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ORIGINAL ARTICLE

Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs

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Abstract The aim of this study was to analyze and characterize the diversity of culturable thermotolerant bacteria in Manikaran hot springs. A total of 235 isolates were obtained employing different media, and screened for temperature tolerance (40 °C–70 °C). A set of 85 isolates tolerant to 45 °C or above were placed in 42 phylogenetic clusters after amplified ribosomal DNA restriction analysis (16S rRNA-ARDRA). Sequencing of the 16S rRNA gene of 42 representative isolates followed by BLAST search revealed that the majority of isolates belonged to Firmicutes, followed by equal representation of Actinobacteria and Proteobacteria. Screening of representative isolates (42 ARDRA phylotypes) for amylase activity revealed that 26 % of the isolates were positive, while 45 % exhibited protease activity, among which one amylase and six protease producers were tolerant up to 70 °C. BIOLOG-based identification of 13 isolates exhibiting temperature tolerance up to 70 °C, using carbon utilization patterns and sensitivity to chemicals, revealed a high degree of correlation with identification based on 16S rRNA gene sequencing for all isolates, except one (M48). These promising isolates showing a range of useful metabolic attributes demand to be explored further for industrial and agricultural applications.

Keywords Culturable bacteria · 16S rRNA gene · ARDRA · Sequencing · BIOLOG analysis · Enzymes

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Introduction

Exotic niches, such as thermal springs, harbor populations of microorganisms that can be a source of commercially important products like enzymes, sugars, compatible solutes and antibiotics (Satyanarayana et al. 2005). Thermal springs are a manifestation of geological activity and represent aquatic microcosms that are produced by the emergence of geothermally heated groundwater from the Earth's crust. Prokaryotes are the major component of most ecosystems, being ubiquitous in nature because of their small size, easy dispersal, metabolic versatility, ability to utilize a broad range of nutrients, and tolerance to unfavorable and extreme conditions. Thermal springs are, therefore, no exception to colonization by prokaryotes. Diversity analysis of such extreme environments has grown in significance because of their diverse and unusual chemistry and the opportunity they provide to identify rare compounds and genes (Kuddus and Ramtekke 2012).

In the past, phylogenetic- combined with cultivation- and in situ microbial physiological- and ecological-studies have revealed the abundant diversity of thermophilic microorganisms inhabiting hot springs around the world in locations like Japan, New Zealand, Iceland and Yellowstone National Park in the US (Tobler and Benning 2011). Physiologically diverse microorganisms are likely to exhibit diverse chemistry, hence increasing the chance of finding novel compounds. Several hot springs in different regions of India have been known to geologists for years (Ghosh et al. 2003). Although a few attempts have been made to study and utilize microorganisms from hot springs in India (Nawani and Kaur 2000; Ghosh et al. 2003), most such studies are fragmented in nature.

Manikaran hot springs are located in the Beas and Parvati valley geothermal system, at an elevation of 1,760 metres (Cinti et al. 2009). These hot springs are the hottest in the country, with a temperature range of 89–95 ° C (Dwivedi et al. 2012). Despite intensive studies on terrestrial thermal springs, very little is known about the bacterial diversity of thermal

springs at high elevation (Huang et al. 2011). Hence, a comprehensive approach is needed to analyze the bacterial diversity of such a niche, in terms of its taxonomic and biochemical attributes.

Studies on the diversity of bacterial communities from various environments have been undertaken mainly using traditional methods of isolating and culturing microorganisms. Such methods involve isolation of microbes on standard cultivation media and characterization of microbes (Ranjard et al. 2000). Culture-based methods of analyzing microbes have been the mainstay of microbiology since their origin in the pioneering works of Robert Koch and Louis Pasteur. However, culture-based techniques have many limitations when analyzing the bacterial diversity of a particular environmental niche. Aerobic and anaerobic organisms cannot be cultured together; fastidious organisms will often not grow, because essential nutrients for growth or optimal environmental conditions such as temperature, pH, essential mixtures of gases may not be present (Piterina and Pembroke 2010). Despite these limitations, there is immense scope for culture-based diversity studies because of the advantages they offer in the generation of valuable germplasm. Working with strains isolated from hot springs offers the major advantage of preserving those strains for future studies and exploring them in due course for potential biotechnological applications (Akanbi et al. 2010; Acharya and Chaudhary 2012).

In the 1980s, a new tool for identifying bacteria and evaluating their phylogenetic relationships was developed in the laboratories of Woese and other researchers. It was found that all life forms could be identified by comparing a stable part of their common genetic code. Candidate genes used for these evolutionary studies numbered as many as 20; including 5S rRNA, 16S rRNA, 23S rRNA and the 16-23S rRNA internal transcribed spacer (ITS) region. Among these latter, the 16S rRNA gene is considered the best evolutionary chronometer because of its universal presence in all bacteria, its relative stability over evolutionary time, and its appropriate size (1,500 bp), i.e., large enough for bioinformatic analyses (Patel 2001). Amplified ribosomal DNA restriction analysis (ARDRA), which involves the comparison of restriction patterns (Yadav et al. 2010) and analyses using bioinformatic tools, represents a further improvement in the analysis of the 16S rRNA gene. ARDRA is generally considered valuable for strain typing and screening clone libraries to identify phylogenetic clusters within a microbial community (Sklarz et al. 2009).

The present study attempted to decipher the bacterial diversity of culturable thermotolerant bacteria from Manikaran thermal springs employing different media, followed by screening of isolates for temperature tolerance. ARDRA analysis was done for phylogenetic clustering of the thermotolerant isolates. Sequencing the 16S rRNA gene of representative thermotolerant strains was undertaken for identification. BIOLOG and analyses of other important biochemical attributes helped in further screening and selection of promising isolates, as a prelude to their application in agriculture and industry.

Material and methods

Sampling and isolation of culturable bacteria

Water samples were collected from four different sites of Manikaran hot springs. Manikaran is located at 32 ° 02′ N latitude and 77 ° 34′ E longitude. The temperature and pH of Manikaran hot springs ranges from 89 °C to 95 °C and 7.8 to 8.2, respectively. Water samples were collected in thermos flasks and brought to laboratory within 12 h of collection and processed for isolation of culturable bacteria.

