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Cultivable bacterial diversity of terrestrial thermal spring of Unkeshwar, India

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Abstract

Ten different thermotolerent bacteria were isolated from terrestrial thermal spring of Unkeshwar in Nanded district of Maharashtra (India). These isolates were characterized by morphological characters, microscopic features, biochemical pattern and physiological attributes. These isolates were identified as *Bacillus licheniformis* (APP7), *Bacillus megaterium* (APP8), *Actinobacillus hominis* (APP9), *Lysinibacillus sphaericus* (APP10), *Paenibacillus alvei* (APP11), *Bacillus simplex* (APP12), *Actinobacillus seminis* (APP13), *Pseudomonas fragii* (APP14), *Staphylococcus cohnii* (APP15) and *Streptococcus thermophilus* (APP16). These isolates belonged to class Firmicutes and Gamma proteobacteria and showed production of biotechnologically important thermostable hydrolytic enzymes such as caseinase, amylase, gelatinase, urease and lipase.

Keywords: Unkeshwar (India), Diversity, Hot spring, Thermophiles, Thermozymes

Introduction

Thermophiles are found in different biotopes such as hot springs, geothermal sediments and marine solfataras (Rothschild and Manicineli 2001). Thermophiles fulfill demand of different industrially important enzymes that have optimum temperatures ranged from 45 - 110°C (Antranikian et al. 1995). Thermozymes are having industrial importance in some applications because they withstand and are active at temperatures above 60-70°C. In addition, they are more resistant than their mesophilic counterparts to organic solvents, detergents, low and high pH and other denaturing agents (Cowan et al. 1985; Cowan 1997; Gupta and Khare 2006).

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Thermozymes are used in several industries such as detergent, agricultural, starch liquefaction, saccharification, textile, paper, food, baking, novel food applications and analysis in medical and clinical chemistry in which they replace chemicals or mesophilic enzymes (Pathak and Rekadwad 2013). With the industrially desirable attributes of thermozymes are of considerable biotechnological interest and may open unexplored avenues of bio-catalysis which were otherwise limited due to use of mesophilic enzymes. Therefore there has been a need to explore various thermal habitats like hot water springs for isolation, identification and cultivation of thermophilic organisms and determination of their extracellular thermostable enzyme profile. Similar types of studies were carried out on Soldhar hot spring site of Garhwal Himalaya, India (Sharma et al. 2009) and hot spring of Bakreswar, West Bengal (India) (Hauli et al. 2013).

In present investigation, we have selected Unkeshwar hot water spring (19°51'21.3"N and 78°15'00.9"E) which is located at remote area in Nanded district (Maharashtra, India) and yet no scientific studies have been reported regarding cultivable bacterial diversity of this spring (Figure 1).

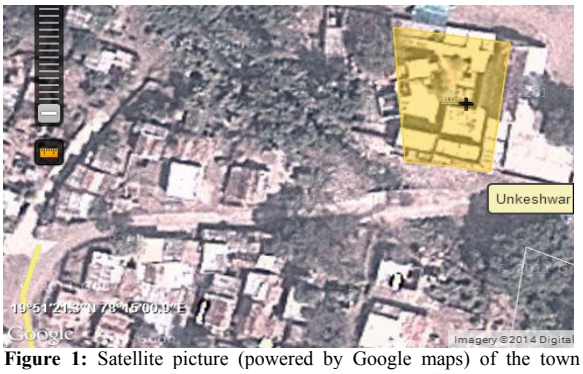

Figure 1: Satellite picture (powered by Google maps) of the town Unkeshwar (19°51'21.3"N, 78°15'00.9"E), district Nanded, Maharashtra, India. The geographical location of hot spring is showed by the symbol '+' in the map marked by yellow shade.

In this context, we have reported isolation of bacterial isolates from Unkeshwar hot spring and identification based on morphological, microscopic and biochemical features. Enzyme profile of the same isolates was also evaluated.

