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EXPERIMENTAL ARTICLES

Culture Dependent Diversity and Phylogeny of Thermophilic Bacilli from a Natural Hot Spring Reservoir in the Gir Forest, Gujarat (India)[1]

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Abstract—The thermophilic bacteria, isolated from a natural hot spring reservoir, Tulsi Shyam (Gir Forest, Gujarat, India) were characterized and diversified using the conventional approaches; Gram reaction, cell morphology, growth patterns, biochemical properties and antibiotic sensitivity. The bacteria were Gram positive, rod shaped and catalase positive. Majority of them produced amylase, indicating their ecological and biotechnological significance. The bacteria were further categorized on the basis of the amplified ribosomal DNA restriction analysis (ARDRA) patterns, generated by a tetracutter RE, HaeIII. As a polyphasic taxonomy approach, the isolates were clustered into 9 different groups, based on the conventional approaches and the ARDRA patterns. The further analysis was based on the 16S rRNA gene sequencing. The trend suggested that the phenotypic and phylogenetic diversity data considerably coincided. Overall, the part of Tulsi Shyam hot spring bacterial community represented by cultivated aerobic organotrophs can be clustered into 3 Genera, *Anoxybacillus*, *Geobacillus/Aeribacillus* and *Bacillus*. They can further be grouped into sub-clusters at the species level to establish phylogenetic and phenotypic relationship. Further, the diversity indices; Simpson's index, Dominance index and Shannon index indicated the species diversity, species richness and distribution evenness, respectively. A lower Simpson's index (0.1905) and higher Dominance index (0.8095) revealed significant bacterial diversity. While a higher Shannon index (2.987) suggested evenness in the bacterial distribution in different soil and water samples. The lower values of Berger-Parker Dominance index and Ginni coefficient supported the trends of the Shannon index. As the studied habitat was not earlier explored for the bacterial diversity, the findings would significantly add to the knowledge of the cultivation based microbial diversity.

Keywords: Thermophilic bacteria, polyphasic taxonomy, ARDRA, 16S rRNA sequencing, phylogeny, microbial diversity, hot springs, diversity indices

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INTRODUCTION

The life thrives even in the most challenging environmental conditions prevailing on the Earth, such as extremes of salinity, acidity, alkalinity, temperature and pressure. This has fascinated the research community to explore the microbial diversity and phylogeny under these inhospitable habitats. Similarly, various adaptive measures may also provide some clues to understand various evolutionary pathways. Besides, the extremophilic microorganisms may also possess great biotechnological potentials. Among the extremophiles, the thermophilic bacteria are common in soil and volcanic habitats, having very limited species composition. Yet, they possess all the major nutritional categories and metabolize various substrates. The ability to grow optimally at >60°C is associated with the extremely thermophilic groups, owing primarily to their thermally stable macromolecules (Brock, 1986; Austin, 1988; Singh, 2006; Kikani et al., 2010).

Among the genus *Bacillus* and related genera, thermophilic, psychrophilic, acidophilic, alkalophilic and halophilic bacteria are widely distributed in the nature, able to utilize a wide range of carbon sources for the heterotrophic or autotrophic growth. The genus *Bacillus* is a phenotypically large, diverse collection of Gram positive, spore forming, aerobic or facultatively anaerobic, rod-shaped bacteria. As a result of the advanced molecular techniques and bioinformatics tools, they were subjected to the considerable reclassification in recent past, revealing vast phylogenetic heterogeneity (Nazina et al., 2001). The phylogenetic divergence of the genus *Bacillus* has pointed to the unavoidable need of further studies to classify some of the members of the bacilli in appropriate taxonomic group (Nazina et al., 2001; Rainey et al., 1994; Logan et al., 2009). With the help of the 16S rRNA gene sequencing data, the genus *Bacillus* has been divided into more manageable and defined

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groups (Logan et al., 2009). Based on such studies, the facultative thermophilic species, such as *Bacillus* smithii, B. *coagulans* and *B. licheniformis* fall into the genus *Bacillus* genetic group 1, together with the other mesophilic species, such as *B. subtilis* (Rainey et al., 1994). However, the thermophilic bacteria belonging to the Bacillus genetic group 5 have been re-classified (Nazina et al., 2005). The thermophilic members, having growth optimum between 45 and more than 70°C are classified into the genera *Bacillus, Aeribacillus, Anoxybacillus, Geobacillus, Cerasibacillus, Caldalkalibacillus, Alicyclobacillus, Sulfobacillus, Brevibacillus, Ureibacillus, Thermobacillus* and *Thermoactinomyces* (Nazina et al. 2005; Logan et al., 2009).

In general, the 16S rRNA gene sequencing technique is used to determine the genus affiliation. However, for species differentiation DNA-DNA hybridization is needed. These data can be further used for the phylogenetic relatedness and species delineation. The amplified ribosomal DNA restriction analysis (ARDRA) can be used to analyze the culture-dependent diversity of a habitat. ARDRA is a commonly used technique to study the microbial diversity that depends on the DNA polymorphism. The 16S rDNA genes are amplified, using the universal or the genus-specific primers. Then they are digested by the tetracutter restriction endonucleases (REs), followed by the separation of the fragments on the high-density agarose or acrylamide gels. The emerging profiles are used either to cluster the community into genotypic groups or for the strain typing. Reportedly, ARDRA was used in the analysis of the mixed bacterial populations, belonging to various environmental niches (Cihan et al., 2012; Singh et al., 2012). Consequently, the polyphasic approaches are gaining attention in the field of microbial ecology to screen the microbes producing novel enzymes, having unique properties for the newer applications (Derekova et al., 2008; Kikani et al., 2010; Singh et al., 2012). The proposed habitat, a natural hot spring reservoir in Tulsi Shyam, Gujarat has not been earlier explored systematically. However, our research group at the Saurshtra University, Rajkot has reported on the thermostable α-amylases from the same habitat during last few years (Kikani and Singh 2011, 2012; Kikani et al., 2013). In the present report, we described the diversity and phylogeny of the thermophilic bacteria, using the polyphasic taxonomic approaches.

MATERIALS AND METHODS

The Natural Thermal Habitat, Tulsi Shyam

The research habitat, Tulsi Shyam is a natural hot spring reservoir located in the middle of the Gir Forest in the Gujarat State, India (Latitude: 21.051; Longitude: 71.025). As discussed, the proposed site has never been explored earlier. Besides, it is the only natural thermal habitat in the Saurashtra region in the Gujarat State, having religious significance too. In order to explore the bacterial diversity and phylogeny, three soil and two water samples were collected. The temperature and pH were 50°C and 6.5, respectively. As the hot spring reservoirs were spatially adjacent to each other, the physicochemical properties did not alter much.

