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Identification of Thermophilic *Flavobacterium* and *Anoxybacillus* in Unexplored Tatapani Hot spring of Kishtwar District of Jammu and Kashmir: A North Western Himalayan State

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ABSTRACT

Two industrially important thermophilic bacteria were isolated from Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India and were named as KSI and KSII. Water sample from Tatapani hot spring was collected for screening of industrially important thermophillic bacteria. Only two thermophilic bacteria (KSI and KSII) were isolated from Tatapani hot spring water sample. The strict thermophilic nature of both the bacterial isolates (KSI and KSII) was tested by growing them at different temperatures ranging from 40 °C-70 °C. KSI and KSII bacterial isolates showed their optimum growth at temperature 60 °C and at pH 9.0. Morphological and biochemical analysis of both the bacterial isolates was carried out. Results revealed that KSI bacterial isolate was non-motile, Gram negative, large rods and whitish in color. While, KSII bacterial isolate was non-motile, Gram positive, medium rods and creamish in color. Molecular identification of both the bacterial isolates (KSI and KSII) was done by 16s rDNA sequencing. Phylogenetic analysis of 16s rDNA sequences of KSI and KSII bacterial isolates obtained after sequencing revealed that KS1 (GenBank accession no KU248487) had closest homology (99%) with *Flavobacterium thermophilum* G-21 (GenBank accession no NR 104891.1) and bacterial isolate KSII (GenBank accession no KU248486) showed 99% homology with *Anoxybacillus sp.* DR01 (GenBank accession no EU621359.1). Thermophillic bacteria (KSI and KSII) were tested for production of industrially important thermophilic enzymes. Both KSI and KSII bacterial isolates could produce catalase enzyme (catalase positive), nitrate reductase enzyme (nitrate positive) and could produce acetoin (Voges Proskaue positive). KSI was efficient in producing enzymes such as amylase and cellulase, while KSII was efficient in producing glutaminase. The bacteria (KSI and KSII) isolated in the present study are thermophilic in nature and could produce industrially important thermophilic enzymes. Thus, these microorganisms can be used commercially for large scale production of industrially important thermostable catalase, nitrate reductase, amylase, cellulase and glutaminase enzymes.

Keyword (s): Kishtwar, Himalayas, *Flavobacterium thermophilum, Anoxybacillus*

INTRODUCTION

Thermophiles show optimum growth between 60-70°C and a very little growth below 45 °C (1). Thermophilic bacteria are being explored extensively for production of novel thermostable

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enzymes as compared to the plant and animal sources. Thermostable enzymes can withstand high temperature during their large scale production and processing in industries as compared to the mesophilc enzymes. Variety of thermophilic enzymes including proteases, amylase, isomerase and lipase are being utilized in beverages, food, and detergent industries. Whereas, ribonuclease, malate dehydrogenase, Taq DNA polymerase, T4 DNA ligase, and lysozyme are useful in research laboratories (2). Thus, these thermophilic microbial sources fulfill the industrial demands of detergents, pharmaceuticals, food, textiles, and research and development industries. Thermostable enzymes are stable and active under harsh conditions of high temperature thus provide new opportunities for biocatalysis and biotransformation (3).

Geothermally heated regions of the Earth like hydrothermal vents, hot springs, geysers and compost are the main sources of thermophiles. According to the geological survey of India, there are more than over 350 hot springs in India and are classified on the basis of their geo-tectonic setup (4, 5). Most of the Indian hot springs are being explored for microbial diversity. Cellulase and amylase producing thermophilic bacteria named as *Geobacillus sp*. have been isolated from Tattapani hot spring of Himachal Pradesh India (6, 7); Panamik (Ladakh, Jammu and Kashmir). There is also a report on cellulase producing *Thermophilic bacilli* from Tattapani Hot Spring sediment in North West Himalayas (8); two alkaline Indian hot springs, Yumthang (Sikkim) and Jakrem (Meghalaya) are enriched with bacterial and archaeal diversity e.g., *Firmicutes, Chloroflexi and Thermi* dominant in Jakrem and *Proteobacteria* in Yumthang (9).

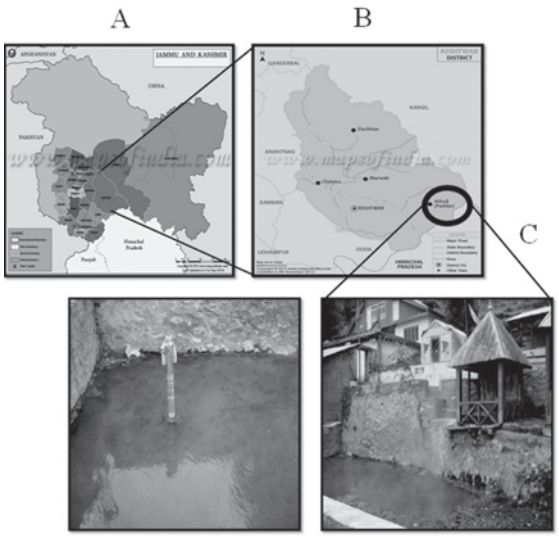

Fig. 1: Geographical location of Tatapani hotspring. (A) Map showing Jammu and Kashmir. (B) Enlarged view of Kishtwar district showing location of Tatapani hotspring circled (black) and (C) Actual view of Tatapani hotspring.