Materials and Methods

Water samples of hot springs of Unkeshwar (India) were collected in April 2011 and temperature of water was recorded at the site. Collected water samples in presterilized polypropylene bottles were placed in ice bags and transported in laboratory within 24 h. For the isolation of bacterial colonies, 0.1 ml of composite sample was spread on Nutrient agar, Tryptone-yeast extract agar, Tryptone-yeast glucose agar, Vogel Johnson Agar, Glucose sodium azide glycerol Agar, Thiosulphate agar, J agar, Brain heart infusion agar, Gram negative agar and Bacillus agar plates in triplicates (Aneja 2007; Krieg and Holt 1984; Ronald 2005; Sneath et al. 1984).

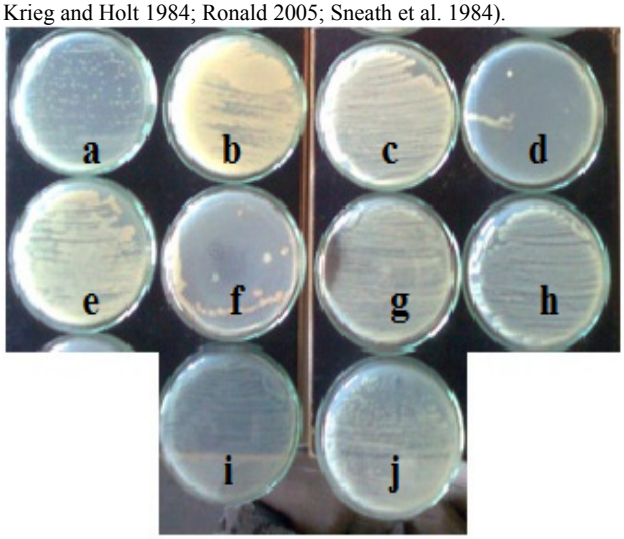

Figure 2: Selected bacterial isolates from Unkeshwar (India) hot water spring. Abbreviations: a: APP9, b: APP7, c: APP11, d: APP8, e: APP10, f: APP12, g: APP14, h: APP15, i: APP16, j: APP13

Appropriate positive and negative controls were used in all these tests. Antibiotic susceptibility was tested by disc diffusion method (Pathak and Rathod 2013; Pathak and Sardar 2012). Effect of temperature on growth of organism was determined by varying temperature in the range of 25 to 75°C with an increment of 10°C. Optimization of pH for growth was carried out by growing organism in media having different pH in the range 4 to 10 with an increment of 1 unit. Optimum incubation period required for growth was determined by incubating cultures up to 96 h. Optimum NaCl concentration required for growth was determined by incubating cultures in media having 0 to 5% NaCl (Aneja 2007; Krieg and Holt 1984; Sneath et al. 1984).

Results and Discussion

Temperature and pH of collected water sample of Unkeshwar (India) hot spring was 47°C and 7.0 respectively. Of the total 10 different medium used, Nutrient agar (95 CFU) showed luxuriant growth of bacteria followed by Tryptone-yeast glucose agar (90 CFU), Brain heart infusion agar (88 CFU), Tryptone-yeast extract agar (70 CFU), J agar (68 CFU), Bacillus agar (62 CFU), Thiosulphate agar (54 CFU), Gram negative agar (51 CFU), Vogel Johnson Agar (23 CFU) and Glucose sodium azide glycerol Agar (13 CFU). The calculated CFU values are average of three replicates. Morphologically distinct ten colonies were selected from all media used and designated as APP7 to APP16 for further characterization (Figure 2). Morphological characters, microscopic features, biochemical pattern, physiological attributes and sensitivity to antibiotics of APP7 to APP16 isolates are illustrated in table 1, 2, 3, 4 and 5 respectively.

