.127
MKAS - RW18	Pseudomonas alcaligenes	2.331	Pseudomonas alcaligenes	2.258

Table 7. MALDI-TOF based identification by Ethanol: formic acid extraction method

Strain ID	Organism identified	Cut off value	Organism identified	Cut off value
MKAS - RS2	Corynebacterium mucifaciens	1.976	Corynebacterium mucifaciens	2.146
MKAS - RS9	Enterobacter kobei	2.292	Enterobacter asburiae	2.115
MKAS - RS17	Escherichia coli	2.455	Escherichia coli	1.972
MKAS - RS19	Staphylococcus capitis	2.2	Staphylococcus capitis	1.519
MKAS - RS22	Pseudomonas oleovorans	1.934	Pseudomonas oleovorans	1.938
MKAS - RS23	**Micrococcus luteus**	2.078	**Micrococcus luteus**	1.403
MKAS - RS41	**no peaks found**	< 0	**no peaks found**	< 0
MKAS - RS53	**not reliable identification**	1.825	**not reliable identification**	1.803
MKAS - RW5	**Kocuria palustris**	2.119	**Kocuria palustris**	2.101
MKAS - RW7	**Pseudomonas alcaligenes**	2.214	**Pseudomonas alcaligenes**	2.151
MKAS - RW8	Staphylococcus hominis	2.233	Staphylococcus hominis	2.011
MKAS - RW9	**Moraxella_sg_Moraxella osloensis**	1.937	**Moraxella_sg_Moraxella osloensis**	1.805
MKAS - RW12	Bacillus sp.	1.897	Bacillus subtilis	1.83
MKAS - RW17	Enterobacter cloacae	2.258	Enterobacter kobei	2.128
MKAS - RW18	Enterobacter cloacae	2.193	Enterobacter ludwigii	2.161

3.3. DNA Isolation:

DNA was successfully isolated from both sediment and water samples of Rajapur-Unhale hot spring. All the 55 isolates were subjected to DNA isolation, the **c**oncentration and purity of the samples were checked in Nano-Drop. The DNA of all the isolates were of good quantity and quality was confirmed in agarose gel electrophoresis with 0.8% agarose (fig.4). The quantification of isolated DNA further subjected to nanodrop in which the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~1.8 was generally accepted as "pure" for DNA; a ratio of ~2.0 was generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The genomic DNA of the isolates were dissolved in TE or ultra-pure water and stored at -20^{O}C for further experiments especially Polymerase chain reaction (PCR).

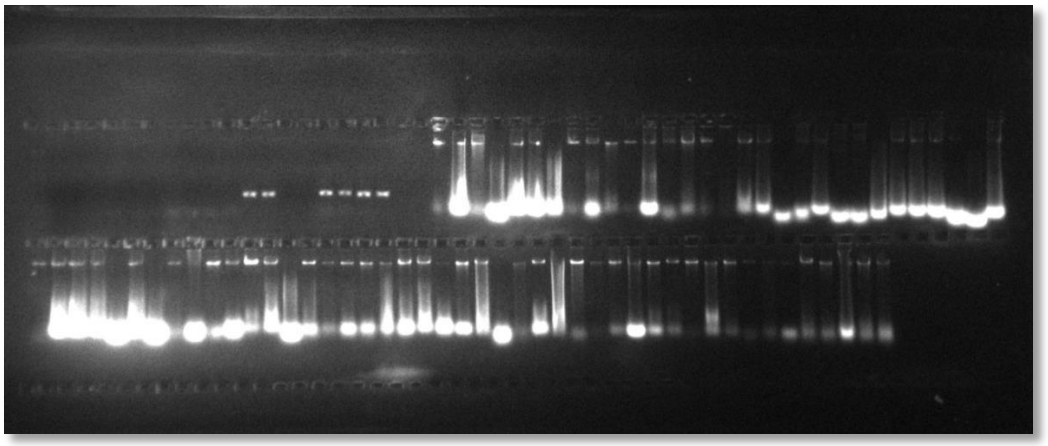

Fig.4. DNA isolation of the representative isolates

3.4. 16S Rrna Gene Amplification

The 16S rRNA was the gold standard method to identify the bacterial isolates. 16S rRNA genes were amplified using universal eubacterial primers 27f (59-AGAGTTTGATCCTGGCTCAG-39) and 1492r (59-AAGTCGTAACAAGGTAACCGTA-39). The amplified DNA fragment was confirmed in agarose gel electrophoresis with 1% agarose concentration and purified using PEG purification. The purified PCR product was sequenced with primers 27f, 357f, 518r, 926f, 1100r and 1492r (*Escherichia coli* numbering system) shown in fig.5.