Isolation of the Thermophilic Bacteria

The samples were serially diluted in the sterile distilled water. 1 g of each soil sample was diluted into 10 mL sterile distilled water, while 2 mL of the water sample was diluted into the sterile distilled water to a final volume of 10 mL. From the diluted samples, 5% (vol/vol) was inoculated into the modified medium, containing 0.7% (wt/vol) peptone, 0.5% (wt/vol) yeast extract, 0.3% (wt/vol) malt extract and 0.5% (wt/vol) NaCl, along with 1% (wt/vol) glucose, at different medium pH between 7 and 9. The medium components were purchased from Hi Media Ltd, India. The cultures were incubated at 60°C for 24–48 h. Thereafter, 0.1 mL of the enriched culture was inoculated on the modified thermophilic agar plates containing the same ingredients with 5% (wt/vol) agar at pH 7–9. The inoculated plates were incubated at the temperatures between 37 and 70°C for 24–48 h. The colonies were subsequently streaked repeatedly until pure colonies were obtained. The colony characterization, Gram reaction and the morphological diversity were carried out. The growth patterns and the optimum growth parameters were evaluated for the individual isolate. The biochemical characteristics and the antibiogram of the thermophilic bacteria were assessed later on for further diversification. The thermophilic bacterial isolates were also screened for various enzymes, such as protease, amylase, cellulase and lipase on the Gelatin, Starch, Dubo's cellulose and Tributyrin agar plates, respectively. Additionally, the data of the classical approaches were arranged in a matrix of the binary data, which were further used to create the phenogram, using Jaccard's similarity index by PAST software (Hammer et al., 2001).

Isolation of the Genomic DNA

The bacterial cultures were grown under their respective optimum conditions. The activated cultures were used for the isolation of the genomic DNA. The centrifuged bacterial pellet was treated with the STE buffer, pH 8 (containing 25 mM NaCl and 1 mM EDTA in 10 mM tris-Cl) and GET buffer, pH 8 (containing 50 mM glucose, 10 mM EDTA in 25 mM tris-Cl), followed by the cellular lysis using the chemical lysis buffer (1 mL, containing 10 mg of lysozyme and 0.1 g SDS). Later, it was incubated at 65°C for 2 h. After sufficient cooling, it was treated with the mixture of phenol, chloroform and iso-amyl alcohol in the ratio of 25 : 24 : 1. The supernatant was further treated with the mixture of choloroform and iso-amyl alcohol

in the ration of 24 : 1. To the treated sample, 3 M potassium acetate was added, followed by the addition of double volume of the chilled ethanol for the precipitation of DNA. The spooled or centrifuged DNA was air-dried and dissolved in the minimal volume of the TE buffer, pH 8 (containing 1 mM EDTA in 10 mM tris-Cl).

Determination of the DNA Yield and Purity

The purity and yield of the extracted DNA was estimated spectrophotometrically by calculating the ratio of the absorptions at 260 and 280 nm. Additionally, the DNA preparation was also analyzed on the Agarose gel electrophoresis, with 0.8% (wt/vol) Agarose.

The DNA concentration was calculated, in μg/mL as follows:

$$\text{DNA concentration} = 50 \times A_{260} \times \text{Dilution Factor.}$$

Amplified 16S rDNA Restriction Analysis (ARDRA)

The genomic DNA from each bacterial isolate was amplified, using the 16S universal bacterial forward and reverse primers. To the 100 ng of DNA template, 25 pmol of each, Forward (AGA GTT TGA TCC TGG CTC AG) and Reverse (ACG GCT ACC TTG TTA CGA CTT) oligonucleotide primers (Imperial Life Sciences, India) and 25 μL of 2X Red Mix Plus (Merck, India) were added. The amplification protocol consisted of step-1, the initial denaturation at 94°C for 1 min; step-2, denaturation at 94°C for 30 s; step-3, annealing at 54°C for 30 s; step-4, extension at 72°C for 2 min. Subsequently, the steps 2, 3 and 4 were repeated for 29 cycles, followed by a final elongation step at 72°C for 2 min. Besides, two negative controls, one without template DNA and another without any primer were also included. The amplified products and the 500 bp DNA marker (Merck, India) were loaded onto the agarose gel of 1.2% (wt/vol) Agarose concentration. The amplified products were stored at −20°C till further use.

The amplified products were digested, using the tetra-cutter restriction enzyme, Hae-III (Fermentas, India), which cuts at GG/CC. To the 15 μL of the amplified product, 2 μL of Hae-III and 2 μL of the digestion buffer (Fermentas, India) were added. The final volume was adjusted to 35 μL with the Nuclease free sterile MiliQ grade water (Fermentas, India). The reaction mixture was gently mixed and incubated at 37°C for 2 h. The digested products and 100 bp DNA ladder (Merck, India) were loaded onto the agarose gel of 3% (wt/vol) agarose concentration (Merck, India) to visualize the variations in the digestion patterns, reflecting the diversity of the thermophilic bacteria from Tulsi Shyam. The trends were later compared with those obtained by the *In-Silico* ARDRA by Hae-III, using the software, CLEAVER (Jarman 2006).

16S rRNA Gene Sequencing

Based on the information from the conventional microbiological approaches and the molecular techniques, the bacterial isolates with different properties and diverse band patterns were subsequently identified by the 16S rRNA gene sequencing. The genomic DNA was isolated from the pure culture and used to amplify the ~1.5 kb 16S rRNA fragment using consensus primers and high-fidelity PCR polymerase. The PCR product was bi-directionally sequenced using a forward, reverse and internal primer. The sequence was subsequently aligned and analyzed to determine the closest homolog to the microbe.

Construction of the Phylogenetic Tree

The 16S rRNA gene sequences were analyzed, using the NCBI BLAST search program at http://www.ncbi.nlm.nih.gov/BLAST within the GenBank database. The phylogenetic tree was constructed, using the software Clustal W (http://www.genome.jp/tools/clustalw/). The bacterial diversity of the natural thermal habitat was further analyzed statistically, in terms of the species richness and evenness by calculating various diversity indices (Hammer et al., 2001). The significant differences in the microbial diversity between soil and water samples were analyzed by one-way ANOVA.