On the basis of morphological characters, microscopic features, biochemical pattern, physiological attributes and sensitivity to antibiotics APP7, APP8, APP9, APP10, APP11, APP12, APP13, APP14, APP15 and APP16 isolates were identified as *Bacillus licheniformis*, *Bacillus megaterium*, *Actinobacillus hominis*, *Lysinibacillus sphaericus*, *Paenibacillus alvei*, *Bacillus simplex*, *Actinobacillus seminis*, *Pseudomonas fragii*, *Staphylococcus cohnii* and *Streptococcus thermophilus* respectively. Out of 10 identified isolates seven isolates belongs to class Firmicutes and three belongs to class Gamma proteobacteria. Six isolates were Gram positive, three were Gram negative and one isolate was Gram variable. Out of six Gram positive isolates, five were spore former and one was non spore former as well four were motile and two were non motile (Table 2). Of the total 10 isolates, seven were caseinase and urease producers, six were amylase and oxidase producers,

Table 1: Morphological characters of bacterial isolates from Unkeshwar hot water spring (India)

Characters	APP7	APP8	APP9	APP10	APP11	APP12	APP13	APP14	APP15	APP16
Shape	Round	Round	Round	Round	Circular	Round	Round	Round	Circular	Round
Size	4 mm	3 mm	1 mm	3 mm	1 mm	3 mm	4 mm	2 mm	1 mm	2 mm
Margin	Undulate	Undulate	Regular	Regular	Regular	Regular	Entire	Regular	Entire	Entire
Elevation	Semi-raised	Elevated	Flat	Raised	Semi-raised	Semi-raised	Convex	Convex	Convex	Convex
Consistency	Non-sticky	Non - sticky	Semi-sticky	Dry	Dry	Dry	Dry	Butyrous	Dry	Non - sticky
Opacity	Opaque	Opaque	Opaque	Opaque	Trans-lucent	Opaque	Opaque	Opaque	Trans-lucent	Opaque
Surface	Dull	Smooth	Semi-mucoid	Glossy	Shiny	Umbonate	Smooth	Smooth	Glistening	Smooth
Color	White	Pale white	White	Pale white	Pale white	Creamy	White	White	Pale white	Dirty white
Pigmentation	-	-	-	-	-	-	-	-	-	-

Morphological characters recorded for selected isolates were colony shape, size, margin, consistency, opacity, surface, color and pigmentation. Microscopic features of selected isolated recorded were cell shape, cell length, cell width, cell motility, spore staining, Gram staining and flagella staining (Aneja 2007). Catalase, oxidase, indole and H_2S production, citrate utilization and hydrolysis of gelatin, casein, starch, cellulose, urea, pectin and tween-80 were observed. Methyl red, Vogues-Proskauer tests and carbohydrate fermentation tests were performed using standard procedures.

Table 2: Microscopic features of bacterial isolates from Unkeshwar hot water spring (India)

Characters	APP7	APP8	APP9	APP10	APP11	APP12	APP13	APP14	APP15	APP16
Cell shape	Straight rods	Straight rods	Pleo-Morphic	Rod	Rod	Rod	Coco-bacillary	Rods	Coccoid	Cocci in chains
Cell length	1.5 µm	2.0 µm	0.8µm	1.5 µm	3.0 µm	1.0µm	1.2µm	0.5µm	-	-
Cell width	0.6 µm	0.8 µm	0.2µm	0.6 µm	0.5µm	µm	0.5µm	1.5µm	0.8µm	0.6µm
Sporulation	+	+	-	+	+	+	-	-	-	-
Position of spore	ST	T	-	C	T	T	-	-	-	-
Motility	M	M	NM	M	M	M	NM	M	NM	NM
Grams nature	Gram positive	Gram positive	Gram negative	Gram variable	Gram positive	Gram positive	Gram negative	Gram negative	Gram positive	Gram positive
Flagella arrangement	PT	PT	-	PT	PT	PT	-	Polar	-	-

Abbreviations: PT: Peritrichous flagella, **ST**: Sub-terminal, **T**: Terminal, **C**: Central, **M**: Motile, **NM**: Non motile, **+**: Present, **-**: Absent