The Himalayan regions of Jammu & Kashmir State (A North Western Himalayan state) posseses more than 20 hot spring sites. In this study, a hot spring located in Himalayan sub range of Kishtwar district of Jammu and Kashmir, India (Fig. 1A-C) was explored for isolation of industrially important thermophilic bacteria. This hot spring is not even in the list of thermal hot springs of India and has not been explored for microbial diversity.

Two thermophilic bacteria were isolated from Tatapani hot spring and were named as KSI and KSII. Both the thermophilic bacterial isolates were analyzed morphologically and by biochemical/ molecular characterization. After molecular characterization, KSI and KSII were identified as thermophilic bacteria of genus *Flavobacterium* and *Anoxybacillus,* respectively. Temperature and pH optimization for KSI and KSII bacterial isolates was carried out. Thermophilic isolates were further analyzed for thermophilic hydrolytic enzyme production. In this study, authors have also compared the characteristics of KSI and KSII thermophilic bacterial isolates with that of PW4, isolated from Tattapani hot spring Himachal Pradesh, India. Identification of PW4 was also done by 16s rDNA sequencing in the present study.

MATERIALS AND METHODS

Isolation and screening of thermophilic bacteria : Water sample was collected aseptically

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in sterile falcon tube (50 ml) from Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India (33° 19' 0" North, 75° 46' 0") East. For isolation of thermophilic bacteria, Tatapani hot spring water sample was 10 fold serially diluted and plated on nutrient broth medium containing 2 % agar and the plates were incubated for 24 h at 60 °C. After incubation, morphologically distinct colonies were selected and re-streaked on a nutrient broth medium containing 2 % agar to get pure colonies. Purified colonies were preserved in 50 % glycerol at -80 °C.

Microscopic analysis of thermophilic bacterial isolates *:* Morphology of the isolated bacteria was studied by Gram's staining (10) and the bacteria were visualized under light microscope.

Effect of temperature and pH on the growth of thermophilic bacterial isolates : The optimum temperature and pH for the growth of thermophilic bacterial isolates were studied by streaking and by quantitative measurements either in nutrient broth medium containing 2 % agar or in only nutrient broth medium, respectively. The cultures were grown at different temperature in the range of 40-70 °C. The pH of medium was adjusted to different pH ranges (5-12). Glacial acetic acid was used to adjust the acidic pH 5 and 6; whereas, alkaline pH of 8.0-12.0 was adjusted by 5N NaOH. Cultures were incubated at 60 °C for 24 hours. After incubation, plates were observed for growth of the bacterial isolates and growth was also observed by quantitative estimation of the culture medium by monitoring the optical density (OD) of liquid culture at A_{600} nm on a double beam UV/ VIS spectrophotometer.

Biochemical characterization *:* In order to test the production of industrially important enzymes by thermophilic bacteria isolated from Tatapani hot spring water sample, various biochemical tests were performed as described earlier (11, 12). For catalase test, a small amount of 24 h old grown culture of thermophilic bacterial isolates was placed aseptically on the clean surface of glass slide. A drop of H_2O_2 (3 %) was placed over bacterial culture on the slide. The slide was observed for release of oxygen bubbles. In glutaminase test, thermophilic bacterial isolates were inoculated in nutrient broth medium (10 ml) supplemented with 1 % glutamine and a drop of phenol red. Culture medium was incubated for 72 h at 60 °C and was observed for change in color from yellow to pink.

Methyl red test was performed by inoculating fresh thermophilic bacterial cultures into Methyl Red Voges Proskauer (VP) broth (10 ml) followed by incubation at 60 °C for 72 h. After incubation, 3-4 drops of methyl red indicator solution was added to the test tubes containing bacterial isolates. The test tubes were observed for change in color of the culture medium from yellow to red. Voges Proskauer test was carried out by inoculating fresh thermophilic bacterial cultures into Methyl Red Voges Proskauer broth (10 ml) followed by incubation at 60 °C for 72 h. After incubation, 1 ml of 5% alpha naphthol and 0.5 ml of 40% KOH were added to the culture medium followed by gentle shaking. The test tubes were observed for change in color of the culture medium from yellow to red.

