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ISOLATION AND CHARACTERIZATION OF THERMOPHILIC BACTERIA IN NATURAL HOT WATER SPRINGS OF HIMACHAL PRADESH (INDIA)

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ABSTRACT
In the present investigation, four thermophilic bacterial strains isolated from Manikaran hot water spring of northern Himalayan region of Himachal Pradesh has been studied. Based on morphological and biochemical characters, four isolates viz., PS1, PS2, PS3 and PS4, were identified as thermophilic bacteria and the optimal temperature for growth of these isolates was 65ºC and the optimal pH was 6-8. Molecular community were determined by phylogenetic analysis of 16S rRNA gene sequences and this analysis showed that all phylotypes retrieved from enrichment cultures were affiliated to *Firmicutes*, belonging to the genera *Brevibacillus*, *Paenibacillus*, and *Bacillus*. The 16S rRNA gene sequences of these four isolates were then submitted to NCBI under these accession numbers. JN 559848.1 (*Brevibacillus thermoruber,* PS1), JN 559849.1 (*Brevibacillus thermoruber,* PS2), JN 559850.1 (*Paenibacillus sp.,* PS3) and JN 559851.1 (*Bacillus licheniformis,* PS4).

INTRODUCTION

The microbial world is the largest unexplored reservoir of biodiversity on the earth. It is an important frontier in biology under intensive investigations. The vast array of microbial activities and their importance to the biosphere and to human economics provide strong rationale for understanding their diversity, conservation and exploitation. It has been speculated that the thermophiles were among the first living organisms on this planet, developing and evolving during the primordial birthing days of the earth when surface temperatures were quite hot and thus, have been called as "Universal ancestor'' (Doolittle, 1999). Thermophilic microorganisms prefer living at higher temperatures in hot springs and these organisms not only survive but might even thrive in boiling water. Isolation of thermophiles has received considerable attention among the scientific community in whole world because of their biotechnological importance which are usually not denatured by high temperature and even remain active at elevated temperature (Becker *et al.,* 1997; Beg *et al.,* 2000; Lee *et al.,* 1999; Sonnleitner and Fiechter, 1983). Scientists are taking great interest in the study of thermophiles ever since the discovery of *Thermus aquaticus* from the Great Fountain region of Yellowstone National Park (Brock and Freeze, 1969) and generated a multimillion dollar biotechnology industry. Studies in the last three decades have revealed that 95% of bacteria present in the environment are still unexplored or overlooked in laboratory cultivation and hence remain obscure for their ecological functions and unexploited for biotechnological applications.

For several decades, thermophilic bacteria have attracted the interest of many scientists due to their biotechnological potential (Adiguzel *et al.,* 2009). In particular, phenotypic and genotypic characterization of thermophilic bacteria has been done for many geothermal areas in different parts of the world, including Turkey (Gul-Guven *et al.,* 2008; Adiguzel *et al.,* 2009), Italy (Maugeri *et al.,* 2001), Bulgaria (Derekova *et al.,* 2008), Greece (Sievert *et al.,* 2000), China (Lau *et al.,* 2009), India (Sharma *et al.,* 2008) and Iceland (Takacs *et al.,* 2001). Advances in molecular biology techniques, such as 16S rRNA sequencing have provided excellent opportunity for identification and characterization purposes of microorganism at species and subspecies levels (Adiguzel, 2006; Zaliha *et al.,* 2007). These methods have been used also for studying the diversity in ecosystem, presenting the phylogenetic relation between strains and discriminating the microorganism, which are genetically close to each other (Adiguzel, 2006). Phylogenetic research showed that thermophiles are abundant in many more extreme environments (Hugenholtz *et al.,* 1998; Skirnisdottir *et al.,* 2000; Hjorleifsdottir *et al.,* 2001). Thermophilic strains have been recovered from thermal springs in China (Guo *et al.,* 2003), Japan (Kurosawa *et al.,* 2005), Antarctica (Imperio *et al.,* 2008), Saudi Arabia (Amjad khalil, 2011), Jordan (Obeidat *et al.,* 2012) and India (Bisht and Panda, 2011; Acharya and Chaudhary, 2012; Panda *et al.,* 2013) . More recently, study of bio-chemical and molecular characterization of thermophiles from hot water springs became of interest, therefore many studies devoted to the comprehension of molecular basis of the adaptation to high

temperature (Panda *et al.,* 2013).