For enumeration and isolation from water, samples were plated on five different media using a standard spread plate technique and incubated at 37 °C in an incubator for 48–72 h. The different media used were, nutrient agar (peptone 0.5 %, beef extract 0.3 %, NaCl 0.5 % and agar 1.8 %), thermus agar (peptone 0.5 %, yeast extract 0.2 %, beef extract 0.4 %, NaCl 0.5 % and agar 1.8 %), R2A medium (proteose peptone 0.05 %, casamino acid 0.05 %, yeast extract 0.05 %, dextrose 0.05 %, soluble starch 0.05 %, dipotassium hydrogen phosphate 0.03 %, sodium pyruvate 0.03 %, magnesium sulfate heptahydrate 0.005 %), King's B medium (protease peptone 2 %, dipotassium hydrogen phosphate 0.15 %, magnesium sulfate 0.15 % and agar 1.8 %) and thermus peptone meat extract yeast extract medium (TPMY: peptone 0.35 %, meat extract 0.5 % yeast extract 0.2 % NaCl 0.15 % and agar 1.8 %). After incubation, plates were observed for colony morphology and the total viable count was recorded for each sample in the different media employed. Based on differences in colony morphologies, 235 morphotypes were picked up from different plates and axenized.

Screening of isolates for temperature tolerance

All 235 isolates were screened for temperature tolerance by incubating the culture spot inoculated plates at different temperatures (40, 45, 50, 55 and 60 °C) for 72 h. Cultures tolerant to 60 °C were screened further by inoculating in broth and incubating in a shaker at 150 rpm for 72 h at 70 °C. The optical density of the broth was measured at 600 nm and compared with growth at 37 °C.

Genomic DNA extraction

Isolates were grown in specific broth, until they reached an $OD_{600\ nm} > 1.0$. The cells were pelleted from 5 ml culture, washed thrice with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and the pellet was resuspended in 750 μl TE buffer.

Genomic DNA was isolated from the suspended pellet using Zymo Research Fungal/Bacterial DNA MicroPrep™ following the standard protocol prescribed by the manufacturer.

PCR amplification of 16S rRNA gene

The primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used for amplification of 16S rRNA gene from the genomic DNA of isolates (Edwards et al. 1989). Amplification was carried out in a 100 μl reaction volume containing 50–100 ng template DNA, primers pA and pH (100 ng each), dATP, dCTP, dTTP and dGTP (200 μM each), *Taq* polymerase reaction buffer (10X) 10 μl and 1.0 U *Taq* polymerase. Conditions used for amplification were as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were resolved by electrophoresis at 60 V for 1 h in 1.2 % agarose gel in 1X TAE buffer. Gels were then stained with ethidium bromide (10 mg/ml) and visualized on a gel documentation system (Alpha Imager, Alpha Innotech, Santa Clara, CA).

Amplified rDNA restriction analysis

Purified PCR products were digested separately with three restriction endonucleases - *Alu* I, *Msp* I and *Hae* III in a 30 μl reaction volume, using recommended buffers at 37 °C. Restricted PCR products were resolved by electrophoresis at 45 V for 1.5 to 2 h in 2.5 % agarose gels in 1X TAE buffer for amplified rDNA restriction analysis (ARDRA). Gels were then stained with ethidium bromide and visualized. Strong and clear bands were scored as binary data (presence and absence of bands). The numerical taxonomy analysis program (NTYSIS) package (version 2.02e, Exeter Software, Setauket, NY) was used to score similarity and clustering analysis using the binary data. Jaccard's coefficient was used to calculate the similarity among the isolates and dendrogram was constructed using the UPGMA method (Nei and Li 1979).

16S rRNA gene sequencing and phylogenetic analysis

PCR amplified 16S rRNA genes were purified and sequenced using both pA and pH primers for forward and reverse reactions, respectively. Sequencing employed a dideoxy cycle with fluorescent terminators and was run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer (Applied Biosystems, Foster City, CA). The partial 16S rRNA gene sequences were compared with sequences available in the NCBI database. Isolates were identified to species level on the basis of 16S rRNA gene sequence similarity of ≥97 % with the sequences in GenBank. Sequence alignment and comparison used the multiple sequence alignment tool CLUSTAL W2 (Thompson et al. 1994) with default parameters. The phylogenetic tree was constructed on aligned data sets using the neighbour joining (NJ) method (Saitou and Nei 1987) and the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed on 1,000 random samples taken from multiple alignments (Felsentein 1981).

Amylase production

Amylase production was tested qualitatively on Starch Agar (peptone 0.5 %, yeast extract 0.2 %, soluble starch 2 %, NaCl 0.5 %). Isolates were streaked on starch agar plates and incubated at 45 °C for 24–36 h and after incubation period plates were flooded with Lugol's iodine solution (10 g potassium iodide, 5 g iodine crystals, 100 ml distilled water). Isolates showing a zone of clearance against a dark blue background were recorded as positive for amylase production.

Protease production

Protease production was tested qualitatively by placing isolated colonies of distinct cultures on 10 % skim milk agar plates and incubating at 45 °C for 24–36 h. A zone of clearance against an opaque background indicated a positive result for protease production.

BIOLOG analyses of selected isolates

The set of selected isolates tolerant to 70 °C was characterized using the GEN III MicroPlate™ (http://www.biolog.com), to generate a profile based on 92 biochemical tests (70 Carbon sources and 22 Chemical sensitivity tests). The NTYSIS package (Exeter software, version 2.02e) was used to score similarity and clustering analysis using the binary data generated through BIOLOG. Jaccard's coefficient was used to calculate the similarity among isolates along with their reference strains, and a dendrogram was constructed using the UPGMA method (Nei and Li 1979). Microbial Identification System software of BIOLOG was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

Results and discussion

Thermal springs represent extreme niches whose pristine quality is maintained over a period of time. The terrestrial hot springs that exist on Earth (Tobler and Benning 2011) represent hot spots for unusual forms of life, genes and metabolites. In the last two decades, a number of researchers have investigated various facets of the bacterial diversity in hot springs of different parts of world (Kanokratna et al. 2004; Meyer-Dombard et al. 2005; Pagaling et al. 2012). The diversity of bacterial

communities in various ecologies has been investigated mostly using culture-dependent approaches, although it is estimated that this may represent 0.1 to a maximum of 10 % of the total population (Ranjard et al. 2000). 16S rRNA gene sequencing, in combination with ARDRA analyses was used effectively for diversity studies in the past (Yadav et al. 2010). Our investigation therefore focused towards the isolation of microbes using different media and growth at high temperature so as to generate diverse nutritional types, undertake ARDRA analyses, evaluate the biochemical characterization of representative isolates and identify the thermotolerant isolates by BIOLOG analyses and 16S rRNA gene sequencing.