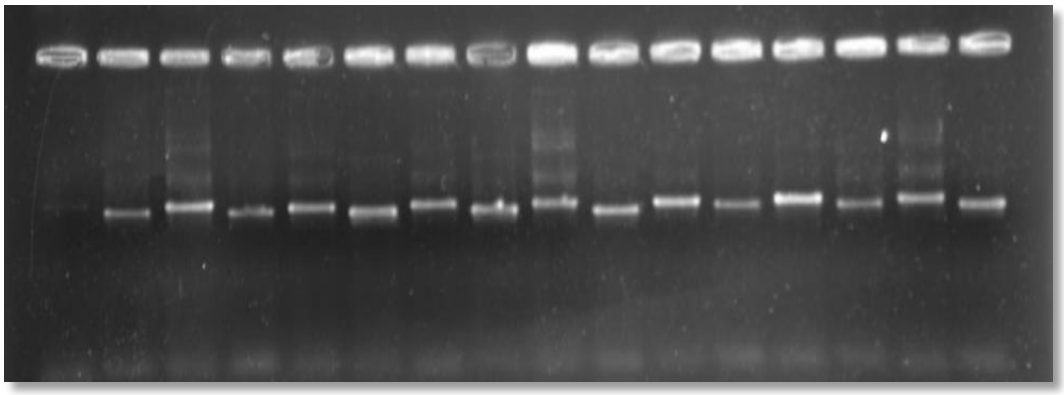

Fig.5. 16S rRNA gene amplification of the representative isolates from hotsprings

3.5. PEG Purification Of PCR Product

In this figure the bands signify the presence of amplified product. Those of which were not amplified were again processed for PCR amplification and then all the samples were processed for PEG purification.

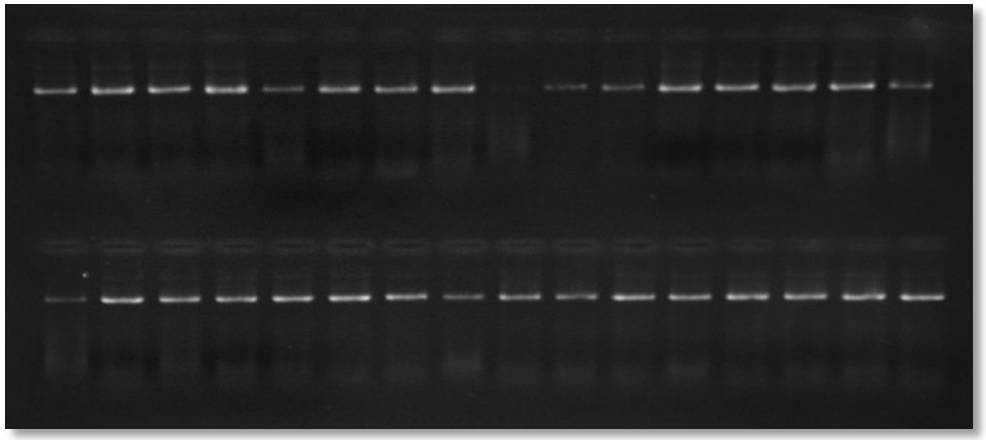

Fig.6. PEG purification of 16S rRNA gene amplification product of the representative isolates from hotsprings

In this figure the bands signify the PEG purification of amplified product. Those of which were not purified were again processed for PCR amplification and then all the samples were further subjected to PEG purification. The purified products were further subjected to 16S rRNA gene sequence which was determined by the dideoxy chain-termination method using the Big-Dye terminator kit with an ABI 310 Genetic Analyzer (Applied Biosystems).Samples showing PCR amplification, checked on 1% agarose gel is shown in the fig.6.

3.6. Identification Of The Hot Spring Isolates

The hotspring isolates were sequenced, assembled and identified through Ez-Taxon server by blast search analysis of the sequences in fasta format. Fifty five isolates were identified through 16S rRNA gene sequencing.