The state of Himachal Pradesh, situated in northern region of India, is also rich in hot springs. One such hot water spring is Manikaran and the microbial diversity of this hot spring has not yet been fully explored due to difficulties in isolation, maintenance of pure culture and thus, their diversity and biotechnological potential remains to explored. The aim of this study was to isolate and identify thermophilic bacteria from Manikaran hot water springs, determine the thermostability of the isolates and study the phylogenetic affiliation of the thermophilic bacterium in comparison with other thermophilic bacterial isolates.

MATERIALS AND METHODS

Sampling

Samples in the form of water, soil, pebbles and rock mattings from different sites of Manikaran hot water springs were collected in sterilized screw capped vials and jars and were brought to the laboratory and kept at 4ºC in refrigerator till further processing. The temperature and pH were recorded at the time of sampling.

Culture media

Culture media tried for the isolation of thermophilic bacteria were Castenholz TYE medium (Castenholz, 1969), and had the following composition**:** $NaNO_3$ 1.4g, Na_2HPO_4 0.22g, KNO_3 0.21g, Nitrilotriacetic acid 0.2g, $MgSO_4.7H_2O$ 0.2g, $MgSO_4.7H_2O$ 0.2g, $CaSO_4.2H_2O$ 0.12g, NaCl 0.016g, $FeCl_3$ (0.03%) 2.0ml, Nitsch's trace elements ($MnSO_4$ 2.2g, H_3BO_3 0.5g, $ZnSO_4$ 0.5g, $CoCl_2.6H_2O$ 0.046g, Na_2MoO_4 0.025g, $CuSO_4$ 0.016g, H_2SO_4 0.5g) 2.0mL, Casein 10.0g, Yeast extract 10.0g and distilled water to make one liter having pH 7.0. All the media components were procured from SRL, Mumbai (India)

Incubation conditions

All the incubations were done in covered water bath incubator at 70ºC for 3-4 days in Castenholz TYE broth. Growth was measured turbidimetrically at 600 nm by use of a Bausch & Lomb Spectronic 20 colorimeter. Agar plates were wrapped in Saran Wrap (Dow Chemical Co.) to prevent drying and incubated just above the surface of the water in a covered water bath.

Optimization of growth conditions

Growth conditions were optimized for temperature, pH and time. The temperature range for incubation investigated varied from 40, 50, 60, 70, 80, 90 and 100ºC and pH range was optimized at 70ºC for 24 hrs using Castenholz TYE medium adjusted from 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 separately whereas effect of different incubation times for the growth of these thermophilic bacteria was studied for 24, 48, 72, 96, 120 and 144hrs. In all cases optical density was monitored at 600nm on a double beam UV/VIS scanning spectrophotometer.

Morphological and biochemical characterization

Thermophilic bacterial isolates were studied for various morphological characteristics viz., colour, gram reaction, shape, spore formation and motility (Kristjansson *et al.,* 1986). Various biochemical tests were carried out for the biochemical characterization of selected bacterial isolates viz., catalase test, urease test, oxidase test, indole test, citrate test, MR-VP test and fermentation of sugars (Case and Johnson, 1984), (Harrigan, 1998), (Aneza, 2003). All the biochemical test reagents were procured from Himedia, Mumbai (India).

Genomic DNA extraction

Thermophilic bacterial cultures were inoculated into 20mL Castenholz TYE broth and incubated at 70ºC overnight. Cultures were centrifuged at 14000 rpm for 5min; cell pellets were washed two times with distilled water and then used for DNA isolation using Genomic DNA extraction Mini-Kit (Real Genomics) according to manufacturer's instructions.