Manikaran hot springs are located at Manikaran village in Kullu district of Himachal Pradesh, India, which represents a high altitude environment. The thermal discharges of Manikaran hot springs are classified as the Na-HCO_3-Cl type. Chemical analysis of thermal waters from these hot springs indicated the presence of calcium, magnesium, sodium, potassium, sulphate, chloride and carbonate at concentrations 38–70 ppm, 4.6–10.4 ppm, 75–106 ppm, 15–20 ppm, 55–70 ppm, 87–117 ppm and 120–200 ppm, respectively (Chandrasekharam et al. 2005).

Enumeration and isolation of culturable bacteria

In order to obtain a diverse range of nutritional types and bacteria with various metabolic requirements, five types of media were employed. These included a nutrient-rich medium (nutrient agar) for isolating a larger proportion of the available diversity, a medium suitable for oligotrophs (R2A medium), specific media designed for thermophiles (thermus agar, TPMY medium) and a medium used mostly for *Pseudomonas* and related genera (King's B medium). The population of aerobic heterotrophic bacteria in the water samples of Manikaran thermal springs ranged from 72 to 241×10^4 cfu/ml. Among the media used, the highest population of 241×10^4 cfu/ml was recorded on nutrient agar medium. A total of 235 isolates was selected based on colony morphology, among which 137 isolates were from nutrient agar medium, 47 from thermus agar medium, 13 from King's B medium, and 19 each from TPMY and R2A media (Table 1).

Screening for temperature tolerance

All 235 isolates were screened for temperature tolerance of which 85 were found to tolerate 45 °C, and 13 isolates could tolerate 70 °C. Isolate M7 was found to be most tolerant, exhibiting more than 70 % growth when compared to its growth at 37 °C. Evaluation of temperature tolerance of the isolates revealed that only 13 isolates were thermotolerant up to 70 °C. While studying the archaeal and bacterial diversity in 12 hot springs of Tibetan Plateau of China, Huang et al. (2011), reported that one of the springs (GL81) with the highest temperature among the 12 studied was dominated by a mesophilic microbial community. Lau et al. (2006) and Mitchell (2009) also reported that there is no monotonic relationship between microbial diversity and thermal stress. Based on the temperature tolerance, 85 isolates were selected for molecular characterization.

Table 1 Media-based quantification of bacterial morphoytpes from Manikaran hot spring samples. *TPMY* Thermus peptone meat extract yeast extract

Medium	Number of morphotypes	Total viable count (10^4 CFU/ml)			
		MI	MII	MIII	MIV
Nutrient agar	137	214	241	198	182
Thermus agar	47	226	207	234	221
TPMY	19	116	148	121	128
King's B	13	88	104	72	85
R2A	19	133	106	144	115

Amplified rDNA restriction analysis

A set of 85 isolates that were tolerant to temperatures 45 °C or above were analyzed using ARDRA—an important technique for distinguishing closely related taxonomic groups, especially for screening endospore-forming bacilli (Ettoumi et al. 2009). PCR amplification of the 16S rRNA gene followed by ARDRA with three restriction enzymes was carried out to analyze the variation among the 85 isolates. PCR amplification of 16S rRNA gene yielded a single amplicon of 1.5 kb from all the isolates. When the amplicons were digested with restriction enzymes, the different patterns comprising three to six fragments ranging in size from 100 to 800 bp, were characterized. ARDRA revealed that restriction digestion with *Alu* I (31 groups) was more discriminative as compared to *Msp* I and *Hae* III. A combined dendrogram (based on patterns generated using three enzymes individually) was constructed to determine the percent similarity among the isolates (Fig. 1). The isolates were grouped into 42 clusters at 95 % similarity level, based on the combined dendrogram (Table 2).

16S rRNA gene sequencing and phylogenetic analysis

Sequence data from the 16S rRNA gene of 42 representative isolates were analyzed by BLAST. The nearest match from the GenBank database for each of the 42 representative isolates is reported (Table 2). A phylogenetic tree was constructed using these 42 isolates along with the closest sequences in the NCBI GenBank (Fig. 2a, b). Of the 42 sequences obtained in this study, 11 were 100 % identical to those already reported in the GenBank (NCBI) database, and the remaining isolates had 98–99 % identity to reported sequences in database

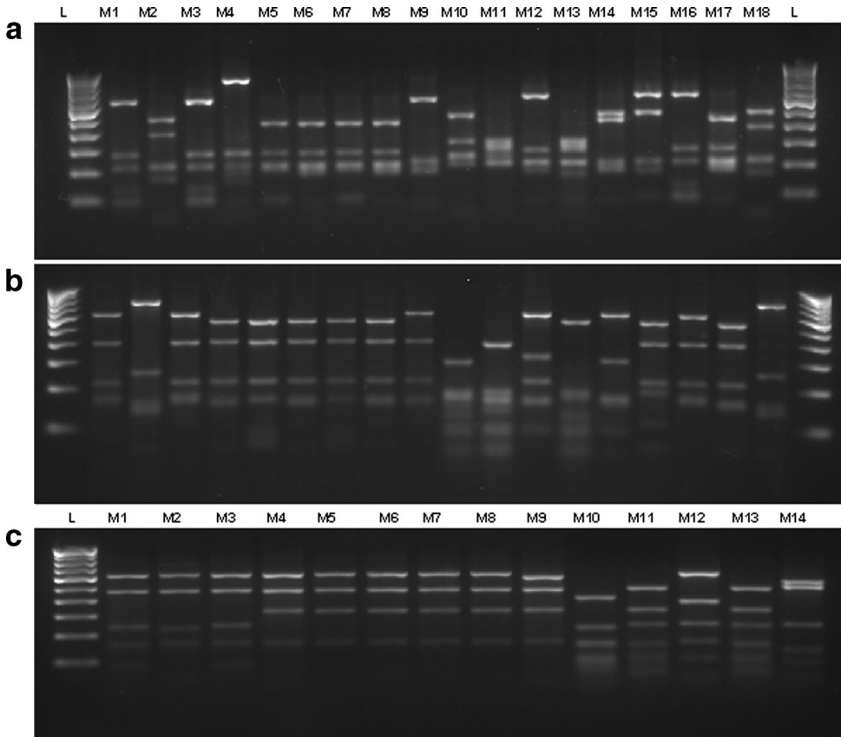

Fig. 1a–c Representative restriction patterns generated using three different restriction enzyme digests of amplified 16S rRNA gene of thermotolerant isolates. **a** *Alu*I **b** *Msp*I **c** *Hae*III

(as consulted in September 2012). Among the 42 isolates 72 % (30 isolates) belonged to Firmicutes, which is in accordance with Sayeh et al. (2010). BLAST analysis on the sequences of 42 representative isolates revealed the majority of isolates showing relatedness to the genus *Bacillus*. Apart from this, many *Bacillus* derived genera like *Brevibacillus*, *Aneurinibacillus*, and *Lysinibacillus* were also recorded.