RESULTS AND DISCUSSION

The Conventional Approaches for the Bacterial Diversity

To explore the culture-dependent diversity, a total of 21 thermophilic bacterial isolates were obtained. Each grew within the temperature range of 45–70°C and the medium pH, 7–9. Evidently, none could grow at 37°C, indicating their true thermophilic nature. Although majority of the bacteria were neutrophilic in nature, only limited alkali tolerance was apparent. The bacterial isolates were Gram positive, displaying variation in their cell morphology, cell size and arrangements (Table 1). Similar studies have been reported on the culture-dependent diversity by Cihan et al. (2012), where the bacterial isolates, belonging to the genus *Geobacillus*, grew between 50 and 70°C and also displayed Gram positive or Gram variable features, endospore formation, catalase activity and motility (Cihan et al., 2012).

Remarkably, the bacteria in the present study displayed positive test for the catalase production, while few of them were oxidase positive. The patterns generated on other biochemical tests were also documented in Table 2. The sugar utilization profile of the studied bacterial isolates can be compared with the other report on the alkalithermophilic bacteria from the hot springs in Buryatia (Zaitseva et al., 2004), where all the isolates were catalase positive and could utilized glucose and sucrose, while most could ferment mannitol

Table 1. The properties of the thermophilic bacteria isolated from Tulsi Shyam (India)

No.	Isolate	Growth pH	Growth Temperature	Gram Reaction and Morphology	Cell size
1	TSWK1-1	7 (6–9)	50°C (45–70°C)	Gram-positive, long thin rods, arranged singly and in chains	Width: 0.13 μm Length: 3.77 μm
2	TSWK2-1	7 (6–9)	45°C (45–60°C)	Gram-positive, very short thin rods, arranged in chains	Width: 0.04 μm Length: 2.48 μm
3	TSSA-1	7 (6–9)	50°C, 55°C (45–70°C)	Gram-positive short thin rods, arranged singly	Width: 0.18 μm Length: 3.06 μm
4	TSSA-2	7, 8 (6–9)	55°C (45–70°C)	Gram-positive short thin rods, arranged singly	Width: 0.11 μm Length: 2.90 μm
5	TSSA-3	7 (6–9)	50°C (45–60°C)	Gram-positive, short thick rods, arranged singly	Width: 0.25 μm Length: 3.26 μm
6	TSSB-1	7 (6–9)	55°C (45–55°C)	Gram-positive, long thick rods, arranged singly and in chains	Width: 0.23 μm Length: 3.75 μm
7	TSSB-2	7 (7–10)	50°C (45–60°C)	Gram-positive, very short thin rods, arranged in chains	Width: 0.08 μm Length: 2.54 μm
8	TSSB-3	7 (7–9)	50°C (45–55°C)	Gram-positive, long thick rods, arranged singly and in chains	Width: 0.3 μm Length: 3.65 μm
9	TSSB-4	8 (6–10)	45°C (45–55°C)	Gram-positive, thin coccobacilli, arranged singly	Width: 0.05 μm Length: 2.54 μm
10	TSSB-5	8, 9 (6–10)	45°C (45–60°C)	Gram-positive, long thin rods, arranged in singly	Width: 0.08 μm Length: 3.69 μm
11	TSSB-6	8 (7–10)	50°C (45–60°C)	Gram-positive, long thin rods, arranged in chains	Width: 0.11 μm Length: 3.62 μm
12	TSSC-1	7, 8 (6–9)	60°C (45–70°C)	Gram-positive, very long thin rods, arranged in chains	Width: 0.2 μm Length: 4.56 μm
13	TSSC-2	7 (6–9)	55°C (45–70°C)	Gram-positive, very long thin rods, arranged singly	Width: 0.18 μm Length: 4.35 μm
14	TSSC-3	7 (6–9)	55°C (45–70°C)	Gram-positive short thin rods, arranged singly	Width: 0.15 μm Length: 3.17 μm
15	TSSC-4	7 (6–9)	55°C (45–60°C)	Gram-positive, thick cocobacilli, arranged singly and in chains	Width: 0.25 μm Length: 2.25 μm
16	TSSC-5	8 (8–10)	45°C (45–55°C)	Gram-positive, Thin short rods, arranged singly and in chains	Width: 0.11 μm Length: 2.07 μm
17	TSSC-6	7, 8 (6–9)	55°C (45–70°C)	Gram-positive, very long thin rods, arranged in chains	Width: 0.2 μm Length: 4.5 μm
18	TSSC-7	8 (8–9)	50°C (45–55°C)	Gram-positive, Thin short rods, arranged singly and in chains	Width: 0.15 μm Length: 2.22 μm
19	TSSC-8	7, 8 (6–9)	50°C (45–60°C)	Gram-positive, short thick rods, arranged singly	Width: 0.25 μm Length: 3.17 μm
20	TSSC-9	7 (6–8)	55°C (45–70°C)	Gram-positive short thin rods, arranged singly	Width: 0.17 μm Length: 3.42 μm
21	TSSC-10	7 (7–8)	50°C (45–60°C)	Gram-positive, short thick rods, arranged singly	Width: 0.38 μm Length: 3.15 μm

Table 2. The biochemical tests of the thermophilic bacteria isolated from Tulsi Shyam (India)

Biochemical Tests	Thermophilic bacterial isolates																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Gelatin hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Starch hydrolysis	+	+	–	–	+	–	+	–	–	+	+	+	+	+	–	–	+	–	+	+	–
Cellulose hydrolysis	–	–	+	+	–	–	–	–	–	–	+	+	+	+	–	–	+		–	+	–
Lipid hydrolysis	–	–	–	–	–	+	–	+	+	–	–	–	–	–	+	–	–	–	–	–	–
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	–	–	–	–	+	–	–	–	+	–	–	+	+	–	–	–	+	–	+	–	+
Sugar fermentation test																					
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	+	–	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Fructose	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+
Mannitol	–	+	+	+	+	–	+	–	–	+	+	+	+	+	–	–	+	–	+	+	+
Galactose	+	+	–	–	–	–	+	–	–	+	+	+	+	–	–	–	+	–	–	–	–
Phenyl alanine deamination	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Citrate utilization	+	+	+	+	+	–	+	–	–	+	+	+	+	+	–	–	+	–	+	+	+
TSI test																					
Acid formation in slant	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid formation in butt	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas production	+	+	–	–	–	+	+	+	+	+	+	–	–	–	+	+	–	+	–	–	–
H_2S production	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Indole formation	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Urea degradation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red test	+	+	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–
Voges–Proskauer test	+	+	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–
Nitrate reduction to Nitrite	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ammonia production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Abbreviations: "+" indicates the positive test; "–" indicates the negative test Isolate indications: 1: TSWK1-1, 2: TSWK2-1, 3 to 5: TSSA1 to TSSA-3, 6 to 11: TSSB-1 to TSSB-6, 12 to 21: TSSC-1 to TSSC-1.