PCR amplification of the 16S rRNA gene

The PCR amplification of the 16S rRNA gene from purified genomic DNA was carried out in 0.2mL PCR tubes with 20 μL reaction volume by using the forward primer (5′-GGTCAGCGGCGGACGGGTGAGTAAC-3′) and the reverse primer (5′-GACGGGCGGTGTGTACAGAGGCCCG-3′) and all the amplifications were performed using thermal cycler (MultiGene PCR system, Labnet). PCR and molecular biology reagents were procured from Bangalore Genei, (Bangalore) and primers were custom-synthesized and supplied in lyophilized form by Integrated DNA Technologies Inc., USA.

Sequencing analysis

The PCR products obtained through amplification with specific primers targeting rRNA gene were sequenced, using same upstream and downstream primers, by a commercial sequencing facility (Xcleris lab). The sequences of these four bacterial isolates after sequencing were blasted using online NCBI BLAST program, http://www.ncbi.nib.gov/blast (Altschul *et al.,* 1997). Phylogenetic analyses were used for comparative genomics to show evolutionary relationships. The analysis began with aligning of sequences using tools like Clustal W and after alignment, phylogenetic tree was constructed using MEGA 6.0 software.

RESULTS AND DISCUSSION

In the present study, the occurrence of thermophilic bacteria belonging to genus *Brevibacillus, Paenibacillus* and *Bacillus* were investigated in thermal springs of Manikaran, Himachal Pradesh. The water temperature was found to be ranged from 80-105ºC, with pH ranging from 4.0-7.0. Out of all samples collected from the different sites of Manikaran thermal spring, four thermophilic bacteria were isolated. The isolates were given codes viz., PS1, PS2, PS3 and PS4. Colonies of thermophilic bacterial isolates PS1 and PS2 were creamish circular on Castenholz TYE medium and cells were gram positive, rod shaped, arranged singly, nonmotile and sporulating (Table 1). These isolates were positive for Nitrate reduction test, Catalase test, Voges-Proskauer test and for Starch hydrolysis where as rest of biochemical descriptors viz., Indole test, Oxidase test, Methyl Red test, Gelatin liquefaction, Citrate utilization, Lysine utilization, Phenylalanine deamination, Urease production, H_2S production and fermentation of sugars were found to be negative (Table 2). Optimal temperature observed for their maximum growth was 65ºC, optimal pH was 6.0-7.0, and optimal time was 96 hrs. Colonies of

Table 1: Morphological characterization of isolates

Strain	Colony size	Colony morphology	Gram reaction	Cellular morphology	Spore formation	Motility
PS1	2	Creamish, circular	+ve	Rods	+ve	Nonmotile
PS2	2	Creamish, circular	+ve	Rods	+ve	Nonmotile
PS3	1.5	Yellowish-white, spreading growth	+ve	Rods	+ve	Motile
PS4	2	Creamish- white, circular	+ve	Rods	+ve	Motile

Table 2: Biochemical characterization of the bacterial isolates

Biochemical tests	PS1	PS2	PS3	PS4
Citrate utilization	-ve	-ve	-ve	-ve
Indole	-ve	-ve	-ve	-ve
Nitrate reduction	+ve	+ve	-ve	-ve
Lysine utilization	-ve	-ve	-ve	-ve
Ornithine Utilization	-ve	-ve	-ve	-ve
Phenylalanine deamination	-ve	-ve	-ve	-ve
Urease production	-ve	-ve	-ve	-ve
H_2S production	-ve	-ve	-ve	-ve
Catalase	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	+ve	-ve
Methyl Red	-ve	-ve	+ve	+ve
Voges Proskauer	+ve	+ve	-ve	-ve
Starch hydrolysis	+ve	+ve	-ve	-ve
Gelatin liquefaction	-ve	-ve	+ve	-ve
Fermentation of sugars				
Glucose	-ve	-ve	-ve	-ve
Sucrose	-ve	-ve	-ve	-ve
Lactose	-ve	-ve	-ve	-ve