Table 3: Biochemical pattern of bacterial isolates from Unkeshwar hot water spring (India)

Tests	APP7	APP8	APP9	APP10	APP11	APP12	APP13	APP14	APP15	APP16
Dextrose	+	+	-	w+	+	w+	w+	+	-	+
Fructose	-	+	-	-	+	-	-	-	-	+
Lactose	+	+	-	-	-	-	-	-	+	+
Sucrose	+	-	+	-	-	w+	-	-	-	+
Mannitol	+	+	+	+	-	-	-	-	-	-
Maltose	+	-	-	-	-	w+	-	-	-	-
Xylose	-	-	+	-	+	w+	-	-	-	-
Arabinose	-	+	-	-	+	-	-	+	-	-
Galactose	+	+	+	-	-	-	-	+	-	-
Glycerol	+	-	-	-	-	-	-	-	-	-
Cellobiose	-	+	-	-	+	-	-	-	-	-
Sorbitol	+	-	-	-	+	-	-	-	-	-
Mellibiose	-	-	+	-	-	-	-	-	-	-
Mannose	+	-	-	-	-	-	-	-	+	+
Trehalose	+	-	-	-	-	-	-	-	+	-
Ribose	+	+	+	-	-	+	-	-	-	+
Salicin	+	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-
Inulin	-	+	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-
Raffinose	-	+	+	-	-	-	-	-	-	-
Gas and H_2S production	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-
MR test	-	-	-	-	-	-	-	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-
Citrate test	-	+	-	+	-	-	-	-	-	+

Abbreviations: w+**:** weakly positive**, +:** acid production or positive test**, -:** No acid production or negative test

Table 4: Enzyme profile of bacterial isolates from Unkeshwar hot water spring (India)

Enzymes	APP7	APP8	APP9	APP10	APP11	APP12	APP13	APP14	APP15	APP16
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	+	+	-	+	-	+	-	+
Caseinase	+	+	+	+	+	+	-	+	-	-
Amylase	+	+	-	+	+	+	-	-	-	+
Gelatinase	+	+	-	+	+	+	-	-	-	-
Cellulase	-	-	-	-	-	-	-	-	-	-
Urease	+	+	+	-	+	+	-	-	+	+
Lipase	-	w+	-	w+	w+	-	-	w+	-	-
Pectinase	-	-	-	-	-	-	-	-	-	-

Abbreviation: w+**:** weakly positive**, +:** Positive**, -:** Negative

Table 5: Physiological attributes of bacterial isolates from Unkeshwar hot water spring (India)

Characters	APP7	APP8	APP9	APP10	APP11	APP12	APP13	APP14	APP15	APP16
Temperature growth range (^oC)	25-55	25-55	25-55	25-55	25-45	25-45	25-45	25-45	25-45	25-55
Optimum temperature (^oC)	45	45	45	45	35	35	45	35	35	35
pH growth range	5-8	5-8	4-8	5-9	6-8	6-9	6-8	6-8	6-8	6-8
Optimum pH	7.0	7.0	7.0	8.0	7.0	7.0	7.0	7.0	7.0	7.0
NaCl tolerance (%)	0-4	0-5	0-5	0-5	0-2	0-2	0-2	0-3	0-3	0-2
Optimum growth period (h)	48	48	72	48	72	48	48	48	72	72

Table 6: Bacterial isolates from Unkeshwar hot water spring (India) showing sensitivity to various antibiotics