Indole test was done by inoculating the tryptophan broth (10 ml) with fresh bacterial culture followed by incubation at 60 °C for 24 h. After

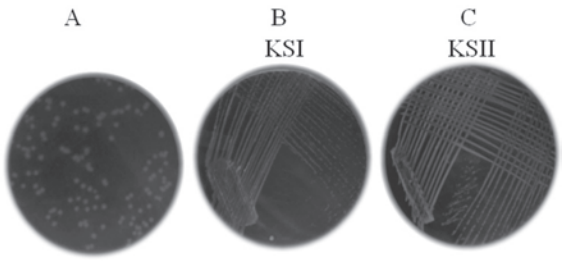

Fig. 2: Growth of bacterial isolates from the water sample of Tatapani. Growth of bacterial colonies from water sample of Tatapani hot spring on LB agar (2 %) medium. (A) 10^{-4} dilution was plated on LB agar medium. Two bacterial isolates (B) KSI and (C) KSII were streaked on LB agar (2 %) medium. Petriplates were incubated at 60 °C for 12 h and observed for growth.

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incubation, 0.5 ml of Kovac's reagent was added to the culture medium. The test tubes were observed for formation of pink colored ring. In nitrate reductase test, nitrate broth (10 ml) was inoculated with fresh bacterial culture followed by incubation at 60 °C for 24 h. After incubation, one drop of sulfanilic acid and one drop of α-naphthylamine was added to the culture medium. The test tubes were observed for change in color of the culture medium from yellow to red.

The amylase and cellulase activity of the thermophilic bacterial isolates was tested by spotting equal number of cells on nutrient broth medium containing 2 % agar and starch (1 %) or caboxy methyl cellulose (CMC) (1 %) plates respectively. Plates were incubated at 60 °C for 24 h. After incubation plates were flooded with Gram's iodine and the plates were observed for zone of clearance around bacterial colonies.

Thermophilic bacterial isolates were also screened for protease activity based on the hydrolysis of casein protein at 60 °C. For this test, equal number of the bacterial cells were spotted on nutrient broth medium containing 2 % agar and casein (1 %). Plates were incubated at 60 °C for 24 h. After incubation, plates were observed for zone of clearance around the bacterial colonies. For lipase activity equal number of cells of thermophilic bacterial isolates were spotted on nutrient broth medium containing 2 % agar and tributyrin (1 %) and observed for the zone of clearance around bacterial colonies after incubation at 60 °C for 24 h. Thermophilic bacteria named as PW1, PW4 PW12, PW10 and PS3 isolated previously from Tattapani hot spring, Himachal Pradesh, India (6, 7) were used as either positive or negative controls in the present study.

Genomic DNA preparation and PCR amplification of 16s rDNA : For the molecular identification of thermophilic bacterial isolates, genomic DNA extraction and purification from each sample (KSI, KSII and PW4) was done as described (13). Each genomic DNA sample (100 ng) was used as template for 16s rDNA gene amplification using universal primer 17F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-TACCTTGTTACGACTT-3´). The amplification was done by initial 2 min denaturing at 94 °C , while 30 sec at every cycle of denaturation at 94 °C , annealing at 40 °C for 30 sec, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. The PCR products were resolved on 1% agarose gel and visualized under UV gel documentation system (Alpha Innotech, USA). PCR products were purified by using a gel extraction kit (Thermoscientific). Gel purified PCR products of 16s rDNA of all bacterial isolates (KSI, KSII and PW4) were sequenced on both the strands using 27 F and 1492 R primers at Eurofins, Bangalore, India (https://www.eurofins.com).

The complete 16s rDNA sequence for each bacterial isolate was generated manually by removing overlapping sequences. The 16s rDNA gene sequence of each bacterial strain was compared against other bacterial 16s rDNA sequences available in the Gene bank data base by using BLAST (blastn) search (14). The nucleotide sequences were aligned using Clustal W 1.74 (15). Phylogenetic tree was constructed by neighbor joining using MEGA4 (http://www.megasoftware.net) (16) and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1,000 replicates). The nucleotide sequences were submitted in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

RESULTS

Water sample of Tatapani showed the presence of thermophilic bacteria : The Tatapani hot spring water sample was plated on nutrient broth medium containing 2 % agar. Whitish and creamish colored colonies were observed after incubation for 24 h at 60 °C. To estimate the total bacterial count, bacterial colonies were counted using colony counter and the colony forming unit (CFU) was determined. CFU count of water sample was 3×10^4 ml^{-1} (Fig. 2A). Both the bacterial isolates showed good visible growth at 60 °C and pH 9.