Table 3. The antibiotic sensitivity profiles of the thermophilic bacteria isolated from Tulsi Shyam (India)

Antibiotics used (Concentration/ disc)	Thermophilic bacterial isolates																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Amikacin (30 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefotaxime (30 μg)	S	S	S	I	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S
Ampicillin (10 μg)	I	S	I	I	S	R	S	R	S	S	I	S	I	S	S	S	S	S	S	I	I
Cefoperazone (75 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin (10 μg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Clarithromycin (15 μg)	R	I	I	R	I	R	I	R	I	I	S	I	I	R	R	S	I	S	I	I	I
Sparfloxacin (5 μg)	R	I	R	I	I	R	I	R	R	I	I	I	I	I	R	S	I	S	I	R	I
Ciprofloxacin (5 μg)	R	I	I	I	R	R	I	R	R	I	I	R	R	I	I	S	R	S	R	I	R
Cefadroxil (30 μg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefuroxime (30 μg)	I	I	R	R	S	R	I	R	S	I	S	R	R	S	S	S	R	S	S	R	S
Azithromycin (15 μg)	R	I	R	R	I	R	I	R	I	I	I	R	I	R	S	S	R	S	I	R	I
Roxithromycin (30 μg)	R	S	I	I	S	R	S	R	S	S	S	I	S	I	S	S	I	S	S	I	I

Abbreviations:
S: Sensitive; I: Intermediate; R: Resistance. Isolate indication by numbers: 1: TSWK1-1, 2: TSWK2-1, 3 to 5: TSSA1 to TSSA-3, 6 to 11: TSSB-1 to TSSB-6, 12 to 21: TSSC-1 to TSSC-1.

as well. The antibiotic sensitivity of the thermophilic bacteria highlighted that all the bacterial strains were sensitive to the Gram positive-specific antibiotics (Table 3).

The thermophilic bacteria were screened for the production of the extracellular enzymes; amylases, proteases, cellulases and lipases (Table 2), where majority of them secreted amylases at a broad range of temperature (Fig. 1) and pH (Fig. 2). While majority of them grew and secreted amylases optimally at pH 7, the alkali-tolerance nature was apparent in few cases. Most of the cellulase producers also produced amylases. However, only few produced lipases, while none could secrete proteases significantly. Overall, amylase may be regarded as marker enzyme for the proposed natural thermal habitat. Similar results were obtained by the thermophilic bacilli, isolated from the Bulgerian hot springs (Derekova et al., 2008). On the same note, Singh et al. (2012) emphasized on the biocatalytic potential of the bacteria and the application of the conventional approaches in conjunction with the ARDRA profiling to explore the diversity of the bacteria, associated with the early phase of the mushroom composting. In another study, Babavalian et al. (2013) also explicated bacterial diversity based on various their ability to secrete hydrolytic enzymes.

ARDRA

The polyphasic approach has focused attention in the microbial ecology and taxonomical investigations. This approach includes the phenotypic and genotypic information, based on various molecular fingerprinting methods to generate various clusters of the bacteria, belonging to the same genus (Vaneechoutte et al., 1992; Cihan et al., 2012; Singh et al., 2012). Presently, RFLP-16S rDNA is recognized as a powerful and rapid method to identify relatives of a large number of the newer isolates (Vinuesa et al., 2005); (Appunu et al., 2008). Yet, ARDRA is an extension and advancement of the molecular technique, Restriction Fragment Length polymorphism (RFLP).

The genomic DNA was isolated from the bacterial isolates with good purity and yield. The ratio >1.8 at

