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Research Article

Culturable Diversity of Thermophilic Microorganisms Found in Hot Springs of Northern Himalayas and to Explore Their Potential for Production of Industrially Important Enzymes

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Abstract: Thermophiles are the microorganisms that live and grow in extremely hot environment that would kill most other microorganisms. They can be isolated from a number of marine and terrestrial geothermally heated habitats including shallow terrestrial hot springs, hyderothermal vents, sediment from volcanic islands and deep sea hyderothermal vents. Thermophiles have been documented as a very good source of industrial enzymes which are thermostable. So in the present investigation, cultivable diversity of 101 thermophilic microbial strains isolated from hot springs of northern Himalayan region of Himachal Pradesh has been studied. Amongst these strains, only 2 were fungi and rest were bacteria. These isolates have been found to be aerobic and sustaining a temperature of or above 50 ^{0}C. All the isolates were checked with respect to their industrial enzyme production potential and were found to be a good source of amylase, cellulase and xylanase. Maximum enzyme producing strains were selected based on the enzyme units of amylase, cellulase and xylanase produced and were identified on the basis of 16S ribosomal RNA gene sequence analysis. Selected isolates were identified mainly as *Bacillus subtilis* NM (G), *Bacillus megaterium* NM 9, *Paenibacillus* N1 and a fungal isolate as *Myceliophthora thermophila* SH1.
Keywords: Amylase, Cellulase, Xylanase, *Bacillus, Myceliophthora thermophila* SH1.

INTRODUCTION

Microbial life does not seem to be limited to specific environments. During the past few decades it has become clear that microbial communities can be found in the most diverse conditions, including extremes of temperature, pressure, salinity and pH [1]. Microorganisms requiring extreme environments for growth are called extremophiles.

Temperature is one of the most important factor controlling the activity and evolution of microorganisms. Microorganisms that have been found to be growing in extremely hot environments i.e. at a temperature range of 55 to 121 °C are known as thermophiles. The cellular components of thermophiles are extremely thermostable and these together with their unique metabolic capabilities, offer considerable promise for biotechnological applications. Such environments are thus of great interest to microbiologists and biotechnologists, as the organisms isolated from these environments are a good source of thermostable enzymes.

In northern Himalayas, 21°57' – 37°5' N latitudes and 72°40' – 97°25' E longitudes with boiling hot water is famous among local people due to their thermophilic potential. Thermal hot springs represent extreme niches that have maintained some degree of pristine quality and their biotechnological potential has remained unrealized. In the last few decades, several attempts have been made for phylogenetic characterization of microflora from thermal springs in different parts of the world *viz.* New Zealand, Iceland, Thailand and Japan etc. [2, 3]

The use of enzymes, obtained from microorganisms as biotransformation catalysts for the formation of various products is well established and well documented [4,5]. As industrial process conditions are harsh, there are demands for biocatalysts that can withstand the process conditions. The majority of the enzymes used to date originate from mesophilic organisms and, despite their many advantages; the application of these enzymes is restricted due to their limited stability at the extremes of temperature, pH and ionic strength. On the other hand, thermophiles are a potent source of thermozymes, which show utmost stability under conditions of high temperature. Thus, biocatalysis using thermophiles as well as thermozymes is rapidly being transformed from an academic science to an industrially viable technology. Each group of the thermophiles has unique features, which can be harnessed to provide enzymes with a wide range of application possibilities [6-8]. Extremophiles are a source of enzymes (extremozymes) with extreme stability and the application of these enzymes as biocatalysts is attractive because they are stable and active under conditions that were previously regarded as incompatible with biological materials. The ability to grow at high temperatures is associated with thermally stable macromolecules. As a consequence of growth at high temperature and unique macromolecular

properties, thermophilic bacteria can possess high metabolic rates, physically and chemically stable enzymes and lower growth but higher end product yields than mesophilic species. Therefore, thermophilic processes are more stable, rapid and facilitate reactant activity and product recovery [8].

Among industrially important enzymes, amylases, cellulases and xylanases have attracted the major attention globally because of their wide application in many fields. Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. Fungal and bacterial amylases could be potentially useful in the pharmaceutical and fine-chemical industries [9]. The application of enzyme increases more with the discovery of thermostable enzymes because thermophilic process is more stable, faster, needs lower costs [10] .Liquid sugar industry needs thermostable α-amylase that can maintain its activity at high temperature, such as in the gelatinization (100-110 °C) and liquefaction process (80-90 °C). Therefore, continuous researches to obtain many more thermostable α-amylases are needed [11].