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Microscopic and biochemical characterization of KSI and KSII : Two different thermophilic bacterial isolates were observed on the basis of colony color and were named as KSI and KSII. Both the bacterial isolates (KSI and KSII) were then selected for their biochemical characterization and for their thermophilic enzyme producing abilities, (Fig. 2B-C). Bacterial isolate KSI showed whitish colony and was observed as Gram negative. On the other hand, KSII isolate showed creamish coloration and was Gram positive in nature (Fig. 3A-B). KSI bacteria were large rod shaped as compared to KSII. Moreover, both the bacterial isolates were non-motile in nature (Table 1).

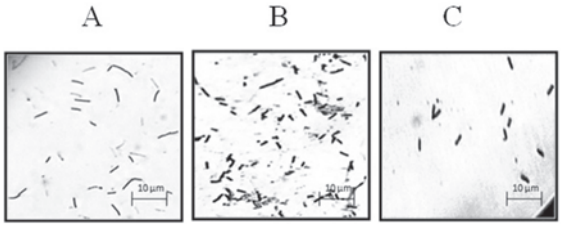

Fig. 3: Light micrograph (100X) of the isolated thermophilic bacteria. (A) KSI, (B) KSII, and (C) *Bacillus sp.* as known control.

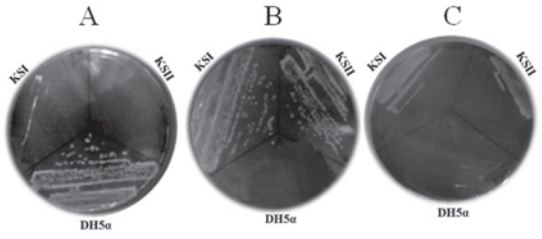

Fig. 4: Growth of thermophilic bacterial isolates at different temperature. Thermophilic bacterial isolates were streaked on LB agar (2 %) medium and incubated for 24 h at different temperatures ranging from 40 °C to 70 °C. (A) Only *E. coli* showed observable growth 40 °C (B) Both KSI and KSII were able to grow at 60 °C and (C) Little growth of KSI and KSII was observed at 65 °C. No growth of KSI and KSII was observed at 50 °C, 55 °C, 70 °C (Data not shown).

Strict thermophilic bacterial isolates were identified from Tatapani hot spring of Kishtwar : To study the effect of temperature on growth, thermophilic bacterial isolates were streaked on nutrient broth medium containing 2 % agar or were grown in only nutrient broth medium parallely. Both the culture medium were incubated at different temperatures (40 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C) for 24 h. Both the bacterial isolates showed detectable growth between 60-65 °C when grown either in nutrient broth medium containing 2 % agar or in only nutrient broth medium. Whereas, none of the bacterial isolates could grow at 40 °C, 50 °C, 55 °C, 70 °C (data not shown), even when incubated for 72 h. In contrast, a mesophile *E. coli* strain DH5α showed detectable growth at 40 °C and no growth at high temperature (Fig. 4A-C and Fig. 5). This indicates the strict thermophilic nature of the bacteria (KSI and KSII) isolated from Tatapani hot spring water sample.

Further, the growth of thermophilic bacterial isolates (KSI and KSII) was tested by streaking of the bacterial isolates on nutrient broth medium containing 2 % agar or by growing the bacterial isolates in only nutrient broth medium parallely. Both the media, either nutrient broth medium containing 2 % agar or only nutrient broth medium

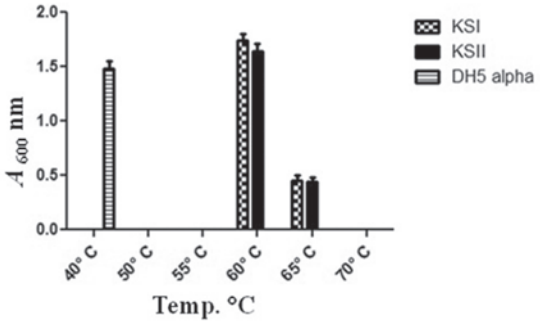

Fig. 5: Effect of temperature on the growth of thermophilic bacterial isolates. Equal number of cells were inoculated in LB medium and incubated at different temperature (40-70 °C) as indicated for 24 h. Cell density was measured by measuring absorbance at A_{600} nm and plotted against the incubation temperature. Data of three independent experiments was plotted with standard deviation.

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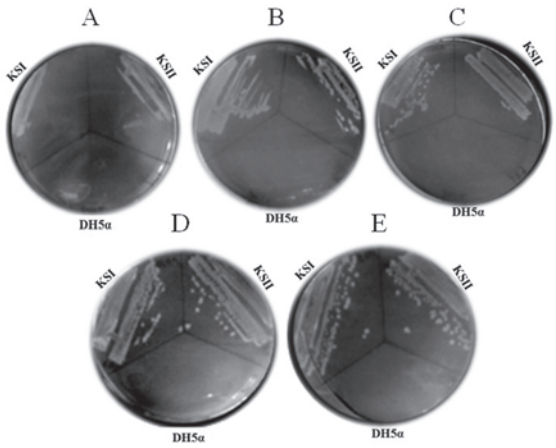

Fig. 6: Growth of thermophilic bacterial isolates at different pH. Thermophilic bacterial isolates were streaked on LB agar medium adjusted at (A) pH 5.0 (B) pH 6.0 (C) pH 7.0 (D) pH 8.0 (E) pH 9.0 (F) pH 10.0 (G) pH 11.0 (H) pH 12.0. Petri plates were incubated at 60 °C for 24 h. Mesophilic bacterial strain DH5α was used as control.