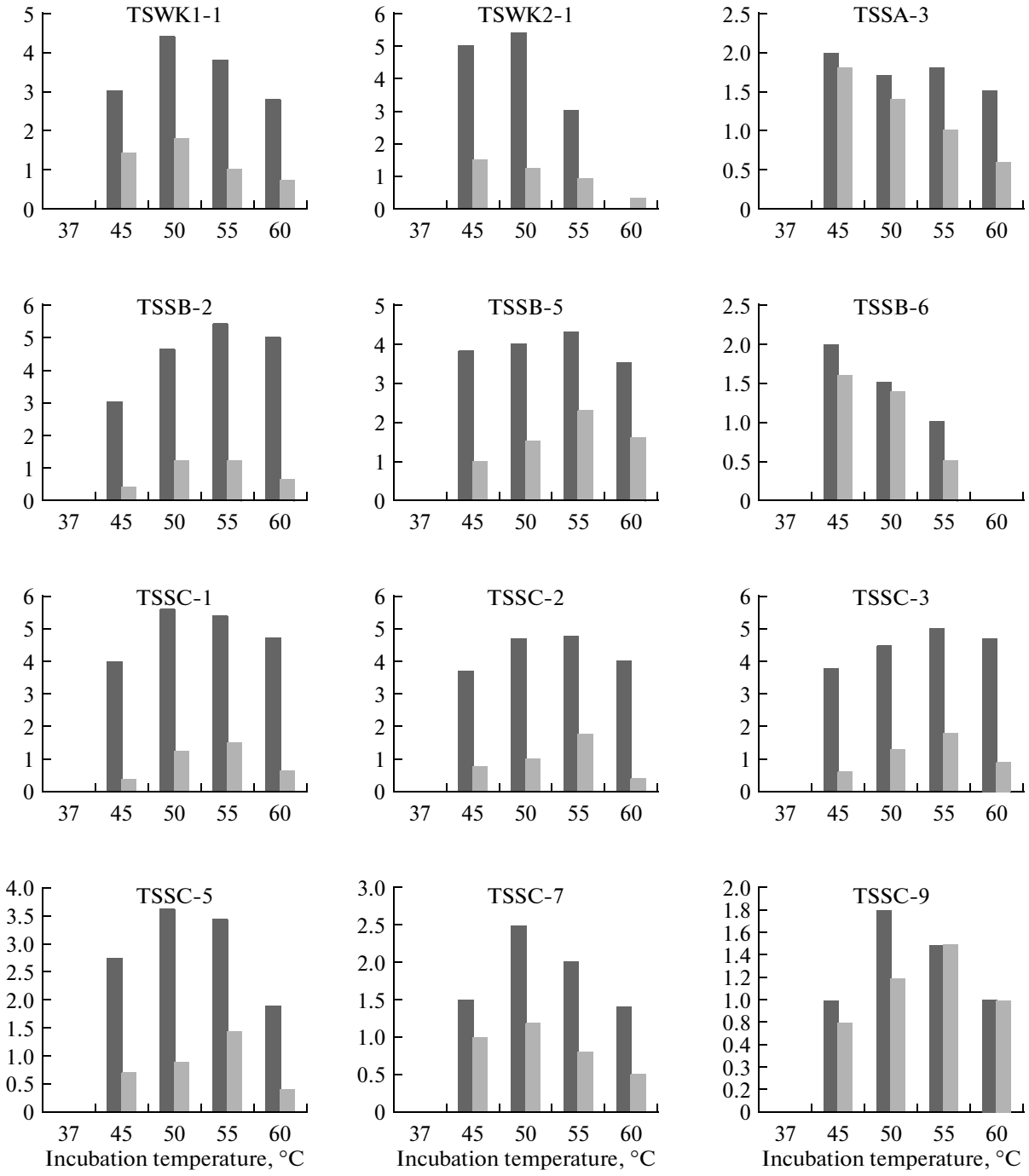

Fig. 1. Effects of the incubation temperature (37–60°C) on the amylase secretion and the bacterial growth; Zone ratio (dark bars) and colony diameter, cm (light bars).

260/280 nm, indicated good purity of the extracted DNA. In order to explore the culture-dependent diversity more precisely, the 16S rDNA gene (~1500 bp) was amplified from the DNA of the bacterial isolate. The amplified product was analyzed on 1.2% (wt/vol) agarose gel electrophoresis (Fig. 3). Later, the amplified products were digested with the tetracutter restriction enzyme (Fig. 4). The restriction profiles were analyzed further to explore the diversity. The reason for using tetra-cutter RE was to obtain higher probability of the restriction sites in the amplified product, which would thereby be reflected in the varied restriction profiles even among the closely related bacterial isolates. Thus, even a minor difference in the sequence would lead to variable fragment pattern, leading to the comprehensive and accurate

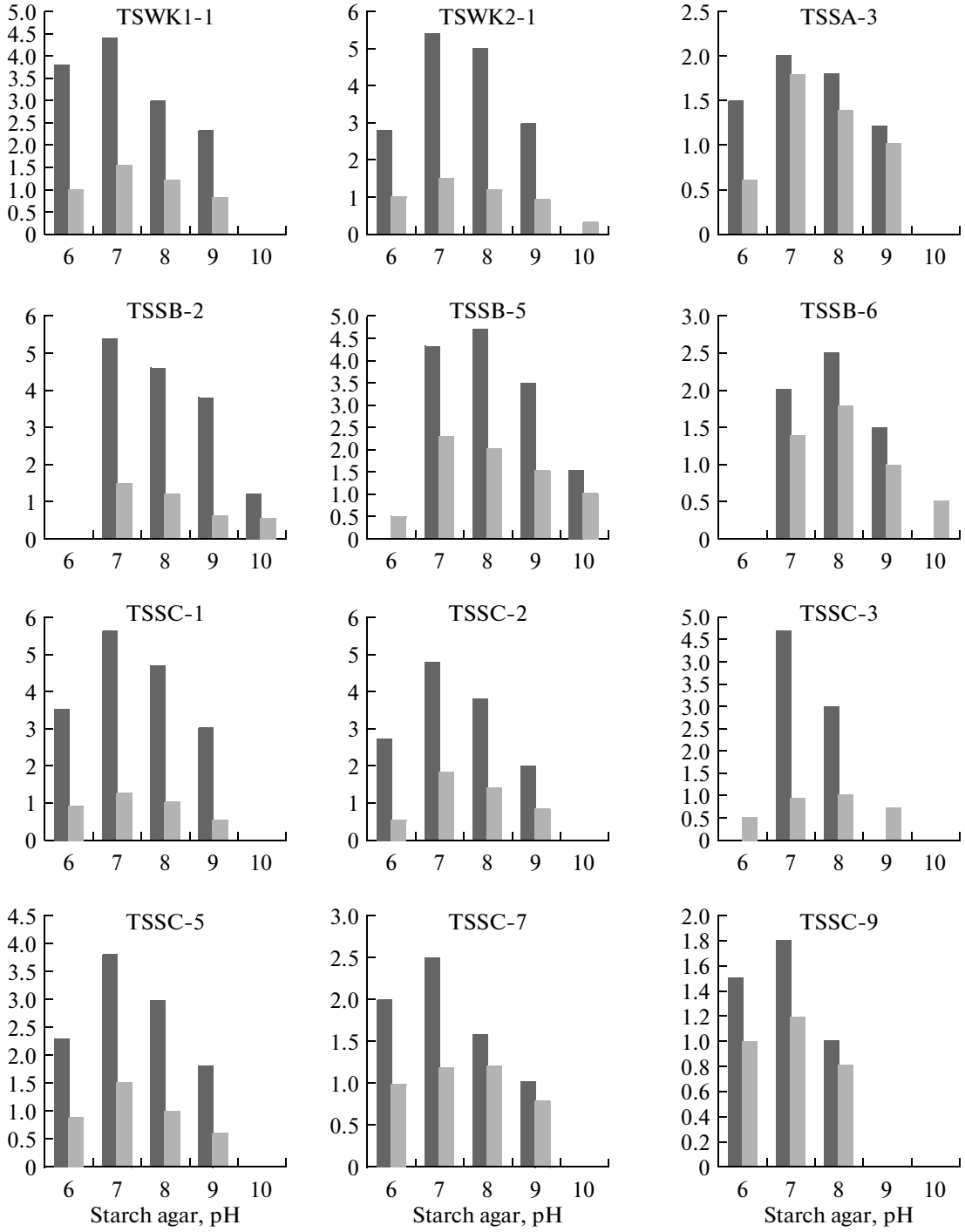

Fig. 2. Effects of the medium pH (6–10) on the amylase secretion and the bacterial growth; Zone ratio (dark bars) and colony diameter, cm (light bars).

information on the diversity. As previously reported, the restriction endonucleases, AluI and HaeIII were most efficiently and frequently used in the ARDRA technique to differentiate the genus *Geobacillus* (Kuisiene et al., 2007). Reportedly, the ARDRA profiles of the genus *Bacillus* and *Geobacillus* have been studied among the thermophilic, spore-forming bacteria, an approach proved to be valuable, easy and accurate technique for the identification of genus *Geobacillus*; (Caccamo et al., 2001; Kuisiene et al., 2002;