Gram positive prokaryotes, especially the Firmicutes and Actinobacteria, are known to be comparatively stress resistant, besides being long range migrants (Cerritos et al. 2011). Because of their temperature tolerance, which is an inherent trait also attributed to this genus (Archana and Satyanarayana 2003), the genus *Bacillus* and its related genera have been explored extensively for their applications in industry and agriculture, especially as a source of hydrolytic enzymes. The members of this genus are spore formers and also produce a number of biocidal metabolites/enzymes, which makes them a common inhabitant of diverse extreme habitats (Nicholson et al. 2000). Enough evidence from recent studies has accumulated, based on the data of 16S rRNA gene sequence analysis, for the reclassification of thermophilic bacteria in the genus *Bacillus* into *Brevibacillus*, *Aneurinibacillus*, *Amphibacillus*, *Virgibacillus*, *Alicyclobacillus*, *Paenibacillus*, *Halobacillus*, and *Geobacillus* (Bae et al. 2005). This supports the presence of genera *Brevibacillus* and *Aneurinibacillus* in the hot springs in our study. The presence of genera like *Micrococcus*, *Microbacterium*, *Staphylococcus*, *Kocuria* and *Exiguobacterium* as thermoresistant aquatic bacteria in our investigation is supported by the work of Cerritos et al. (2011). These apart, representatives from *Planococccus*, *Rhodococcus*, *Pseudomonas*, and *Chelatococcus* were also present. A novel species of *Chelatococcus*—*Chelatococcus sambhunathii* sp. nov—was isolated and characterized from hot spring sediments by Panday and Das (2010). Phylogenetic analyses of bacterial communities in hot springs from low and high elevations of the Tibetan peninsula revealed no significant influence of elevation on diversity (Huang et al. 2011). This illustrates the predominance of *Bacillus* as an aggressive colonizer of diverse types of extreme habitats of the world.

Amylase and protease production

All 42 representative isolates were screened for production of the extracellular enzymes amylase and protease. Plate assays revealed that 26 % of the isolates were amylase producers and 45 % were protease producers (Table 2). Amylases hydrolyze starch molecules into dextrins and progressively into small polymers containing glucose units. Amylases are available from different sources and have extensive commercial applications in industrial processes involving starch conversions (Gupta et al. 2003), as well as in the detergent (Olsen and Falholt 1998), fuel ethanol production, food, textile (Ahlawat et al. 2009) and paper (van der Maarel et al. 2002) industries. Unique properties are required for each application; hence, screening of microorganisms for amylase production allows us to discover novel amylases required for specific industrial applications (Abdel-Fattah et al. 2012). Among the many properties of an effective amylase, thermostability is the most

Table 2 Restriction fragment length polymorphism (RFLP) clusters and sequenced isolates along with the closest available sequences in NCBI GenBank and their hydrolytic ability

RFLP pattern	Representative isolate	GenBank accession number	Temperature tolerance	Nearest phylogenetic neighbor	16S similarity (%)	Division	Amylolytic ability	Proteolytic ability
1	M1	JX312613	45 °C	*Bacillus aryabhattai* (JQ904723)	100	Firmicutes	+	+
2	M2	JX312614	45 °C	*Brevibacillus* sp. (AJ313027)	99	Firmicutes	-	-
3	M4	JX312615	70 °C	*Bacillus licheniformis* (GQ280087)	99	Firmicutes	+	+
4	M5	JX312616	70 °C	*Bacillus pumilus* (JQ435673)	99	Firmicutes	-	+
5	M6	KC315773	70 °C	*Bacillus subtilis* (EF442670)	100	Firmicutes	-	+
6	M7	KC315774	70 °C	*Bacillus pumilus* (HF536558)	100	Firmicutes	-	+
7	M8	KC315775	70 °C	*Bacillus* sp. (KC121051)	100	Firmicutes	-	+
8	M9	KC315776	45 °C	*Bacillus cereus* (GU011948)	98	Firmicutes	-	-
9	M10	JX312617	45 °C	*Rhodococcus* sp. (DQ285075)	99	Actinobacteria	-	-
10	M11	JX312618	45 °C	*Kocuria* sp. (DQ448711)	99	Actinobacteria	+	-
11	M12	JX312619	45 °C	*Planococcus* sp. (JX312584)	100	Firmicutes	-	-
12	M13	JX312620	45 °C	*Micrococcus* sp. (JN866765)	98	Actinobacteria	-	-
13	M21	JX312623	45 °C	*Staphylococcus haemolyticus* (JQ624771)	99	Firmicutes	-	-
14	M25	KC315762	45 °C	*Bacillus arbutinivorans* (FJ380988)	99	Firmicutes	-	-
15	M26	KC315763	45 °C	*Bacillus* sp. (FN397517)	99	Firmicutes	-	-
16	M27	KC315764	45 °C	*Bacillus niacini* (AB680904)	99	Firmicutes	-	-
17	M30	JX312624	45 °C	*Bacillus vireti* (HQ397585)	99	Firmicutes	-	-
18	M35	JX312625	45 °C	*Aneurinibacillus danicus* (NR_028657)	99	Firmicutes	-	-
19	M36	JX312626	45 °C	*Brevibacillus* sp. (FJ529026)	99	Firmicutes	-	+
20	M40	JX312627	45 °C	*Staphylococcus succinus* (HQ423378)	99	Firmicutes	-	-
21	M70	KC315777	70 °C	*Staphylococcus succinus* (HQ423378)	99	Firmicutes	-	-
22	M41	JX312628	45 °C	*Bacillus* sp. (JF901703)	99	Firmicutes	+	+
23	M43	KC315765	70 °C	Uncultured *Klebsiella* sp.(GQ416648)	99	Proteobacteria	+	-
24	M44	JX312629	50 °C	*Pseudomonas* sp. (EU680995)	99	Proteobacteria	-	+
25	M45	JX312630	55 °C	*Microbacterium oxydans* (EU714340)	99	Actinobacteria	-	-
26	M46	KC315766	70 °C	*Pseudomonas psychrophila* (JQ782895)	100	Proteobacteria	-	-
27	M49	KC315769	70 °C	*Pseudomonas psychrophila* (JQ782895)	100	Proteobacteria	-	-
28	M47	KC315767	70 °C	*Exiguobacterium acetylicum* (JX307688)	99	Firmicutes	-	+
29	M48	KC315768	70 °C	*Rhodococcus baikunurensis* (JX683682)	100	Actinobacteria	-	-
30	M50	KC315770	70 °C	*Pseudomonas fluorescens* (JX127246)	99	Proteobacteria	-	+
31	M52	JX312631	50 °C	*Lysinibacillus xylaniticus* (JQ739716)	99	Firmicutes	+	-
32	M53	JX312632	55 °C	*Staphylococcus hominis* (JN644561)	99	Firmicutes	+	+
33	M55	JX312633	70 °C	*Bacillus megaterium* (EU931553)	99	Firmicutes	-	+
34	M59	JX312635	45 °C	*Chelatococcus daeguensis* (NR_044297)	99	Proteobacteria	-	-
35	M61	JX312636	45 °C	*Bacillus flexus* (JQ936679)	100	Firmicutes	+	+
36	M63	KC315771	45 °C	*Bacillus* sp. (HE821233)	99	Firmicutes	-	+
37	M65	KC315772	45 °C	*Bacillus subtilis* (JX845578)	99	Firmicutes	+	+
38	M68	JX312637	55 °C	*Lysinibacillus* sp. (FN397524)	99	Firmicutes	-	-
39	M73	JX312638	45 °C	*Brevibacillus agri* (HQ222834)	100	Firmicutes	-	+
40	M74	JX312639	45 °C	*Bacillus beijingensis* (JQ799102)	100	Firmicutes	+	+
41	M75	JX312640	45 °C	*Bhargavaea cecembensis* (JQ071510)	99	Firmicutes	-	+
42	M80	KC315778	45 °C	*Microbacterium* sp.(DQ339613)	98	Actinobacteria	+	-