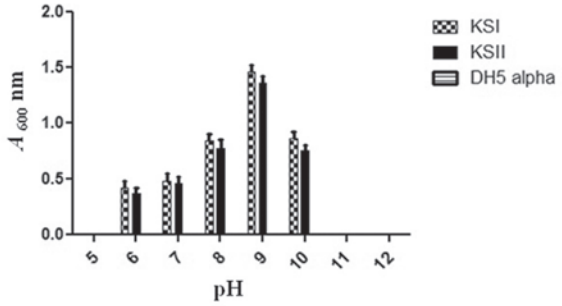

Fig. 7: Effect of pH on the growth of thermophilic bacterial isolates. Equal numbers of cells were inoculated in LB medium adjusted to pH ranging from 5-12. Cultures were incubated at 60 °C for 24 h. Cell density was measured at A_{600} nm and plotted against the different pH. Data of three independent experiments was plotted with standard deviation.

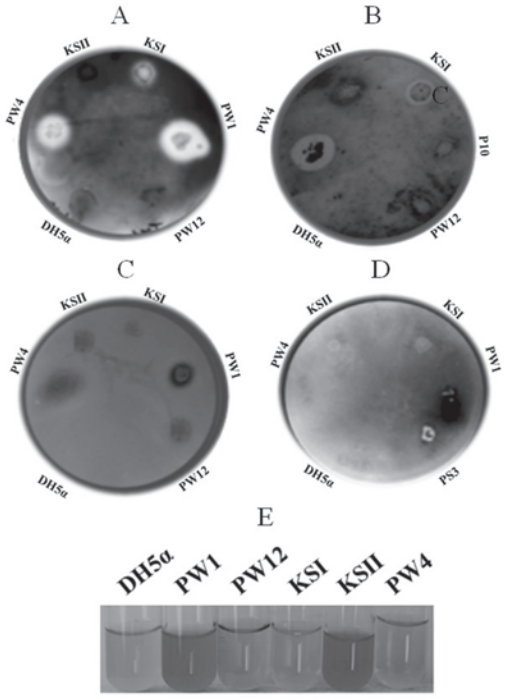

Fig. 8: Qualitative screening of thermophilic bacterial isolates for amylase, cellulase, protease, lipase and glutaminase activity. Equal number of cells of thermophilic bacterial isolates, positive control, negative control and DH5α were spotted on their respective LB agar medium supplemented with (A) 1% starch, (B) 1% CMC (C) 1% skim milk (D) 1% trybutyrin for the detection of amylase, cellulase, protease and lipase respectively. All the cultures were incubated for 24 hr of incubation at 60 °C. After 24 hr of incubation, CMC and starch containing plates were flooded with Gram's iodine and observed for the zone of clearance. Equal number of cells of all thermophilic bacterial isolates, positive control, negative control and DH5α were inoculated in LB medium supplemented with 1% L-glutamine and phenol red. (E) The cultures were incubated at 60 °C for 24 h and visualized for change in color from yellow to pink .

were adjusted to different pH ranging from 5-12 (Fig. 6A-E) and (Fig. 7). Though, the optimum pH for growth of both the bacterial isolates (KSI and KSII) was observed to be pH 9.0, but both the isolates showed detectable growth even at pH 6.0, 7.0, 8.0 and 10.0. No detectable growth was observed at pH 5.0, 11.0 and 12.0 (data not shown).

Production of industrially important thermophilic enzymes by thermophilic bacterial isolates : KSI and KSII bacterial isolates were studied for the production of various industrially important thermophilic enzymes. PW4 a thermophilic bacterial isolate from Tattapani hot spring of Himachal Pradesh, India, was used for

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Table 1. Biochemical analysis of thermophilic bacterial isolates (KSI and KSII)

S. No.	Bacterial strains	Methyl red test	Voges Proskaue test	Indole test	Nitrate reductase test	Catalase test
1.	KSI	-	+++	-	+++	+++
2.	KSII	-	+++	-	+++	+++

comparison of enzyme production. Both the bacterial isolates (KSI and KSII) showed catalase, Voges-Proskauer and nitrate reductase positive reaction. Bacterial isolate PW4 was catalase and Voges-Proskauer positive (Table 1).