In Rajapur, unahale sediment samples, the isolates were identified as Aquaspirillum serpens, Azospira oryzae, Azovibrio restrictus, Bacillus murimartini, Bacillus tequilensis, Cloacibacterium normanense, Cloacibacterium rupense, Corynebacterium mucifaciens, Enterobacter tabaci, Flexibacter flexilis, Gordonia hongkongensis, Macellibacteroides fermentans, Microbacterium aurantiacum, Microbacterium barkeri, Microbacterium dextranolyticum, Microbacterium natoriense, Microbacterium saccharophilum, Micrococcus aloeverae, Ornithinimicrobium pekingense, Porphyrobacter colymbi, Pseudomonas composti, Pseudomonas oleovorans subsp. oleovorans, Rhodococcus kroppenstedtii, Staphylococcus capitis subsp. capitis, Xanthobacter flavus, Xanthobacter tagetidis and Zavarzinia compransoris were mentioned in table 8.

Table 8. 16S rRNA gene sequencing of Rajapur-Unhale sediment sample

S.No	Strain id	16S rRNA based identification	Sequence acession numbers
1	RS1	*Cloacibacterium normanense*	MH715173
2	RS2	*Corynebacterium mucifaciens*	MH715174
3	RS3	*Azovibrio restrictus*	MH715175
4	RS4	*Microbacterium dextranolyticum*	MH715176
5	RS5	*Flexibacter flexilis*	MH715177
6	RS6	*Aquaspirillum serpens*	MH715178
7	RS8	*Microbacterium aurantiacum*	MH715179
8	RS9	*Enterobacter tabaci*	MH715180
9	RS10	*Cloacibacterium normanense*	MH715181
10	RS17	*Cloacibacterium normanense*	MH715182
11	RS19	*Staphylococcus capitis* subsp. *capitis*	MH715183
12	RS22	*Pseudomonas oleovorans* subsp. *oleovorans*	MH715184
13	RS23	*Bacillus murimartini*	MH715185
14	RS26	*Azospira oryzae*	MH715186
15	RS33	*Azospira oryzae*	MH715187
16	RS34	*Enterobacter tabaci*	MH715188
17	RS36	*Azovibrio restrictus*	MH715189
18	RS37	*Micrococcus aloeverae*	MH715190
19	RS38	*Porphyrobacter colymbi*	MH715191
20	RS39	*Zavarzinia compransoris*	MH715192
21	RS40	*Aquaspirillum serpens*	MH715193
22	RS41	*Microbacterium barkeri*	MH715194
23	RS42	*Ornithinimicrobium pekingense*	MH715195
24	RS43	*Ornithinimicrobium pekingense*	MH715196
25	RS44	*Rhodococcus kroppenstedtii*	MH715197
26	RS46	*Gordonia hongkongensis*	MH715198
27	RS47	*Microbacterium aurantiacum*	MH715199

28	RS48	*Zavarzinia compransoris*	MH715200
29	RS51	*Cloacibacterium normanense*	MH715201
30	RS53	*Bacillus tequilensis*	MH715202
31	RS54	*Macellibacteroides fermentans*	MH715203
32	RS55	*Cloacibacterium rupense*	MH715204
33	RS56	*Azovibrio restrictus*	MH715205
34	RS57	*Microbacterium natoriense*	MH715206
35	RS58	*Microbacterium saccharophilum*	MH715207
36	RS59	*Microbacterium saccharophilum*	MH715208
37	RS60	*Xanthobacter flavus*	MH715209
38	RS61	*Xanthobacter tagetidis*	MH715210
39	RS62	*Azovibrio restrictus*	MH715211
40	RS63	*Pseudomonas composti*	MH715212

In Rajapur, unahale water samples, the isolates were identified as Bacillus subtilis subsp. inaquosorum, Bacillus tequilensis, Elstera litoralis, Enhydrobacter aerosaccus, Enterobacter tabaci, Kocuria palustris, Kocuria rhizophila, Micrococcus aloeverae, Pseudomonas aeruginosa, Pseudomonas alcaligenes and Staphylococcus hominis subsp. hominis were mentioned in table 9.