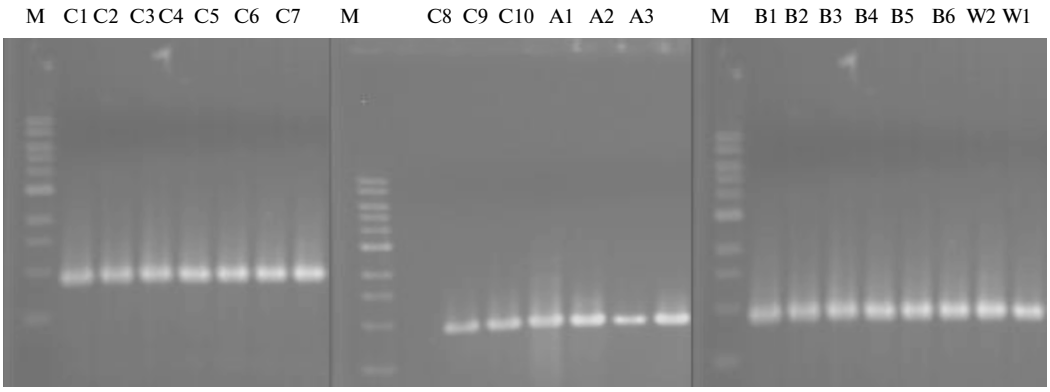

Fig. 3. Analysis of the PCR products, containing the amplified 16S rRNA gene by the agarose gel glectrophoresis; 1.2% (wt/vol) agarose: M (100 bp DNA Ladder, Merck), C1–C10 (thermophilic bacteria, TSSC-1 to TSSC-10), A1-A3 (thermophilic bacteria, TSSA-1 to TSSA-3), B1–B6 (thermophilic bacteria, TSSB-1 to TSSB-6), W1 (thermophilic bacterium, TSSWK1-1) and W2 (thermophilic bacterium, TSSWK2-1).

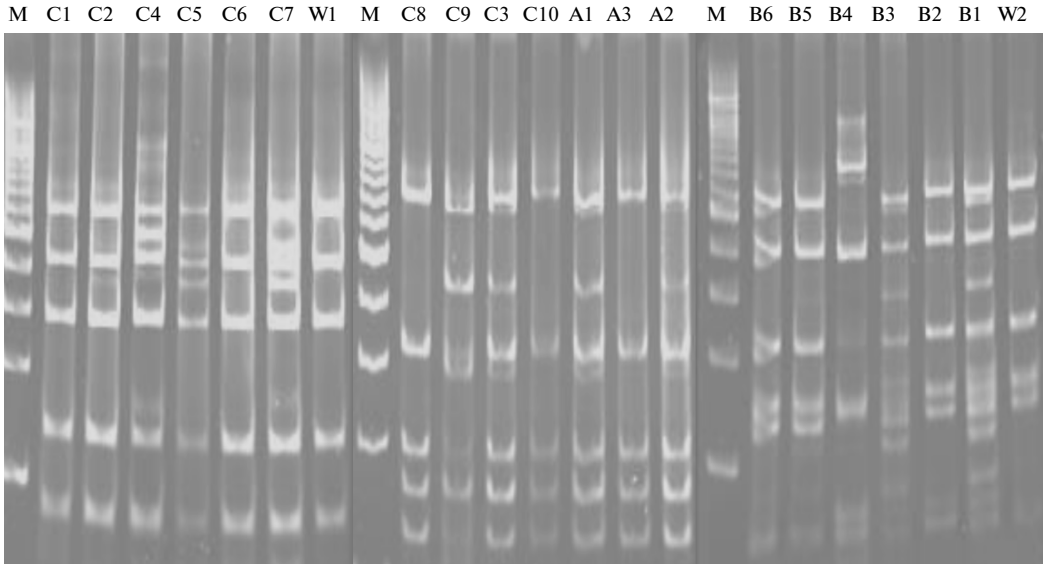

Fig. 4. Analysis of the restriction profiles by the tetra-cutter RE, Hae-III on the agarose gel electrophoresis; 3% (wt/vol) agarose: M (100 bp DNA Ladder, Merck), C1–C10 (thermophilic bacteria, TSSC-1 to TSSC-10), A1–A3 (thermophilic bacteria, TSSA-1 to TSSA-3), B1–B6 (thermophilic bacteria, TSSB-1 to TSSB-6), W1 (thermophilic bacterium, TSSWK1-1) and W2 (thermophilic bacterium, TSSWK2-1).

Kuisiene et al., 2007). Further, it is established that the rDNA genes are organized as a multigene family, expressing with a copy number from 1 to 15. As there might be sequence heterogeneity among the multiple 16S rRNA genes, it may probably affect the recognition sites of the restriction endonucleases. Kuisiene et al. (2007), therefore, recommended that the theoretically and experimentally obtained digestion profiles should be compared. Therefore, we performed *In-Silico* ARDRA using the software, CLEAVER (Jarman, 2006). The *In-Silico* ARDRA results were further compared with the experimental data to confirm the accuracy and efficiency of the results. The common feature in the restriction pattern of each isolate, using Hae-III was the occurrence of a 600 bp fragment. However, as per the *In-Silico* ARDRA patterns, its position in the restriction map (5' to 3' direction) varied considerably, which was used to further diversify the bacterial community in hot spring at the Tulsi Shyam, India (Table 4). Similarly, Cihan et al. (2012) compared the experimental and theoretical ARDRA profiles, for judging the diversity of *Geobacillus* sp. and *Aeribacillus* sp., using the restriction enzymes Hae-III, Alu-I and Taq-I.

Table 4. Comparison of the *In-Silico* ARDRA with the Experimental ARDRA

Identification using 16S rRNA sequencing	Thermophilic bacteria	Theoretical (*In-Silico*)		Experimental	
		Cuts	Fragments (5' to 3' direction)	Cuts	Fragments
Bacillus amyloliquifaciens	TSWK1-1	5	308, 50, 601, 458, 126	5	600, 450, 300, 125, 50
Bacillus licheniformis	TSWK2-1, TSSB-5, TSSB-2, TSSB-6	5	242, 150, 599, 457, 100	5	600, 410, 230, 160, 150
Aeribacillus pallidus	TSSA-3, TSSC-8, TSSC-10	7	216, 78, 22, 301, 110, 184, 603	7	600, 310, 220, 200, 110, 78, 22
Bacillus aerius	TSSB-1, TSSB-3	6	293, 22, 599, 457, 108, 150	7	600, 400, 293, 200, 150, 108, 22
Anoxybacillus voinovskiensis	TSSB-4	6	248, 78, 140, 459, 605, 78	7	700, 600, 400, 248, 140, 89, 78
Anoxybacillus beppuensis	TSSC-1, TSSC-2, TSSC-6	6	321, 78, 140, 459, 600, 96	5	600, 450, 325, 140, 78
Bacillus sp.	TSSC-3, TSSC-9, TSSA-1, TSSA-2	7	208, 78, 22, 301, 110, 184, 606	7	600, 310, 208, 190, 100, 78, 22
Anoxybacillus rupiensis	TSSC-4	6	318, 78, 140, 459, 600, 114	7	600, 500, 459, 300, 150, 120, 78
Anoxybacillus amylolyticus	TSSC-5, TSSC-7	6	312, 78, 140, 459, 600, 148	8	600, 470, 450, 380, 300, 170, 150, 78