To check the amylase and cellulase activity of the thermophilic bacterial isolates, starch and caboxy methyl cellulose (CMC) plates with good bacterial growth were flooded with Gram's iodine. After flooding starch agar plates with Gram's iodine, a varied size clear zone was observed around the bacterial isolate KSI, PW1 (positive control) and PW4. Clear zone of 8 mm size was observed for KSI (Fig. 8A). No zone of clearance was observed around the bacterial isolate KSII, and PW12 (negative control).

Cellulase activity was checked by flooding CMC agar plates with Gram's iodine (17). KSI, PW4 and, PW12 showed zone of clearance of about 8 mm, 16 mm and 8 mm respectively, which indicates production of cellulase (Fig. 8B). Thermophilic bacterial isolates KSII and PW10 (negative control) showed no zone of clearance in CMC agar plates.

Thermophilic bacterial isolates were screened for protease production based on the hydrolysis of casein protein at 60 °C (Fig. 8C). The appearance of zone of clearance due to digestion of casein by the action of extracellular protease indicated the presence of protease activity. Only thermophilic bacterial isolates PW4 and PW1 (positive control) showed protease activity, whereas protease activity was not detected in KSI, KSII and PW12 (negative control) bacterial isolates.

Equal number of cells of thermophilic bacterial isolates were spotted on LB agar medium containing 1 % tributyrin (18) and observed for the zone of clearance as an indication of lipase activity (Fig. 8D). Only the bacterial isolate PW1 (Positive control), showed zone of clearance of 7 mm, while KSI, KSII, PW4 and PS3 (negative control) did not show the zone of hydrolysis.

For glutaminase activity, thermophilic bacterial isolates were inoculated on nutrient broth medium supplemented with 1 % glutamine and a drop of phenol red and observed for change in colour from yellow to pink (19). The bacterial isolates KSII and PW1 (positive control) showed change in color of the medium from yellow to pink (Fig. 8E). No glutaminase activity was detected in KSI, PW4, and PW12 (negative control) isolates.

16s rDNA sequencing identified KSI and KSII as Flavobacterium and Anoxybacillus thermophilic bacteria : For identification of the bacterial isolates, genomic DNA of bacterial isolates was extracted (Fig. 9A). Total genomic DNA of three isolates (KSI, KSII and PW4) which were not identical were subjected to PCR amplification of 16s rDNA using 16s rRNA gene specific primers. 16s rDNA amplified at ~1.5 kbp fragment in all the three isolates as shown in (Fig. 9B). Gel purified DNA fragments were sequenced on both the strands. Overlapped nucleotide sequences obtained by the two primers were removed manually.

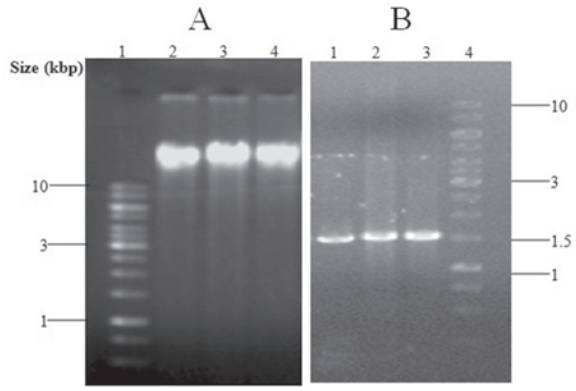

Fig. 9: Genomic DNA isolation and PCR amplification of 16S rDNA gene of KSI, KSII and PW4 bacterial isolates. Total genomic DNA analysis of the thermophilic bacterial isolates as indicated was electrophoresed on 1% agarose gel. (A) Lane 1 indicates 1kb DNA marker, genomic DNA of KSI, KSII and PW4 in lane 2, 3 and 4 respectively. Genomic DNA of the three isolates as indicated was subjected to PCR amplification for 16S rDNA by using primer 1492R and 17F. PCR amplified DNA was electrophoresed on 1 % agarose gel. (B) Lane 1, 2 and 3 represents PCR products of KSI, KSII and PW4 respectively. Lane 4 indicates the molecular size marker (kb).

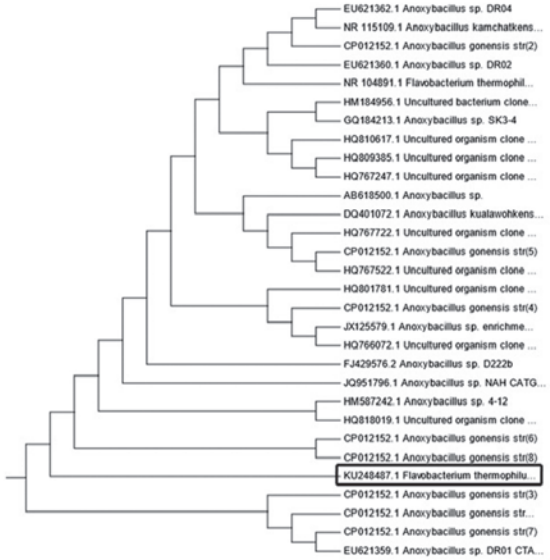

Fig. 10: 16S rDNA based dendrogram showing phylogenetic relationship of newly isolated thermophilie KSI. 16S rDNA sequence of KSI was subjected to BLAST search and hits showing >95% similarity were selected and aligned by using CLUSTAL W. Phylogenetic tree was constructed using MEGA 4 version 3.22. *Flavobacterium thermophilum* KSI KU248487 is indicated in the box.