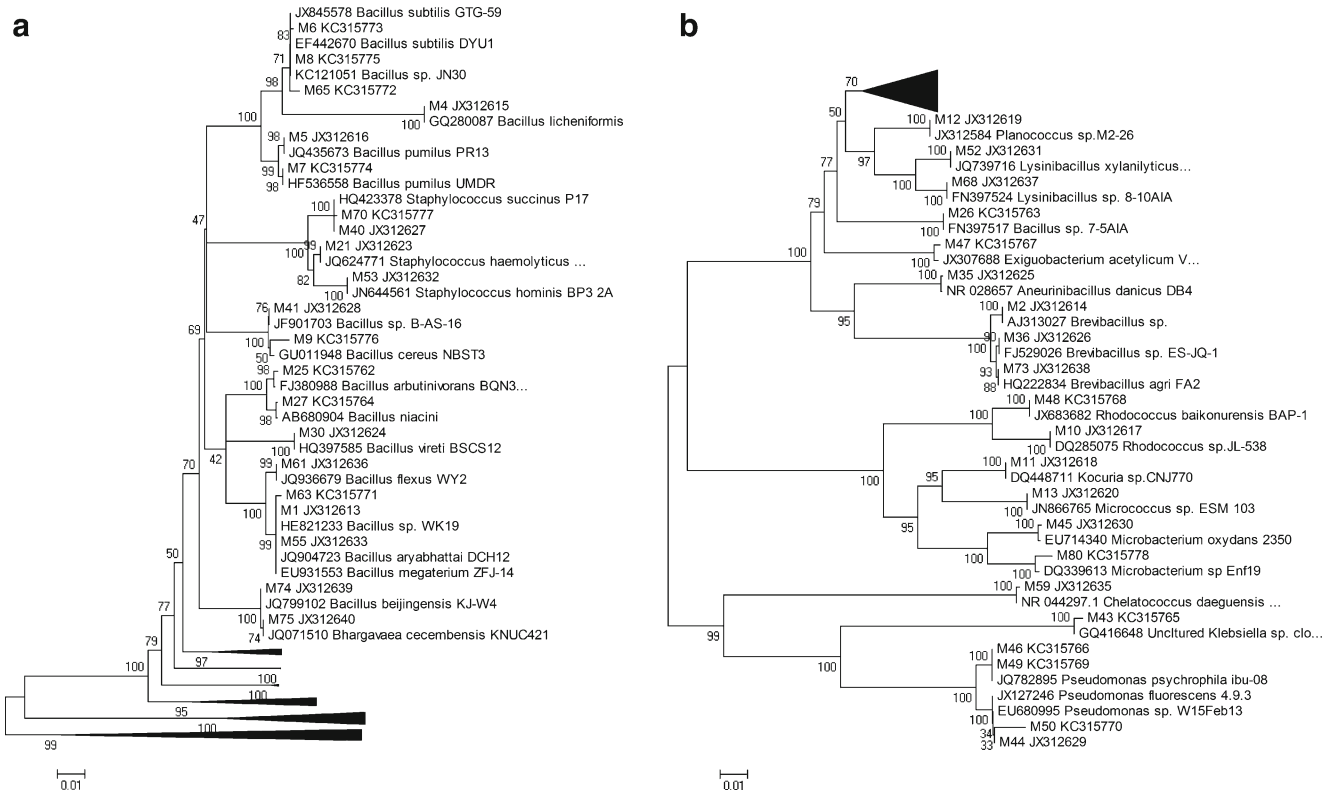

Fig. 2 Unrooted phylogenetic trees based on comparison of 16S rDNA sequences of 42 isolates along with their closest phylogenetic relatives. The phylogenetic tree was constructed based on aligned datasets using the neighbor joining (NJ) method in the program MEGA 4.0.2. Numbers on the tree indicate percentage of bootstrap sampling derived from 1,000 random samples

important, as liquefaction and saccharification of starch are performed at high temperatures. In our present study, out of 42 isolates from Manikaran hot springs, 11 were positive for amylase production. Among the amylase positive isolates, six were identified as belonging to *Bacillus* based on their 16S rRNA gene sequence similarity and one as *Lysinibacillus*, which is again a *Bacillus* derived genus. There was one representative each from the genera *Kocuria*, *Staphylococcus*, *Klebsiella* and *Microbacterium*. Sahay et al. (2011) reported similar results, in which many *Bacillus* sp. tested positive for amylase production. Interestingly, the isolate M4 (*Bacillus licheniformis*), which tested positive for amylase production, also possessed thermotolerance. Considering the requirement for thermostability of amylases in industrial applications, there is the possibility of exploring this isolate further.