Along the lines of the present study, the conventional and molecular methods, such as ARDRA were used to study the fungal diversity of soil, as affected by the land usage (Grantina et al., 2012). On a similar note, the *Robinia pseudoacacia* micro symbionts, isolated from Poland and Japan were identified and characterized by various genomic approaches, mainly RFLP-16S rDNA patterns comparison and the atpD and dnaK gene sequence analysis. The G + C% content and the DNA-DNA hybridization profiles were also compared (Mierzwa et al., 2010). The terminal restriction fragment length polymorphism (T-RFLP) was used to explore the microbial diversity in the sediments of the urban lakes at China (Zhao et al., 2012).

Table 5. Calculation of various Diversity Indices, with respect to the species richness and the evenness in their distribution

Diversity indices	Value
Simpson's index	0.1905
Dominance index	0.8095
Shannon index	2.987
Berger-Parker Dominance index	0.1905
Margalef richness index	2.628
Menhinick index	1.964
Ginni coefficient	4.09
Equitability index	0.9423

Analysis based on the Diversity Indices

Based on the trends emerged by the conventional and molecular approaches, the thermophilic bacteria were stratified in the 9 groups (Table 4). The data was analyzed statistically to explore the diversity. The diversity index is a mathematical measure of the species diversity in a particular habitat or community. It provides information about the community structure and the composition rather than merely the species richness (Table 5). In this context, the Simpson's index describes the probability of the similarity between two randomly selected species. It ranges between 0, highlighting infinite diversity and 1, no diversity. Similarly, the Dominance index signifies the inverse relationship with the Simpson's index. Higher Dominance index indicates greater diversity. The values of the Simpson's index (0.1905) and the Dominance index (0.8095) indicated good diversity among the bacterial species (Table 5). On the same note, Kiel and Gaylarde (2007) reported that the salt concentrations and the seasonal variations affected the microbial diversity in the Southern Brazil, as indicated by Simpson Index. Shannon index is commonly used to characterize the species diversity in a community, with respect to the species richness and the evenness. A low value of the Shannon index indicates uneven species distribution in a particular habitat. Reportedly, the

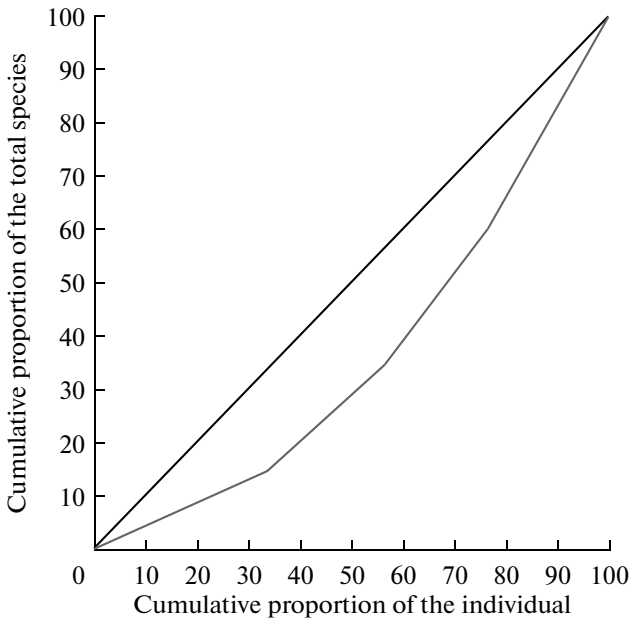

Fig. 5. The lorenz Curve, exhibiting the cumulative proportion between the individuals and total bacterial species.

Shannon index was used to explain the microbial diversity of the Arabian Sea (Kapley et al., 2007) and the Southern West of the China (Chen et al., 2012). More recently, Yousuf et al. (2012) used the Simpson and the Shannon indices, together with Chao and ACE richness estimators to explore microbial diversity and richness. In the similar context, the Menhinick index describes the species richness, while the Equitability index highlights on the evenness in the distribution. Another parameter, Berger-Parker dominance index indicates on the proportional abundance of the most abundant species in comparison to other bacterial species in a particular community. In the present study, the lorenz curve was used to explain the inequality in the biodiversity, where the cumulative proportion of the species was plotted against the total individuals (Fig. 5). The Ginni coefficient is the area between the line of perfect equality and the absolute lorenz curve. The higher values of the Ginni coefficient obviously indicate more unequal distribution (Wittebolle et al., 2009).

16S rDNA Sequencing and Phylogeny

The 16S rRNA gene sequencing is widely used as a reliable technique in the bacterial taxonomy. Evidently, it is routinely used in the polyphasic approach, describing any bacterial species or higher taxa (Ludwig et al., 1999); (Suslova et al., 2012); (Khanaeva et al., 2013). In the present study, the bacterial isolates, exhibiting different profiles, with respect to the conventional microbiological features and the ARDRA band patterns were further identified by the 16S rRNA gene sequencing. The sequences were submitted to NCBI, as *Bacillus amyloliquifaciens* TSWK1-1(GenBank Number, GQ121033), *Bacillus licheniformis* TSWK2-1 (GeneBank Number, JF834890), *Anoxybacillus beppuensis* TSSC-1 (GenBank Number, EU710556), *Bacillus* sp. TSSC-3 (GenBank Number, EU710557) and *Aeribacillus pallidus* TSSA-3 (GenBank Number, KC119567), *Bacillus aerius* TSSB-1 (GenBank Number, KC759318), *Anoxybacillus voinovskiensis* TSSB-4 (GenBank Number, KC759321), *Anoxybacillus rupiensis* TSSC-4 (GenBank Number, KC759325) and *Anoxybacillus amylolyticus* TSSC-5 (GenBank Number, KC759320). The phylogenetic tree was constructed to compare the evolutionary distances among the thermophilic bacteria (Fig. 6). The phenogram is a diagrammatic representation of the taxonomic relationships among the studied bacteria, based on the overall similarity of many characteristics (Fig. 7). The phenogram supported the patterns of the ARDRA and clusters generated by *in-Silico* ARDRA analysis as represented in Table 5. Significantly, the phenotypic data may thus be useful to explore the biotechnological potentials of the thermophilic bacteria using their biochemical properties. Overall, the bacterial community of the studied thermal habitat was grouped into 3 major clusters; *Anoxybacillus* sp., *Geobacillus/Aeribacillus* sp. and *Bacillus* sp. The clusters are further divisible into the sub-clusters to establish phylogenetic and phenotypic relationship among the thermophilic bacteria and their nearest homologs.