thermophilic bacterial isolate PS3 were round, convex with undulating margins, and yellowish-white having spreading growth on Castenholz TYE medium and cells were gram positive, rod shaped, arranged singly, motile and sporulating (Table 1). These isolates were positive for Catalase test, Oxidase test, Gelatin liquefaction and Methyl Red test where as rest of biochemical descriptors viz., Indole test, Nitrate reduction, Citrate utilization, Lysine utilization, Phenylalanine deamination, Urease production, H_2S production and fermentation of sugars were found to be negative (Table-2). Optimal temperature observed for their maximum growth was 50-65ºC, optimal pH was 7.0-9.0 and optimal time was 96 hrs. Whereas colonies of thermophilic bacterial isolate PS4 were circular, creamish-white on Castenholz TYE medium and cells were gram positive, rod shaped, arranged singly, motile and sporulating (Table 1). These isolates were positive for Catalase test and Methyl Red test where as rest of biochemical descriptors viz., Oxidase test, Indole test, Nitrate reduction, Citrate utilization, Lysine utilization, Phenylalanine deamination, Urease production, H_2S production and fermentation of sugars were found to be negative (Table 2). Optimal temperature observed for their maximum growth was 65ºC, optimal pH was 6.0-8.0 and optimal time was 96 hrs. In terms of phenotypic and physiological characters, the identification of thermophilic bacterial isolates, PS1 and PS2 are in agreement with those obtained by Bisht and Panda, 2011, where as thermophilic bacterial isolate PS3 are in line with findings of previous studies by Shida *et al.,* 1997; Saha *et al.,* 2005 and thermophilic bacterial isolate PS4, showed similar phenotypic and physiological characters like those obtained by Khiyami *et al.,* 2012. These thermophilic bacterial isolates also showed consistent results with the results of Rastogi *et al.,* 2009 on the basis of phenotypic and physiological characters. The phenotypic and genotypic characterization of thermophilic bacteria has been done for many geothermal areas in different parts of the world, including Turkey (Gul-Guven *et al.,* 2008; Adiguzel *et al.,* 2009), Italy (Maugeri *et al.,* 2001), Bulgaria (Derekova *et al.,* 2008), Greece (Sievert *et al.,* 2000), China (Lau *et al.,* 2009), India (Sharma *et al.,* 2008) and Iceland (Takacs *et al.,* 2001).

16S rRNA gene sequences of these four isolates were analysed. The 16S rDNA of the isolates was amplified and the amplified genomic DNA of the isolates produced PCR bands of approximately 1300bp in size. PCR products of these isolates viz., PS1, PS2, PS3 and PS4 were then sequenced which contain 1161, 1245, 1153 and 1237 nucleotides having 50-60% G+C content respectively. *Insilico* analysis of these four sequences was carried out using BLASTn analysis and based on BLAST alignment of 16S rDNA sequences of these isolates to GenBank sequences, these isolates were found to belong to genus *Brevibacillus, Paenibacillus* and *Bacillus* with 98-99% identity. The isolate PS1 and PS2 showed 99% similarity with *Brevibacillus thermoruber* (NR 026514.1), isolate PS3 showed 98% similarity with *Paenibacillus barengoltzii* (NR 042756.1) and isolate PS4 showed 99% similarity with *Bacillus licheniformis* (NR 074923.1). These results are in line with those obtained by Maugeri *et al.,* 2001; Saha *et al.,* 2005; Rastogi *et al.,* 2009 and Bisht *et al.,* 2011. A gram positive, rod shaped and sporulating thermophilic *Bacillus licheniformis* has also been isolated from compost (Prema and Devi, 2012). It was also observed in the previous studies that the phylotypes falling in Bacillales formed several monophyletic clusters with known lineages belonging to genera *Brevibacillus, Paenibacillus* and *Bacillus* (Rastogi *et al.,* 2009).