Antibiotics	APP7	APP8	APP9	APP10	APP11	APP12	APP13	APP14	APP15	APP16
Amikacin (30µg)	2.7	2.5	2.5	4.2	1.4	2.5	3.5	3.5	3.1	2.3
Ampicillin (10µg)	2.7	2.4	2.5	4.1	3.8	3.4	3.1	2.4	3.4	3.4
Cefoperazone (75µg)	2.1	3.1	2.6	2.6	2.6	3.6	3.8	2.7	2.8	2.4
Cefoxitin (30µg)	3.2	2.8	3.5	2.5	2.4	3.4	2.6	3.5	2.7	4.2
Ceftazidime (30µg)	3.2	2.5	2.3	2.5	2.6	2.7	2.5	3.6	4.1	2.2
Ceftriaxon (30µg)	2.7	3.0	3.3	2.5	3.6	3.7	3.4	3.6	3.4	2.4
Chloramphenicol (25µg)	2.0	2.5	2.3	2.1	2.2	2.4	2.0	2.1	1.9	3.5
Chloramphenicol (30µg)	3.5	3.1	2.6	2.4	2.8	2.7	2.3	2.5	2.4	3.7
Ciprofloxacin (5µg)	3.9	2.6	4.3	3.5	2.6	2.6	2.7	2.1	2.2	3.4
Cotrimoxazole (30µg)	3.8	3.0	3.6	2.4	2.6	2.6	2.1	2.2	2.6	3.3
Gentamicin (10µg)	2.7	3.1	3.5	3.2	3.3	3.6	3.7	3.6	3.2	2.7
Netillin (30µg)	1.8	2.3	1.6	2.6	1.7	3.6	2.4	2.1	2.5	2.5
Ofloxacin (5µg)	3.2	3.1	1.7	1.3	2.5	2.6	2.8	4.2	3.9	4.0
Penicillin (10U)	2.0	2.9	2.2	1.2	2.4	2.5	0.9	1.1	3.0	3.5
Penicillin G (1U)	1.2	2.7	R	R	1.3	1.6	R	R	2.1	2.2
Piperacillin (100 µg)	1.4	2.7	3.1	2.6	2.6	3.2	2.7	2.3	3.5	2.5
Streptomycin (10µg)	2.5	2.3	2.3	2.4	3.1	3.3	2.1	2.6	3.7	2.6
Sulphatriad (30µg)	2.1	1.6	R	2.1	2.9	3.7	2.2	2.6	R	2.9
Teicoplanin (30µg)	3.1	3.4	2.1	2.0	2.2	2.2	2.1	3.0	2.9	2.7
Tetracycline (30µg)	3.8	2.7	2.6	2.5	2.1	2.6	2.8	2.5	2.1	2.7
Tetracycline (25µg)	2.5	1.5	2.2	2.3	2.1	2.0	2.3	1.6	1.9	2.1
Vancomycin (30µg)	2.1	2.4	2.4	2.5	2.6	2.5	3.0	3.1	2.6	2.4

Abbreviation: R: Resistant; **Size of zone is given in centimeter**

five were gelatinase producers and four were lipase producers. All ten identified isolates were catalase positive (Table 4). All the isolates have showed sensitivity to various antibiotics. APP9, APP10, APP13 and APP14 showed resistance to Penicillin G (1 U/disc). APP9 and APP15 showed resistance to Sulphatriad (30 µg/disc) antibiotic (Table 6).

In an earlier report, Streptococcus thermophilus was isolated from Zerka-Maeen hot spring located along the Jordan Rift Valley (Khalil et al. 2003). Isolation and identification of *Bacillus licheniformis* BT5.9 and *Bacillus megaterium* S_2B_{11} strains were reported from Changar hot spring, Malang, Indonesia (Darah et al. 2013) and Sitakunda hot spring (Hossain and Anwar 2009) respectively. Presence of thermophilic *Actinobacillus* Sp. was reported from cattle compost (Saggu and Shrivastava 2013). Facultative, alkaliphilic and denitrifying *Pseudomonas* strain CRS1 was isolated and characterized from hot spring, Yang-Ming Mountain, Taiwan (Wong and Lee 2014).