Table 9. 16S rRNA gene sequencing of Rajapur-Unhale water sample

S.No	**Strain id**	**16S rRNA based identification**	**Sequence acession numbers**
1	RW1	*Kocuria rhizophila*	MH715213
2	RW2	*Enhydrobacter aerosaccus*	MH715214
3	RW3	*Enhydrobacter aerosaccus*	MH715215
4	RW4	*Micrococcus aloeverae*	MH715216
5	RW5	*Kocuria palustris*	MH715217
6	RW6	*Kocuria rhizophila*	MH715218
7	RW7	*Pseudomonas aeruginosa*	MH715219
8	RW8	*Staphylococcus hominis* subsp. *hominis*	MH715220
9	RW9	*Enhydrobacter aerosaccus*	MH715221
10	RW10	*Elstera litoralis*	MH715222
11	RW12	*Bacillus subtilis* subsp. *inaquosorum*	MH715223
12	RW13	*Kocuria rhizophila*	MH715224
13	RW14	*Bacillus tequilensis*	MH715225
14	RW17	*Enterobacter tabaci*	MH715226
15	RW18	*Pseudomonas alcaligenes*	MH715227

The total diversity of bacterial isolates from sediments and water sample were identified through molecular approach in which the diversity and abundance found to be extreme in Rajapur-Unhale sediment sample compared to Rajapur-Unhale water sample. This might be due to the sediments which persist there in hot springs for longer duration compared to water and moreover the sediments could able to provide maximum nutrients required for the growth of the bacterial isolates than water. The distribution of the isolates were identified and shown in fig.7.

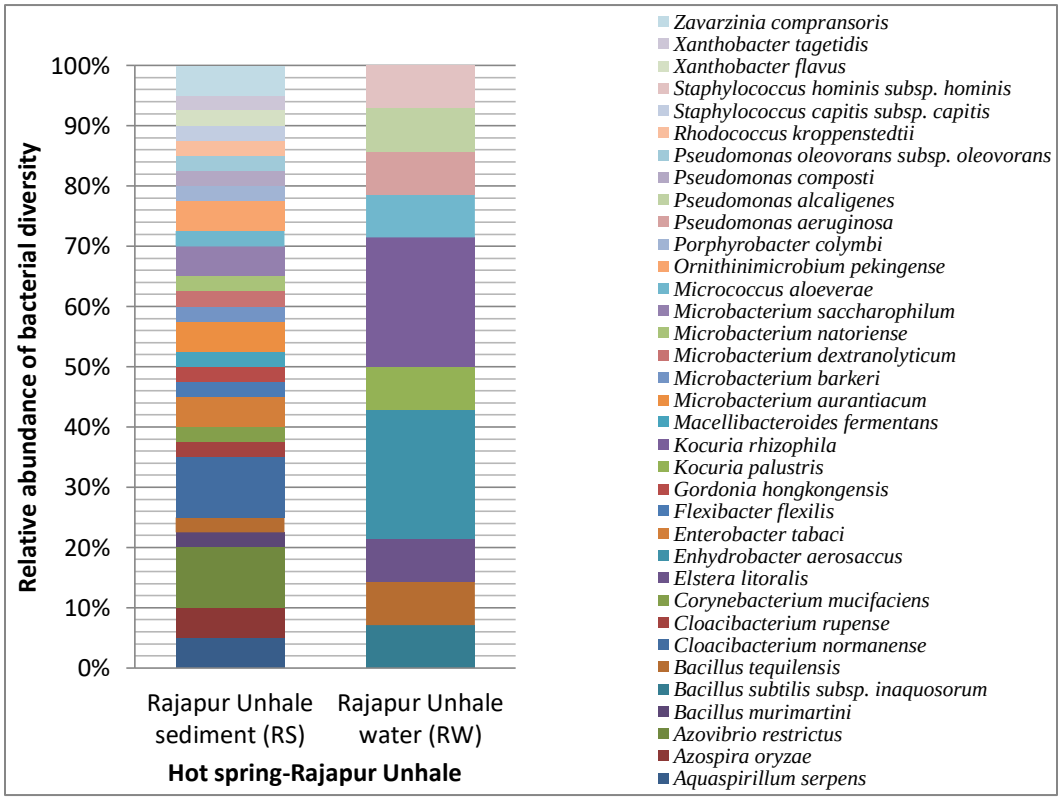

Fig.7. Percentage relative abundance of bacterial Species present in Rajapur-Unhale hot springs.

The Venny (Oliveros, 2015) an interactive tool for comparing lists with Venn's diagrams signifies the number and percentage of the identified isolates shown its similarity and uniqueness among the two samples of hot springs. In Rajapur-Unhale sediment sample, twenty four (68.6%) isolates were found to be unique whereas, in Rajapur-unhale water sample eight (22.9%) isolates were found to be unique which means it specifically confronts the presence of bacterial communities signifies in particular niche. Among 55 isolates three (8.6%) isolates which were common in both Rajapur-Unhale sediment and Rajapur-unhale water samples were mentioned in fig.8.