CONCLUSION

The natural thermal habitat, Tulsi Shyam is an ancient religious place located in the Saurashtra region of the Gujarat State, India. The present study aimed at the culture-dependent diversity of the thermophilic bacteria from this thermal habitat, using the polyphasic taxonomic approach, where the conventional microbiological traits were clubbed with certain molecular approaches, such as 16S rDNA sequences and ARDRA to categorize the bacterial community structure and the assess the phylogenetic relationships. The statistical approaches further explained the microbial diversity, in terms of the species richness and the evenness in the distribution. Noticeably, the proposed research site was never studied earlier for the bacterial diversity and biotechnological potentials. Therefore, the findings emerged from the present research would be a valuable addition to the diversity and phylogeny of the thermophilic bacteria of the natural thermal habitats.

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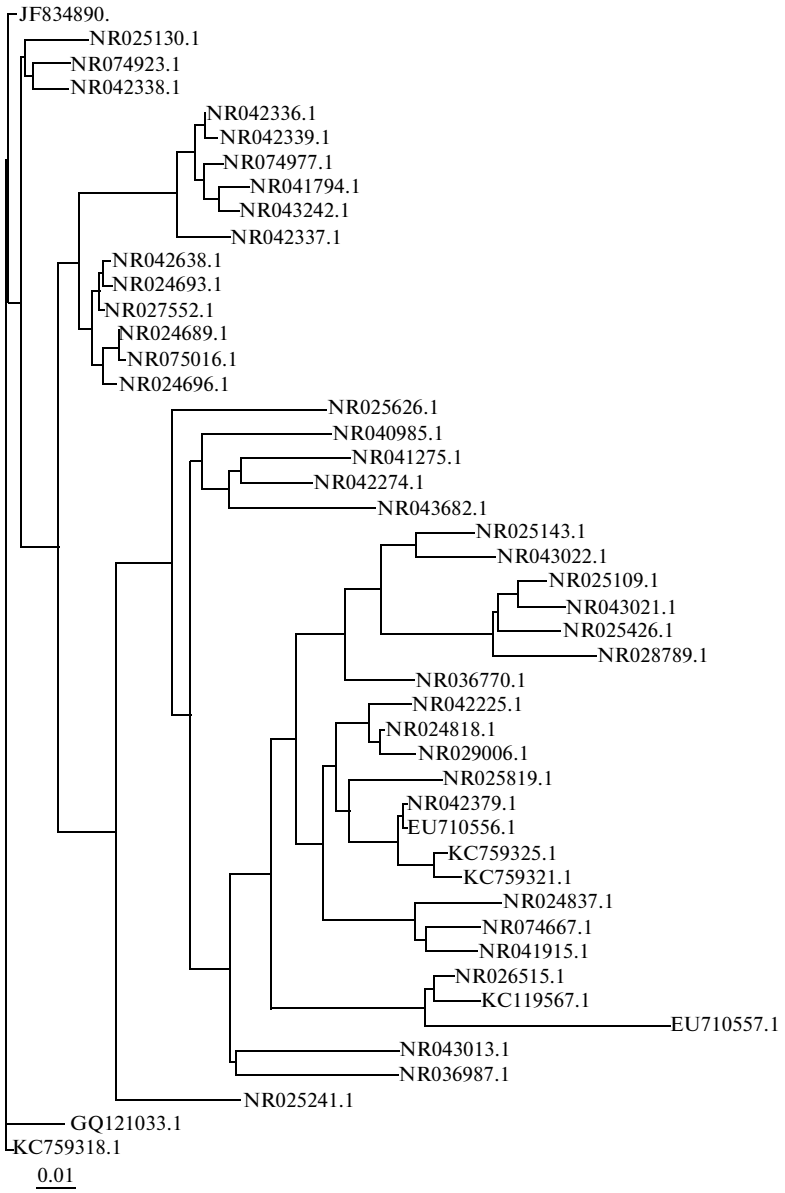
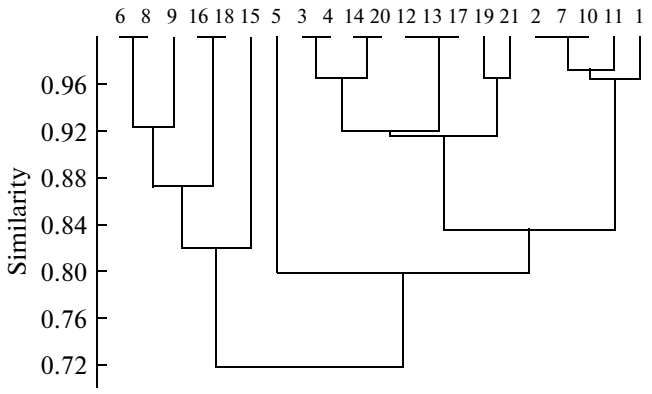


Fig. 6. The phylogenetic tree, exhibiting the phylogenetic relations between the thermophilic bacteria dwelling in the natural hot spring reservoir at Tulsi Shyam with their nearest homologs.



tific and Industrial Research (CSIR), the Government of India, New Delhi is also acknowledged. AKS gratefully acknowledges the Research Fellowship in Sciences for Meritorious Students by UGC, New Delhi, India. BAK also acknowledges the International Travel Grant awarded by the Department of Science

Fig. 7. The phenogram, exhibiting the phenotypic relatedness among the thermophilic bacteria dwelling in the natural hot spring reservoir at Tulsi Shyam with their nearest homologs: 1 (TSWK1-1), 2 (TSWK2-1), 3–5 (TSSA1 to TSSA-3), 6–11 (TSSB-1 to TSSB-6) and 12–21 (TSSC-1 to TSSC-10).

and Technology (DST), the Government of India, New Delhi to present his research in the 9th International Congress on Extremophiles at Sevilla, Spain in 2012.

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