The complete assembled sequence of 1487, 1474 and 1458 bps were obtained for KSI, KSII and PW4, respectively. Similar nucleotide sequences were identified by BLAST (blastn) search. The 16s rDNA nucleotide sequences of all three bacterial isolates were submitted to the NCBI GenBank database under the accession numbers- KU248487 (*Flavobacterium thermophilum* KSI), KU248486 (A*noxybacillus* sp. KSII) and KU248488 (*Bacillus* sp. PW4). The highest level of nucleotide sequence similarity of isolate KSI (GenBank accession no KU248487) was (99%) with *Flavobacterium thermophilum* G-21 (GenBank accession no NR 104891.1) (Fig. 10) whereas KSII (GenBank accession no KU248486) showed 99% similarity with *Anoxybacillus sp.* DR01 (GenBank accession no EU621359.1) (Fig. 11) while PW4 (GenBank accession no KU248488) showed 99% similarity with *Bacillus sp.* SP22 (GenBank accession no JQ808133.1) (Fig. 12).

Discussion

Till date, a number of microorganisms have been isolated from extreme environments like high/ low pH, temperature, salt, pressure for large scale production of industrially important thermophilic enzymes. Among all these, thermophilic bacteria are getting more importance (20). Thermophilic bacteria produce thermozymes, which are highly stable at high temperatures. Hot springs are the main source for thermophilic bacteria. Keeping this into mind, in the present study we initiated the survey of microbiological organisms of Tatapani, an unexplored hot spring of Jammu and Kashmir, India.

In the present study, two industrially important thermophilic bacterial isolates KSI and

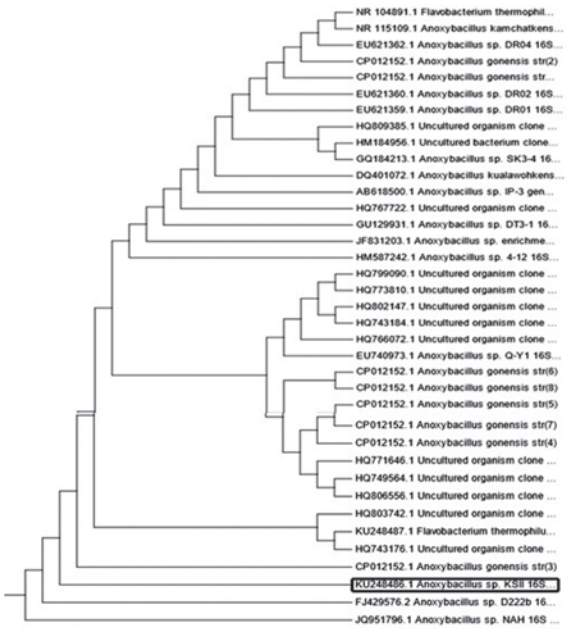

Fig. 11: 16S rDNA based dendrogram showing phylogenetic relationship of newly isolated thermophilie KSII. 16S rDNA sequence of KSII was subjected to BLAST search and hits showing >95% similarity were selected and aligned by using CLUSTAL W. Phylogenetic tree was constructed using MEGA 4 version 3.22. *Anoxybacillus sp.* KSII KU248486 is indicated in the box.

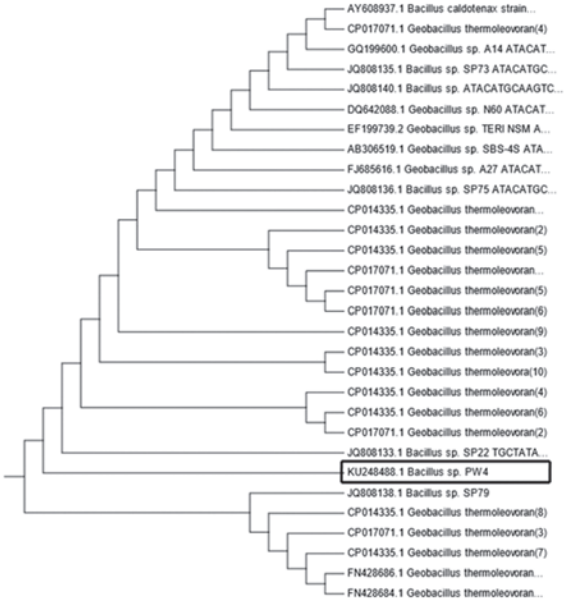

Fig. 12: 16S rDNA based dendrogram showing phylogenetic relationship of newly isolated thermophilie PW4. 16S rDNA sequence of PW4 was subjected to BLAST search and hits showing >95% similarity were selected and aligned by using CLUSTAL W. Phylogenetic tree was constructed using MEGA 4 version 3.22. *Bacillus sp.* PW4 KU248488 is indicated in box.