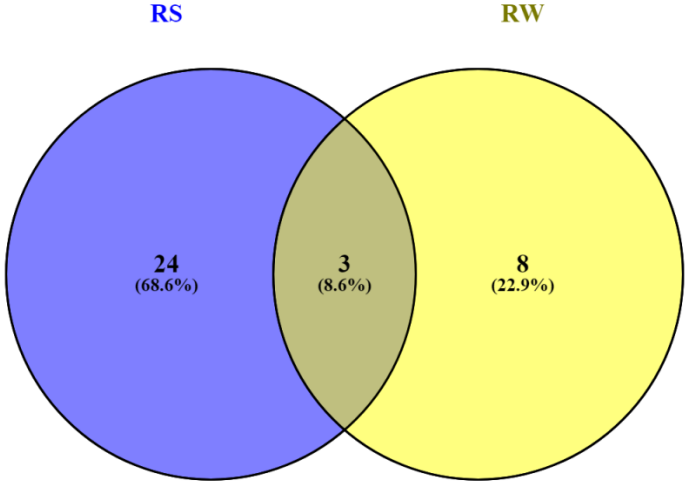

Fig.8. Number and percentage of bacterial species found in Rajapur-Unhale hot springs

3.7. Comparison Of MALDI-TOF And 16S Rrna Gene Sequencing Based Identification.

MALDI-TOF based identification was common in clinical isolates for easy detection and identification of bacterial infection of diseased patient samples. In this study we also used MALDI-TOF approach for the identification of environmental isolates to minimize the identification time and cost of

sequencing. 16S rRNA gene sequencing found to be gold standard for the identification of bacterial isolates from all the niches (Janda and Abbott, 2007). In comparison the MALDI-TOF could identify only 15 isolates among 55 isolates obtained from this study. Out of 15 isolates identified the isolates RS2, RS19, RS22, RW5, RW8, RW12 and RW18 were identified correctly in both genus and species level. The isolates RS9, RS53, RW7 and RW17 were identified genus level similar to 16S rRNA gene sequencing. The isolates RS7, RS23, RS41 and RW9 were found to be mismatched identification compared to 16S identification were mentioned in table 10. Thus, the isolates were identified in 16S were considered to be the best method for the identification of bacterial isolates. Since sequencing remains strenuous and time consuming, more rapidly identification systems have been developed designed for medical purposes. All identification methods depend on the quality of the fundamental database, and achieve very well on bacteria studied from a medical application background (Xiao et al. 2014). The MALDI-TOF database needs to be modernized with environmental isolates for easy identification and cost effective identification.

Table 10. comparison of MALDI-TOF MS and 16S rRNA gene sequencing identification

S.No	Strain id	MALDI-TOF identification	16S rRNA based identification	Sequence acession numbers
41	RS1	Not reliable identification	*Cloacibacterium normanense*	MH715173
42	RS2	*Corynebacterium mucifaciens*	*Corynebacterium mucifaciens*	MH715174
43	RS3	Not reliable identification	*Azovibrio restrictus*	MH715175
44	RS4	Not reliable identification	*Microbacterium dextranolyticum*	MH715176
45	RS5	Not reliable identification	*Flexibacter flexilis*	MH715177
46	RS6	Not reliable identification	*Aquaspirillum serpens*	MH715178
47	RS8	Not reliable identification	*Microbacterium aurantiacum*	MH715179
48	RS9	*Enterobacter kobei*	*Enterobacter tabaci*	MH715180
49	RS10	Not reliable identification	*Cloacibacterium normanense*	MH715181
50	RS17	*Escherichia coli*	*Cloacibacterium normanense*	MH715182
51	RS19	*Staphylococcus capitis*	*Staphylococcus capitis* subsp. *capitis*	MH715183
52	RS22	*Pseudomonas oleovorans*	*Pseudomonas oleovorans* subsp. *oleovorans*	MH715184
53	RS23	Micrococcus luteus	*Bacillus murimartini*	MH715185
54	RS26	Not reliable identification	*Azospira oryzae*	MH715186
55	RS33	Not reliable identification	*Azospira oryzae*	MH715187
56	RS34	Not reliable identification	*Enterobacter tabaci*	MH715188
57	RS36	Not reliable identification	*Azovibrio restrictus*	MH715189
58	RS37	Not reliable identification	*Micrococcus aloeverae*	MH715190
59	RS38	Not reliable identification	*Porphyrobacter colymbi*	MH715191
60	RS39	Not reliable identification	*Zavarzinia compransoris*	MH715192
61	RS40	Not reliable identification	*Aquaspirillum serpens*	MH715193
62	RS41	*Pseudomonas alcaligenes*	*Microbacterium barkeri*	MH715194
63	RS42	Not reliable identification	*Ornithinimicrobium pekingense*	MH715195
64	RS43	Not reliable identification	*Ornithinimicrobium pekingense*	MH715196
65	RS44	Not reliable identification	*Rhodococcus kroppenstedtii*	MH715197
66	RS46	Not reliable identification	*Gordonia hongkongensis*	MH715198
67	RS47	Not reliable identification	*Microbacterium aurantiacum*	MH715199
68	RS48	Not reliable identification	*Zavarzinia compransoris*	MH715200
69	RS51	Not reliable identification	*Cloacibacterium normanense*	MH715201
70	RS53	*Bacillus subtilis*	*Bacillus tequilensis*	MH715202
71	RS54	Not reliable identification	*Macellibacteroides fermentans*	MH715203
72	RS55	Not reliable identification	*Cloacibacterium rupense*	MH715204
73	RS56	Not reliable identification	*Azovibrio restrictus*	MH715205
74	RS57	Not reliable identification	*Microbacterium natoriense*	MH715206