Proteases are hydrolytic enzymes that catalyze total hydrolysis of proteins (Rao et al. 1998). Proteases find commercial applications in the food processing, tannery, detergent, and pharmaceutical industries (Chakrabarti et al. 2000; Kim et al. 2005); microbial proteases dominate commercial applications (Kuddus and Ramtekke 2012). As with amylases, thermal stability is the major requirement for any proteases to find commercial importance (Kristjansson and Kinsella 1990). In our study, among the 42 isolates tested for extracellular protease production, 19 tested positive and a similar trend as for amylase was recorded , as a majority (12) of them were *Bacillus* based genera based on their 16S rDNA sequence similarity. There were also representatives from *Brevibacillus* (2) and *Bhargavaea* (1), both being *Bacillus*-derived genera. Of the remaining four, there were two isolates of *Pseudomonas* and one each of *Exiguobacterium* and *Staphylococcus*. Isolates M4 (*Bacillus licheniformis*), M5 (*Bacillus pumilus*), M6 (*Bacillus subtilis*), M7 (*Bacillus pumilus*), M8 (*Bacillus* sp.) and M47 (*Exiguobacterium acetylicum*), which tested positive for protease production, were also thermotolerant. Hence, these isolates could be explored for thermostable proteases, as there is a need for such enzymes in industrial processes operating at high temperatures (Kristjansson and Kinsella 1990).

Biochemical tests and substrate utilization profiles for selected isolates using BIOLOG

BIOLOG-based phenotyping of the set of 13 highly thermotolerant isolates generated unique fingerprints of the isolates, besides revealing their ability to utilize routine and unusual substrates. This assumes immense importance in the characterization of microbial isolates from extreme environments and their potential utilization in agriculture and industry. The data on substrate utilization patterns and

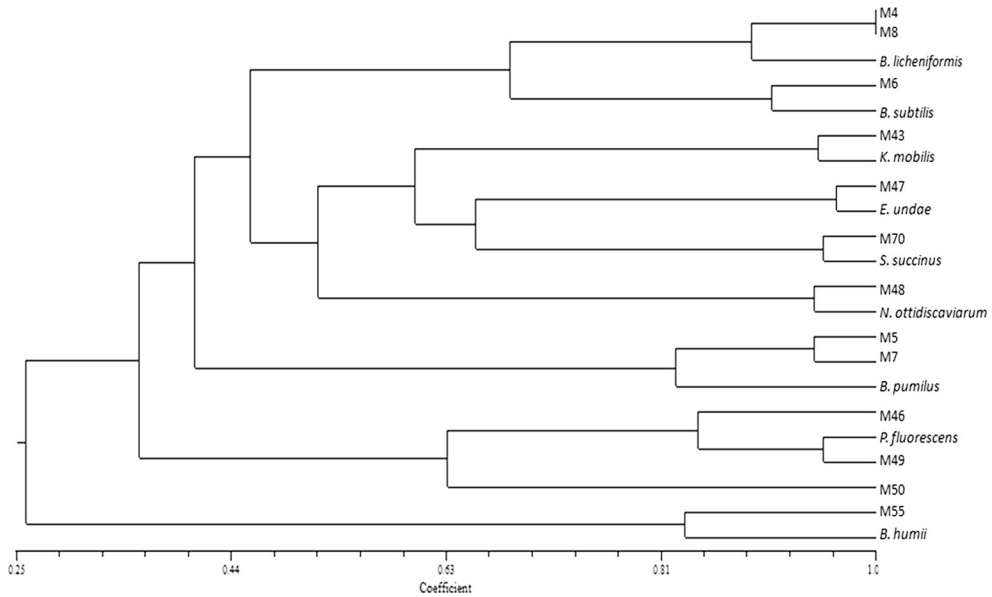

Fig. 3 Dendrogram showing the clustering of thermotolerant isolates along with reference strains based on substrate utilization patterns generated by BIOLOG analyses. The dendrogram was constructed using the UPGMA algorithm and the Jaccard's co-efficient. Distances were calculated based on the utilization of substrates and resistance to chemical agents

sensitivity to chemicals for the 13 temperature tolerant isolates were tabulated (Supplementary Table 1). Significant variation was observed among the isolates with regard to the utilization of sugars, sugar derivatives, metabolic intermediates and amino acids and peptides. A dendrogram was generated based on the data obtained through BIOLOG for the 13 isolates and reference strains available in the database (Fig. 3). Among the 20 sugars tested, D-fructose was utilized by all isolates except M55. Similarly α-D-glucose was utilized by all isolates except M48, while 3-methyl glucose was utilized only by M43. Among the 13 isolates tested for utilization of sugars, isolate M43 was able to utilize all but one sugar (raffinose) and M8 could utilize 17 sugars out of 20 tested. Isolate M55 was able to utilize only two sugars (α-D-glucose and sucrose). Isolates M43 and M48 were able to utilize the highest number of sugar derivatives, 18 and 17, respectively. Among all the sugar derivatives, glucoronamide was utilized by 12 isolates followed by L-galactonic acid and D-gluconic acid (11 isolates). Isolate M55 was able to utilize only one sugar derivative (glucoronamide).

A total of 19 metabolic intermediates was tested for growth of isolates. Isolate M48 was able to utilize the maximum number of metabolic intermediates (15) followed by isolates M8 and M70, which were able to utilize 12 metabolic intermediates each. Isolate M55 utilized the fewest metabolic intermediates (2). The metabolic intermediate, D-fructose-6-PO_4 was utilized by all isolates tested, and L-malic acid was utilized by 11 isolates. p-hydroxy-phenylacetic acid, α-hydroxy-butyric acid and α-keto-butyric acid were the least utilized, and supported the growth of only two isolates. The results on the utilization of amino acids and peptides revealed that isolate M43 was able to use all 12 substrates. Both M8 and M48 utilized ten substrates each. Isolate M55 could not utilize all substrates tested and only two substrates were utilized by isolate M50. Amino acids, L-alanine and L-glutamic acid were utilized by nine isolates while L-arginine and L-serine were utilized by seven isolates. Among the isolates, based on carbon utilization patterns, M55 (*Bacillus* sp.) was able to utilize only 7 %, while M43 *Klebsiella* sp. was able to utilize 85.7 % of the C substrates.