Cellulases are industrially important enzymes that are sold in large volumes for use in different industrial applications *viz.* in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry [12]. There is a growing merit for cellulase in the field of detergents and saccharification of agriculture waste for bio ethanol technology.

Xylanase represents one of the largest groups of industrial enzymes with increasing market demands and its applications in prebleaching of Kraft pulps, bioconversion of lignocelluloses into feed-stocks and fuels [13], extraction of coffee and plant oils, improvement of the nutritional properties of agricultural silages and degumming of plant fibres such as flax, sun hemp and ramie [14-16]. In food industry, the enzyme treatments has favourable effects on dough handing, bread volume, texture and stability [17], in improving digestibility of animal feeds [18]. Xylanase randomly hydrolyze the β-1,4-glucosidic bonds of xylan to produce xylo-oligomers of different length [19].

Present study was therefore undertaken with the main aim of isolating desirable microorganisms from least explored harsh environmental niches and to explore their potential for industrially important thermostable enzyme production showing higher stability to extremes of temperature and pH etc.

EXPERIMENTAL SECTION

Collection of Samples

Water samples were collected in clean and sterilized autoclaved bottles from hot water springs of Tattapani (Distt. Mandi), Manikaran and Vashist (Distt. Kullu) regions of Himachal Pradesh.

Physiochemical Characteristics of Hot Water Springs

Different parameters i.e. pH, temperature, chloride, sulphate, total hardness, calcium hardness, magnesium content and total phosphorous (APHA, 1976) were evaluated for physiochemical analysis of the hot water sample from hot springs.

Isolation of Thermophilic Bacteria

Thermophilic microorganisms were isolated directly from mushroom compost and water samples by serial dilution technique on Glucose Yeast Extract (GYE) medium and incubated at 50 °C for 2-3 days. For enrichment of samples, the samples were grown in modified Thermus broth containing (%w/v) Tryptone 0.8, Yeast extract 0.4, NaCl 0.2 and glucose 1.0 and incubated at 50 °C for 3-5 days. Following incubation the samples were serially diluted and spread on GYE medium and incubated at 50 °C for 24h. Plates were constantly observed for the appearance of bacterial colonies. Single colonies with distinct morphology were selected from each of the plates and were purified by sub-culturing on the same selective media. 101 isolates thus obtained were selected for further studies. The purified bacterial strains were maintained at 4 °C and -20 °C in GYE medium and 30% glycerol, respectively.

Isolation of Thermophilic Fungi

The isolation of fungi was carried out on Czapek dox agar medium (pH 5) by serially diluting samples and spreading over Czapek dox medium. Plates were kept for incubation at 50 ºC for 6 days. The potential fungal isolates so obtained were subcultured and preserved over malt extract agar medium at 4 ºC.

Morphological and Physiological Characteristics of Isolates

Morphological, cultural and biochemical characterization of pure cultures was done by applying standard techniques for bacteria and fungi. Isolated bacteria were identified by using Bergey's Manual of Determinative Bacteriology [20].

Screening of Isolates for Enzyme Production

Thermophilic microbial isolates were screened for industrial enzymes such as amylase, xylanase and cellulase production.

Production of extracellular amylase

For amylase production, isolated bacterial strains were screened on Lama *et al.*, [21] isolating medium by amending it with 1% starch. The bacterial colonies hydrolysing starch were detected by using starch-iodine method [22]. Extracellular production of amylase was done by adding 1% inoculum of each

isolate in Lama *et al*., [21] broth and incubating it at 50 °C for 120 h. The broth was then centrifuged at 5300 g for 10 min and was assayed for quantity of amylase produced.

Amylase assay

Amylase assay was carried out using phosphate buffer (0.1M, pH 7.0), starch solution (1%) and dinitrosalicylic acid reagent and standard maltose (100µg-1000µg). The reaction mixture contained 0.5 ml of starch solution, 0.3 ml of phosphate buffer and 0.2 ml enzyme. The control was run without adding any enzyme. The tubes were incubated at 50 °C for 5 min and then 3 ml DNS reagent was added to each tube. The tubes were immersed in boiling water bath for 15 min and after cooling, they were read spectrophotometrically at 540nm. Concentration of reducing sugars was calculated from the standard curve of maltose.