75	RS58	Not reliable identification	*Microbacterium saccharophilum*	MH715207
76	RS59	Not reliable identification	*Microbacterium saccharophilum*	MH715208
77	RS60	Not reliable identification	*Xanthobacter flavus*	MH715209
78	RS61	Not reliable identification	*Xanthobacter tagetidis*	MH715210
79	RS62	Not reliable identification	*Azovibrio restrictus*	MH715211
80	RS63	Not reliable identification	*Pseudomonas composti*	MH715212
81	RW1	Not reliable identification	*Kocuria rhizophila*	MH715213
82	RW2	Not reliable identification	*Enhydrobacter aerosaccus*	MH715214
83	RW3	Not reliable identification	*Enhydrobacter aerosaccus*	MH715215
84	RW4	Not reliable identification	*Micrococcus aloeverae*	MH715216
85	RW5	*Kocuria palustris*	*Kocuria palustris*	MH715217
86	RW6	Not reliable identification	*Kocuria rhizophila*	MH715218
87	RW7	*Pseudomonas alcaligenes*	*Pseudomonas aeruginosa*	MH715219
88	RW8	*Staphylococcus hominis*	*Staphylococcus hominis* subsp. *hominis*	MH715220
89	RW9	*Moraxella*_sg_*Moraxella osloensis*	*Enhydrobacter aerosaccus*	MH715221
90	RW10	Not reliable identification	*Elstera litoralis*	MH715222
91	RW12	*Bacillus subtilis*	*Bacillus subtilis* subsp. *inaquosorum*	MH715223
92	RW13	Not reliable identification	*Kocuria rhizophila*	MH715224
93	RW14	Not reliable identification	*Bacillus tequilensis*	MH715225
94	RW17	Enterobacter hormaechei	*Enterobacter tabaci*	MH715226
95	RW18	Pseudomonas alcaligenes	*Pseudomonas alcaligenes*	MH715227

4. CONCLUSION

The bacterial diversity and abundance among Rajapur- Unhale sediment and water samples were identified through MALDI-TOF based identification and 16S rRNA gene sequencing based identification. In sediment sample the diversity were found to be maximum compared to water sample. Among 55 isolates obtained only 15 isolates were identified through MALDI-TOF out of which 7 isolates were identified in both genus and species level. Whereas, four isolates were identified in genus level and four isolates were found to be mismatched identification compared to 16S rRNA gene sequencing. The MALDI-TOF database needs to be updated with environmental isolates for the easy and convenient identification. 16S rRNA gene sequencing found to be the gold standard method for the identification of bacterial isolates from all kinds of niche.

ACKNOWLEDGEMENT

The authors are very thankful to the Department of Microbiology, Bharathidasan University Constituent College, Kurumbalur, Perambalur, TamilNadu., India for prviding the facility to conduct our experiments and NCMR, Pune., India for helping us to perform sequencing of the samples.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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