Table 3 BIOLOG-based identification of the thermotolerant isolates

Isolates	BIOLOG identity	16S rRNA gene sequence based identity
M4	*Bacillus licheniformis*	*Bacillus licheniformis* (GQ280087)
M5	*Bacillus pumilus*	*Bacillus pumilus* (JQ435673)
M6	*Bacillus subtilis*	*Bacillus subtilis* (EF442670)
M7	*Bacillus pumilus*	*Bacillus pumilus* (HF536558)
M8	*Bacillus licheniformis*	*Bacillus* sp. (KC121051)
M43	*Klebsiella mobilis*	Uncultured *Klebsiella* sp. (GQ416648)
M46	*Pseudomonas fluorescens*	*Pseudomonas psychrophila* (JQ782895)
M47	*Exiguobacterium undae*	*Exiguobacterium acetylicum* (JX307688)
M48	*Nocardia ottidiscaviarum*	*Rhodococcus baikunurensis* (JX683682)
M49	*Pseudomonas fluorescens*	*Pseudomonas psychrophila* (JQ782895)
M50	*Pseudomonas fluorescens*	*Pseudomonas fluorescens* (JX127246)
M55	*Bacillus humii*	*Bacillus megaterium* (EU931553)
M70	*Staphylococcus succinus*	*Staphylococcus succinus* (HQ423378)

Sensitivity and resistance to antimicrobials

Isolate M50 was found to be resistant to 15 antimicrobials out of 17 tested, closely followed by isolate M49, which was resistant to 13 antimicrobials. Isolate M6 showed resistance to least number of antimicrobial compounds (4) (Supplementary Table 2).

In terms of resistance to chemical agents, sodium lactate, which is known for its bactericidal properties and acts as a preservative, acidity regulator, and bulking agent, did not inhibit any of the isolates tested. This reveals the unusual nature of our isolates. Also, minocycline inhibited the growth of all the isolates, with the exception of M50 (*Psuedomonas fluorescens*), which was found to be most resistant to many of the chemical agents tested. Isolate M47 (*Exiguobacterium acetylicum*) was the only isolate capable of growth on sodium bromate.

Response of selected isolates to abiotic stress

The ability of isolates to show growth at low pH and in the presence of salt was tested and the results are tabulated in Supplementary Table 2. Seven isolates (M4, M5, M6, M7 M8, M49 and M70) were able to show growth at pH values up to 5 and 8 % NaCl. Isolate M47 and M55 were able to grow up to 8 % NaCl concentration and a pH of 6. Isolate M48 was not able to grow either at low pH (5) or at high salt concentration (4/8 % NaCl). Growth at different pH (5 and 6) revealed that all the 13 isolates (originally isolated from a pH of 7.8–8.2) could grow at 6, but three isolates (M47, 48 and 50) were unable to show growth at 5. Salinity up to 1 % NaCl was tolerated by all isolates; however, M48 did not grow at higher salt concentrations (4/8 % NaCl). A total of 92 % isolates and 69 % of the thermotolerant isolates were able to grow at 4 % and 8 % NaCl concentrations, revealing their promise for use in saline environments.

BIOLOG analysis in this study revealed interesting results, especially providing valuable information on the temperature tolerant isolates showing positive results in terms of growth in both low pH (pH up to 5) and high salt concentrations (NaCl concentration up to 8 %). Such isolates could be explored for their potential applications in agriculture and industry.

Identification based on BIOLOG analysis

Using the BIOLOG Microlog 3 software, the 13 temperature tolerant isolates were identified based on the profiles generated using GEN III MicroPlate™. The results of the identification of temperature tolerant isolates are given in Table 3, among which six belonged to the genus *Bacillus*; three to *Pseudomonas* and one each were placed in the genera *Nocardia*, *Staphylococcus*, *Klebsiella* and *Exiguobacterium*. BIOLOG-based identification of the temperature tolerant isolates in our study matched with 16S rRNA gene sequence based identification up to genera level, for all but one isolate (M48). This isolate M48 had a 16S rRNA gene sequence similar to that of *Rhodococcus baikunurensis*; however, this isolate matched with *Nocardia ottidiscaviarum* based on BIOLOG-based analyses. There are reports that C-utilization patterns and identification through BIOLOG have certain limitations, as it is difficult to distinguish closely related organisms (Singh et al. 2010). Also, information on members belonging to Mycobacteria and Corynebacteria in the database is limited.

Rhodococcus is a genus of aerobic, non-sporulating, non-motile Gram-positive bacterium, which is closely related to Mycobacteria and Corynebacteria and thrives in a wide range of environments. Its importance in biotechnological applications comes from its ability to catabolize a wide range of compounds (including harmful pollutants), besides production of thermostable enzymes (Kuddus and Ramtekke 2012). Our study emphasized the novelty of this strain, as it was not capable of utilizing D-glucose or glucuronimide, but was able to utilize α hydroxy-butyric acid. This isolate exhibits unusual properties as, in the published literature to date, the majority of hydroxy-butyrate degrading bacteria belong to Gram negative genera such as *Acidovorax* /*Variovorax* sp. (Mergaert et al. 1993), and *Rhodococcus* is not reported in this context.

The bacterial community composition of Manikaran hot springs exhibited a phylogenetically distinct character, resembling in part that previously reported from other geothermal hot springs (Pagaling et al. 2012; Sayeh et al. 2010), but comprising isolates with novel properties of substrate utilization patterns, tolerance to biocidal agents and abiotic stress such as 8 % NaCl.

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References

Abdel-Fattah YR, Soliman NA, El-Toukhy NM, El-Gendi H, Ahmed RS (2012) Production, purification and characterization of thermostable α-amylase produced by *Bacillus licheniformis* isolate AI20. J Chem. doi:10.1155/2013/673173

Acharya S, Chaudhary A (2012) Alkaline cellulase produced by a newly isolated thermophilic *Aneurinibacillus thermoaerophilus* WBS2 from hot spring, India. Afr J Microbiol Res 6:5453–5458

Ahlawat S, Dhiman SS, Battan B, Mandhan RP, Sharma J (2009) Pectinase production by *Bacillus subtilis* and its potential

application in biopreparation of cotton and micropoly fabric. Process Biochem 44:521–526

Akanbi TO, Kamaruzaman AL, Abu-Bakar F, Hamid SAN, Radu S, Abdul-Manap MY, Saari N (2010) Highly thermostable extracellular lipase-producing *Bacillus* strain isolated from a Malaysian hot spring and identified using 16S rRNA gene sequencing. Int Food Res J 17:45–53

Archana A, Satyanarayana T (2003) Purification and characterization of a cellulase-free xylanase of a moderate thermophile *Bacillus licheniformis* A99. World J Microbiol Biotechnol 19:53–57

Bae SS, Lee JH, Kim SJ (2005) *Bacillus alveayuensis* sp. nov., a thermophilic bacterium isolated from deep-sea sediments of the Ayu trough. Int J Syst Evol Microbiol 55:1211–1215