Molecular community were determined by phylogenetic analysis of 16S rRNA gene sequences and this analysis showed that all phylotypes retrieved from enrichment cultures were affiliated to *Firmicutes*. The phylogenetic analysis of the 16S rRNA gene sequences confirmed the affiliation of these thermophilic isolates with the genus *Brevibacillus, Paenibacillus* and *Bacillus* with 98-99% homology (Fig.1, 2, 3). It was revealed that *Brevibacillus* isolates PS1 and PS2, could be allocated into the species *thermoruber* with homology values ranging from 99-100%. On the other hand, *Paenibacillus* isolate PS3 and *Bacillus* isolate PS4 could be allocated into species *barengoltzii* (98% homology) and *licheniformis* (99% homology) respectively. Several isolates of *Brevibacillus, Paenibacillus* and *Bacillus* were also reported by Kato *et al.,* 2005; Saha *et al.,* 2005; Wang *et al.,* 2008 and Al-Quadan *et al.,* 2009.

The 16S rDNA sequences of these four isolates were then submitted to NCBI under these accession numbers., JN 559848.1 (*Brevibacillus thermoruber,* PS1), JN 559849.1 (*Brevibacillus thermoruber,* PS2), JN 559850.1 (*Paenibacillus*

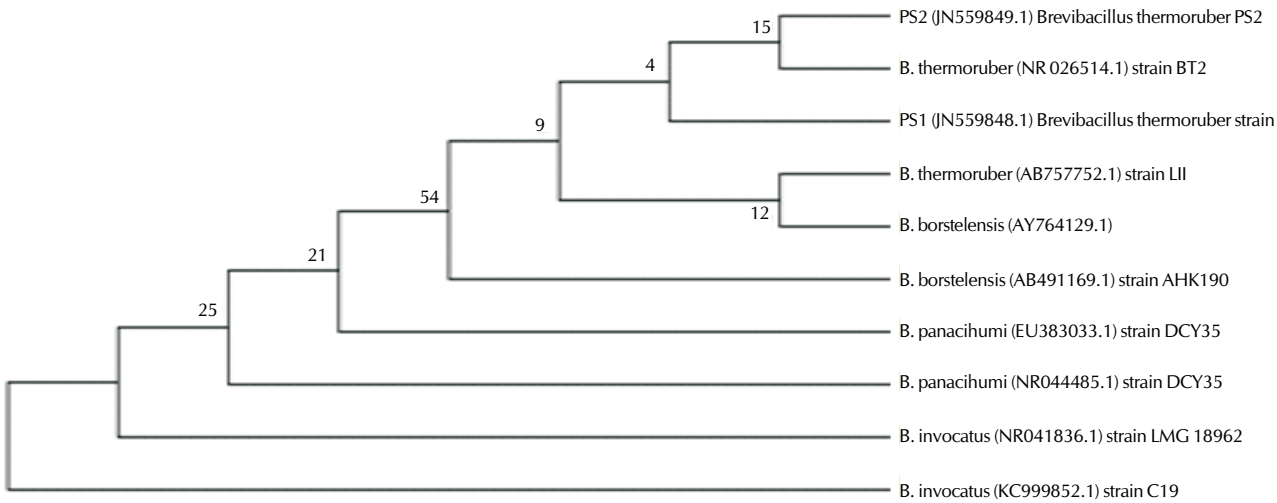

Figure 1: Neighbour joining tree based on 16S rRNA gene sequence showing the phylogenetic relationship of isolates PS1 and PS2 with the analysed sequences