Production of extracellular cellulase and xylanase

Each bacterial culture isolate was grown in 50ml of nutrient broth at 35±2 °C from 24 h. As soon as the substantial growth was observed in the broth, the optical density was set to 1.0 using autoclaved distilled water. For fungus, different fungal cultures were grown in malt extract media by incubating them for 5-7 days. As soon as full plate of fungus was observed, 10 ml of autoclaved distilled water was poured and scratched. The spore suspension thus formed contained 1×10^7 spores/ml.

5ml of inoculum was added to each 45 ml of Reese medium's [23] broth containing 1% cellulose for cellulase and 1% xylose for xylanase in 250ml of Erlenmeyer flasks and the flasks were incubated for 5 days at 50±2 °C for bacteria and at 50±2 °C for fungus at 120rpm.

After the incubation at 50 °C for 5 days, the culture contents were centrifuged at 10,000 rpm for 15 min (4 °C), supernatants were collected and quantitative tests were performed with the supernatants to screen out the hypercellulolytic and hyperxylanolytic producers among different isolates.

Enzyme assays

The sub enzymes of cellulase were quantified by following standard enzyme assays (FPase [23] and β-glucosidase assay [24]. Xylanase activity was measured by employing Miller method [25].

Molecular studies of potential bacterial isolates using 16 S rRNA

Bacterial and fungal isolates which showed best enzyme production (IU/ml) were selected and identified at genomic level using 16 S rRNA technique[26] and laboratory of CABI Bioscience UK centre (Egham), UK International Mycological Institute).

Isolation of genomic DNA (Genei DNA Isolation Kit)

The pure culture of bacterial and fungal strains were inoculated in 10 ml of nutrient broth and grown at 35±2 ºC for 18 h. Isolation of total genomic DNA from the culture was carried out by the following standard procedure.

PCR amplification and sequencing

PCR amplification was done to confirm the identity of the bacterial strain, the small subunit 16S rRNA genes were amplified from the genomic DNA with 16SF (5'AGAGTTTGATCCTGGTCAG3') and 16SR (5'TACCTTGTTACGACTT3') primers to get an amplicon size of 1500 bp. Amplification was carried out in 20µl reaction volume consisting of 10 X buffer, 2.0 µl; 2mM dNTPs, 2.0 µl; 3U/µl Taq DNA polymerase, 0.2 µl; 100ng/µl of each primer, 1µl; template DNA, 1µl and sterilized distilled water 12.8 µl in a ASTEC thermalcycler. DNA template was amplified using PCR amplification reaction conditions specific for each primer set. The amplified product (20 µl) was size separated on 1.0% agarose gel prepared in 1% TAE buffer containing 0.5µg/ml ethidium bromide and photographed with the gel documentation system (alpha Imager 2200). A 100 bp DNA ladder (Genei) was used as molecular weight size markers. The PCR product (1500 bp) was purified from contaminating products by electroelution of the gel slice containing the excised, desired fragment with Qiaquick gel extraction kit (Qiagen, USR). The elution was carried out in 30 µl of nuclease free water.

Nucleotide sequence analysis

The PCR amplicon obtained by amplifying PCR product was diluted in Tris buffer (10 mM, pH 8.5). Dilution used was 1:1000 in order to obtain the DNA concentration required for sequencing (30 mg/ µl), the sequencing required 8µl DNA. The primer used in sequencing reaction was 16SF (5'AGAGTTTGATCCTGGTCAG3') at a concentration of 3 µM. Sequencing was then performed using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA).

BLASTN Analysis

Translated nucleotide sequence was then analyzed for similarities by BLASTN tool.

Molecular studies of potential fungal isolate using ITS (Internal Transcribed Spacer) technique

Isolated fungus was further identified at genomic level using ITS (Internal transcribed spacer) technique as given below:

Fungal cultures were grown initially on Malt extract medium subsequently for 7 days each at 50 °C. Mycelia was filtered from the broth culture through Whatman filter paper, rinsed three times with distilled water, blotted to remove excess water and stored at −70 °C until DNA extraction.

Isolation of genomic DNA

DNA was isolated from fungal mycelia (0.02 g) by grinding with quartz sand and extraction following the Zymo research kit. The restricted DNA was analyzed by using 2 % of agarose gel. Electrophoresis was carried out at 70V for 1h. Ethidium bromide was added in the gel and a pre run was given before loading the samples. The gels were visualized under UV on the gel documentation system (Alpha-imager) and gel image were digitalized.