KSII were isolated from Tatapani hot spring of district Kishtwar, Jammu and Kashmir, India. By 16s rDNA sequencing, it was revealed that the bacterial isolates KSI and KSII were related to genera *Flavobacterium* and *Anoxybacillus* respectively. Previously isolated and characterized thermophilic bacteria PW4 from Tattapani hot spring, Himachal Pradesh, India, was also identified and showed similarity with *Bacillus sp*.

As expected, these thermophilic bacterial isolates (KSI and KSII) produce industrially important thermozymes. *Anoxybacillus* genus was first introduced in 2000 and a number of species are being isolated till now (21). A Novel thermophilic α-Amylase producing *Anoxybacillus flavithermus* SO-13 has been isolated from hot spring mud sample in Afyonkarahisar (Omer) (22). There is also a report on hydrocarbon degrading *Anoxybacillus sp.* isolated from a deep petroleum reservoir (23). KSII showed glutaminase activity and is a glutaminae producing thermophilic bacteria.

Although *Flavobacteroium* is a genus with diverse species, very few reports are available in literature pertaining to the selective isolation and screening of this rare thermophile. *Flavobacterium thermophilum* KSI showed cellulase and amylase activity. KSI is a rare isolate and to our best knowledge, this is the first report on isolation of cellulase producing *Flavobacterium thermophilum* from an Indian hot spring. Amylase producing *Flavobacterium thermophilum* was previously isolated from thermally polluted river in Belgium (24). No such cellulase producing *Flavobacterium*

thermophilum has been reported till now. Oshima and Yamakawa isolated and characterized a novel glycolipid from *Flavobacterium thermophilum,* which was further studied for fatty acid composition (25, 26). Recently, *Flavobacterium arcticum* sp. nov., has been isolated from Arctic seawater (27).

Bacillus sp. survives under wide range of physiological abilities. *Bacillus sp.* PW4 with various enzyme producing abilities was previously isolated from Tatapani hot spring Himachal Pradesh, India. A thermophilic *Bacillus sp*. with protease activity has been isolated from hot spring of Tarabalo, Odisha, India (27). A thermophilic *Bacillus sp*. with extracellular enzymatic activities has recently been isolated from hot spring of Ganeshpuri, Maharashtra, India (28).

Both the bacterial isolates (KSI and KSII) were VP positive, which indicates the production of a compound known as acetoin. Acetoin is an industrially important compound used in food industry as a flavor enhancer and it also gives buttery taste (29). Acetoin is currently produced, commercially by chemical synthesis, which is not safe and not human friendly as it is used mostly in food and cosmetic industry. Thus, production of acetoin by microbial fermentation using KSI and KSII bacterial isolates can replace the chemical synthesis process for natural acetoin production. Moreover, KSI and KSII could produce thermostable acetoin which can withstand high temperature during industrial food processing.

Both the bacterial isolates were also catalase positive, thus can be used for large scale production of thermophilic catalase, which can withstand high temperature treatments in food and textile industries. Nitrate reductase enzyme has important industrial application, as it is mostly used in waste water treatment. In the current study, both the bacterial strains were able to produce thermostable nitrate reductase enzyme and can be used for large scale production of thermostable nitrate reductase enzyme, which can be used further in waste water treatment. Moreover, *Flavobacterium thermophilum* KSI of the present study is a very rare thermophile that can be further explored for the production of new biomolecules of industrial importance.

Conclusion

Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India has never been explored previously for industrially important microbes. In the present study, two industrailly important thermophilic bacteria namely, *Flavobacterium thermophilum* (KSI) and *Anoxybacillus* (KSII) were isolated from the water sample of Tatapani hot spring. This is the first report of isolation of any bacteria from Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India. Both the bacterial isolates (KSI and KSII) are industrially very important, as they can produce industrially important thermostable enzymes viz. catalase, nitrate reductase, cellulose, amylase and glutaminase. In addition, both the bacterial isolates (KSI and KSII) can also be used for large scale production of an industrially important thermostable compound known as acetoin. Moreover, from the present study it was reported that Tatapani hot spring contains a rare and primitive thermophile namely, *Flavobacterium thermophilum*.

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