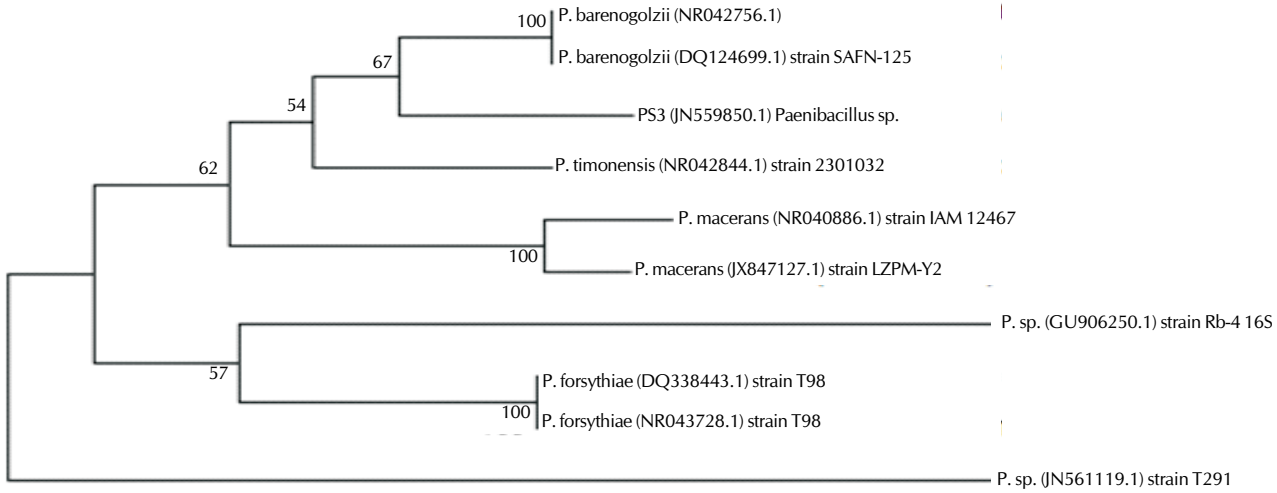

Figure 2: Neighbour joining tree based on 16S rRNA gene sequence showing the phylogenetic relationship of isolate PS3 with the analysed sequences

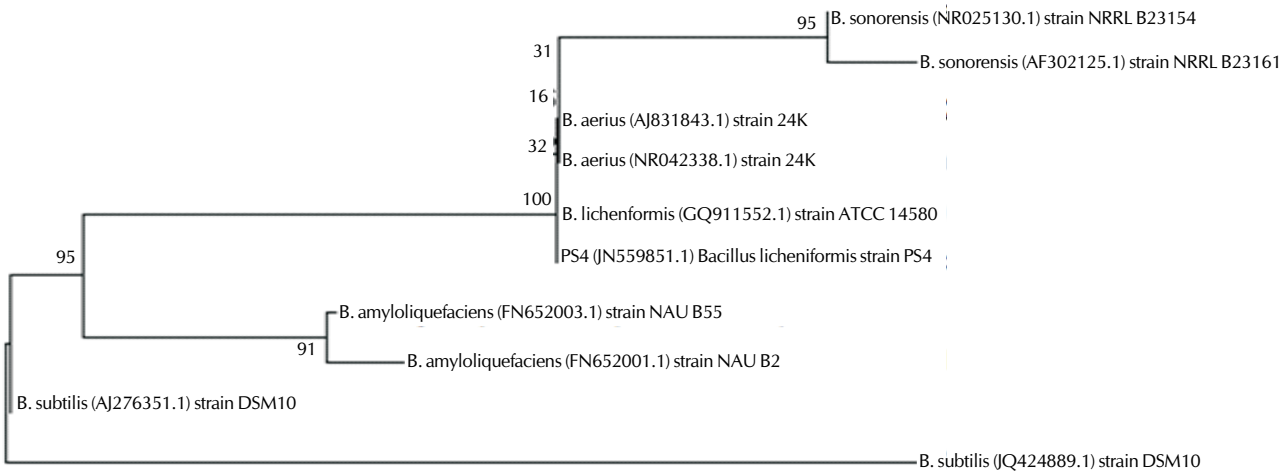

Figure 3: Neighbour joining tree based on 16S rRNA gene sequence showing the phylogenetic relationship of isolate PS4 with the analysed sequences

barengoltzii, PS3) and JN 559851.1 (*Bacillus licheniformis,* PS4).

CONCLUSION

Four thermophilic bacterial isolates viz., (*Brevibacillus thermoruber,* PS1), (*Brevibacillus thermoruber,* PS2), (*Paenibacillus sp.,* PS3) and (*Bacillus licheniformis,* PS4) were isolated and characterized.

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