Cerritos R, Eguiarte LE, Avitia M, Siefert J, Travisano M, Rodriguez-Verdugo A, Souza V (2011) Diversity of culturable thermoresistant aquatic bacteria along an environmental gradient in Cuatro Cienegas, Coahuila, Mexico. Anton Leeuw Int J G 99:303–318

Chakrabarti SK, Matsumura N, Ranu RS (2000) Purification and characterization of an extracellular alkaline serine protease from *A. terreus*. Curr Microbiol 40:239–244

Chandrasekharam D, Alam MA, Minissale A (2005) Thermal discharges at Manikaran, Himachal Pradesh, India. Proceedings World Geothermal Congress Antalya, Turkey, pp 1–4

Cinti D, Pizzino L, Voltattorni N, Quattrocchi F, Walia V (2009) Geochemistry of thermal waters along fault segments in the Beas and Parvati valleys (north-west Himalaya, Himachal Pradesh) and in the sohna town (Haryana), India. Geochem J 43:65–76

Dwivedi V, Sangwan N, Nigam A, Garg N, Niharika N, Khurana P, Khurana JP, Lal R (2012) Draft genome sequence of *Thermus* sp. Strain RL, isolated from a Hot water spring located atop the Himalayan ranges at Manikaran, India. J Bacteriol 194:3534

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Nucleic Acids Res 17:7843–7853

Ettoumi B, Raddadi N, Borin S, Daffonchio D, Boudabous A, Cherif A (2009) Diversity and phylogeny of culturable spore-forming bacilli isolated from marine sediments. J Basic Microbiol 49:S13–S23

Felsentein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376

Ghosh D, Bal B, Kashyap VK, Pal S (2003) Molecular phylogenetic exploration of bacterial diversity in a Bakreshwar (India) hot spring and culture of *Shewanella*-related thermophiles. Appl Environ Microbiol 69:4332–4336

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α-amylse: a biotechnological prospective. Process Biochem 38:1599–1616

Huang Q, Dong CZ, Dong RM, Jiang H, Wang S, Wang G, Fang B, Ding X, Niu L, Li X, Zhang C, Dong H (2011) Archaeal and bacterial diversity in hot springs on the Tibetan plateau, China. Extremophiles 15:549–563

Kanokratna P, Chanapan S, Pootanakit K, Eurwilaichitr L (2004) Diversity and abundance of bacteria and archaea in the Bor khlueng hot spring in Thailand. J Basic Microbiol 44:430–444

Kim JM, Yang MC, Hung JS (2005) Preparation of feather digest as fertilizer with *Bacillus pumillus* KHS-1. J Microbiol Biotechnol 15:427–476

Kristjansson MM, Kinsella JE (1990) Heat stable proteinase from *Thermomonospora fusca*. Int J Peptide Prot Res 36:201–207

Kuddus M, Ramtekke PW (2012) Recent developments in production and biotechnological applications of cold-active microbial proteases. Crit Rev Microbiol 38:380–388. doi:10.3109/1040841X.2012.678477

Lau MCY, Jing H, Aitchison JC, Pointing SB (2006) Highly diverse community structure in a remote central Tibetan geothermal spring does not display monotonic variation to thermal stress. FEMS Microbiol Ecol 57:80–91

Mergaert J, Webb A, Anderson C, Wouters A, Swings J (1993) Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils.Appl Environ Microbiol 59:3233–3238

Meyer-Dombard DR, Shock EL, Amend JP (2005) Archaeal and bacterial communities in geochemically diverse hot springs of Yellowstone national park USA. Geobiology 3:211–227

Mitchell KR (2009) Controls on microbial community structure in thermal environments: exploring bacterial diversity and the relative influence of geochemistry and geography. Dissertation, University of New Mexico

Nawani N, Kaur J (2000) Purification, characterization and thermostability of lipase from a thermophilic *Bacillus* sp. J33. Mol Cell Biochem 206:91–96

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64:548–572

Olsen HSO, Falholt P (1998) The role of enzymes in modern detergency. J Surfactants Deterg 1:555–567

Pagaling E, Grant WD, Cowan DA, Jones BE, Ma Y, Ventosa A, Heaphy S (2012) Bacterial and archaeal diversity in two hot spring microbial mats from the geothermal regions of Tengchong, China. Extremophiles 16:607–618

Panday D, Das SK (2010) *Chelatococcus sambhunathii* sp. nov., a moderately thermophilic alphaproteobacterium isolated from hot spring sediment. Int J Syst Evol Microbiol 60:861–865

Patel JB (2001) 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol Diagn 6:313–321

Piterina AV, Pembroke JT (2010) Preparation and analysis of environmental DNA: optimisation of techniques for phylogenetic analysis of ATAD sludge. In: Mendez-Vilaz A (ed) Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex, pp 1533–1538

Ranjard L, Poly F, Nazaret S (2000) Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Res Microbiol 151:167–177

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635

Sahay H, Singh S, Kaushik R, Saxena AK, Arora DK (2011) Characterization of halophilic bacteria from environmental samples of Pulicat brackish water lake. Biologia 66:741–747

Saitou N, Nei M (1987) The neighbour joining method; a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Satyanarayana T, Raghukumar C, Shivaji S (2005) Extremophilic microbes: diversity and perspectives. Curr Sci 89:78–90

Sayeh R, Birrien JL, Alain K, Barbier G, Hamdi M, Prieur D (2010) Microbial diversity in Tunisian geothermal springs as detected by molecular and culture-based approaches. Extremophiles 14:501–514

Singh AK, Tripathi BM, Sahay H, Singh RN, Kaushik R, Saxena AK, Arora DK (2010) Biochemical and molecular characterization of thermo-alkali tolerant xylanase producing bacteria from thermal springs of Manikaran. Indian J Microbiol 50:S2–S9

Sklarz MY, Angel R, Gillor O, Ines M, Soares M (2009) Evaluating amplified rDNA restriction analysis assay for identification of bacterial communities. Antonie van Leeuwenhoeck 96:659–664

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0.2. Mol Biol Evol 24:1596–1599

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving sensitivity of progressive multiple sequence alignments through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tobler DJ, Benning LG (2011) Bacterial diversity in five Icelandic geothermal waters: temperature and sinter growth rate effects. Extremophiles 15:473–485

van der Maarel MJ, van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L (2002) Properties and applications of starch-converting enzymes of the alpha-amylase family. J Biotechnol 94:137–155

Yadav S, Kaushik R, Saxena AK, Arora DK (2010) Diversity and phylogeny of plant growth-promoting bacilli from moderately acidic soil. J Basic Microbiol 50:1–9