Conclusions

In conclusion, 10 different thermotolerent bacteria were isolated and identified from terrestrial thermal spring of Unkeshwar (India) as *Bacillus licheniformis* (APP7), *Bacillus megaterium* (APP8), *Actinobacillus hominis* (APP9), *Lysinibacillus sphaericus* (APP10), *Paenibacillus alvei* (APP11), *Bacillus simplex* (APP12), *Actinobacillus seminis* (APP13), *Pseudomonas fragii* (APP14), *Staphylococcus cohnii* (APP15) and *Streptococcus thermophilus* (APP16). The results obtained from our studies revealed that, identified bacterial isolates from Unkeshwar (India) hot water spring can also be exploited for production of biotechnologically important and industrially demanding thermozymes like amylase, gelatinase, lipase and urease.

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References

Aneja K, (2007) Experiments in Microbiology, plant pathology and biotechnology. New International Publishers, New Delhi, India.

Antranikian G, Rudiger A, Canganella F et al (1995) Biodegradation of polymers at temperatures up to 130°C. J Macromol Sci 32: 661-669

Cowan D (1997) Thermophilic proteins: stability and function in aqueous and organic solvents. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 1997, 118:429-438.

Cowan D, Daniel R, Morgan H (1985) Thermophilic proteases: Properties and potential applications. Trends in Biotech 3:68–72

Darah I, Han Z, Nuraqilah Y et al (2013) Bacillus licheniformis BT5.9 isolated from Changar Hot spring, Malang, Indonesia, as a potential producer of thermostable α-amylase. Trop Life Sci Res 24(1):71–84

Deshmukh K, Pathak A, Karuppayil M (2011) Bacterial diversity of Lonar Soda Lake of India. Indian J Microbiol 51(1):107-113. doi:10.1007/s12088-011-0159-5

Gupta A, Khare S (2006) A protease stable in organic solvents from solvent tolerant strain *Pseudomonas aeruginosa*. Bioresour Technol 97: 1788-1793. doi:10.1016/j.biortech.2005.09.006

Hauli I, Sarkar B, Mukherjee T et al (2013) Isolation and identification of a novel thermo-alkaline, thermostable, SDS and chelator resistant amylase producing *Anoxybacillus* sp. IB-A from hot spring of Bakreswar, West Bengal (India): First report. Adv Appl Sci Res 4(5):202-212

Hossain M, Anwar M (2009) Characterization of microorganisms from Sitakunda hot spring. The Chittagong Univ J B Sci 4:107-117 doi:10.3329/cujbs.v4i1.13394

Khalil A, Anfoka1 G, Bdour S (2003) Isolation of plasmids present in thermophilic strains from hot springs in Jordan. World J Microb Biot 19:239–241 doi: 10.1023/A:1023692531885

Krieg N, Holt J (1984) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, USA

Pathak A, Rathod M (2013) Production and Characterization of Alkaline Protease by *Bacillus pasteurii*: a Lonar Soda Lake Isolate. Innov Res Chem 1(1):22-26

Pathak A, Rekadwad B (2013) Isolation of thermophilic *Bacillus* Sp. strain EF_TYK1-5 and production of industrially important thermostable α-amylase using suspended solids for fermentation. J Sci Ind Res 72:685-689

Pathak A, Sardar A (2012) Isolation and characterization of carotenoid producing Haloarchaea from solar saltern of Mulund, Mumbai, India. Indian J Nat Prod Resour 3(4):483-488

Ronald M, (2005) Media for environmental microbiology. CRC Press, Boca Raton

Rothschild L, Manicineli R (2001) Life in extreme environments. Nature, 409:1092-1101.

Saggu G, Shrivastava D (2013) study on thermophiles reveals the presence of *Actinobacillus lignieresii* in cattle compost. Int J Appl Sci Biotechnol 1(2):33-41

Sharma A, Pandey A, Shouche Y et al (2009) Characterization and identification of *Geobacillus* spp. isolated from Soldhar hot spring site of Garhwal Himalaya, India. J Basic Microbiol 49:187–194.

Sneath P, Mair N, Sharpe M et al (1984) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore

Wong B, Lee D (2014) *Pseudomonas yangmingensis* sp. nov., an alkaliphilic denitrifying species isolated from a hot spring. J Biosci Bioeng 117(1):71-74