PCR amplification of genomic DNA

Reaction mixture for PCR contained 35 µl of distilled water, 5 µl of PCR buffer (Promega), 4 µl of 25 mM $MgCl_2$, 4 µl of each deoxynucleoside triphosphate at a concentration of 10 nM, 1 µl of each primer (ITS4 and ITS5) at a concentration of 50 pM and 1 µl of isolated DNA (30 ng). The sequences of primers ITS4 and ITS5 were 5'-TCCTCCGCTTATTGATATGC-3' and 5'-GGAAGTAAAAGTCGTAACAAGG-3' respectively (White et al. 1990). The reaction was performed in Personal Cycler Version. The ITS bands were identified by gel electrophoresis on a 1.2 % agarose. DNA template was amplified by using PCR amplification reaction conditions specific for each primer set. An aliquot (8 µl) of positive PCR product was incubated with the addition of appropriate restriction enzyme. After incubation, an aliquot of 40 ng/ µl of brillant blue buffer was added to the reaction mixture and analyzed for products by electrophoresis on 1.2% agarose gel.

BLASTN Analysis

ITS sequence data were analyzed by the Mega 5 software. The forward and reverse sequences for each fungal species were aligned and the identity of the consensus sequence was confirmed using BLAST search to known sequences in NCBI Genbank [27].

RESULTS AND DISCUSSION

Collection of Water Samples

In India, a hillock state Himachal Pradesh-Tattapani (Distt. Mandi), Manikaran and Vashist (Distt. Kullu) are the popular hot water spring pockets which are scarcely being explored for their microbial diversity.

Physiochemical Characterization of Hot Water Springs of HP

Geographically, Himachal Pradesh is located in western Himalayan region. The hot water springs have unique physical location. Tattapani (Distt Mandi) is located 31^0 13' 50" to 32° 04' 30" North and 76° 37' 20" to 77° 23' 15" East whereas Manikaran and Vashist (Distt Kullu) are located 31° 58' N and 77° 06' 4" S.

Physiochemical characteristics of Tattapani, Manikaran and Vashist thermal springs situated in Himachal Pradesh, india include the study of temperature, pH, conductivity, chloride, sulphate, calcium, magnesium and total hardness. Most of the physiochemical characterization of the water samples from these hot pockets has been enlisted in Table-1.

Table 1: Physiochemical analysis of water sample from hot water springs of Himachal Pradesh

Parameter	Vashisht	Manikaran	Tattapani	Mean	S.E. (Mean)
pH	6.590	7.671	6.960	7.071	0.321
Conductivity	782.000	655.000	975.001	804.00	93.030
Total Hardness (mg/l)	60.000	164.000	530.000	399.671	289.231
Ca^{2+} (mg/l)	8.011	40.031	148.120	65.390	42.391
Cl^- (mg/l)	122.200	123.260	385.761	210.41	87.680
Mg^{2+} (mg/l)	9.721	15.550	38.870	21.380	8.911
SO_4^{2-} (mg/l)	57.500	31.681	90.800	59.990	17.110
PO_4^{2-}	0.280	ND	ND	0.091	ND
Temperature	45.000	99.000	52 .000	65.330	16.950
Turbidity	0.021	0.032	0.033	0.033	0.033
Colour	Transparent	Transparent	Transparent	-	-

Tattapani hot spring is located near Sunni in district Mandi on the right bank of Sutlej River at an elevation of 625 m above sea level showed temperature in the range of 53 °C to 60 °C. All the four sites from where the water samples were collected showed the pH in the neutral range i.e. 6.96.

Beas and Parbati valley accounts for 15 of the 30 thermal springs in Himachal Pradesh. In the Parbati valley, Manikaran and in Beas valley, Vashist are the most significant amongst all. The temperature of hot springs varied from 99 °C at Manikaran and 45 °C in Vashist. Maximum temperature (99 °C) was reported among these three hot springs. pH of the hot springs both in Manikaran and Vashist fell into neutral/near neutral range i.e. 7.67 and 6.59 respectively.

Physiochemical analysis of the water from Manikaran and Vashist had total hardness i.e. 164 mg/ ml and 60 mg/ l are considered as soft and moderant

water when compared with tap water having total hardness of 0-100 mg/l as soft water and 100-200 mg/l as moderate water [28]. Whereas water from Tattapani contained total hardness of 530.000 mg/l and thus considered as extremely hard water containing bicarbonates, sulphates, chloride, and nitrates of Ca and Mg. Maximum permissible limit for total hardness is 600 mg/l as per Indian standards [29].

Isolation of Thermophilic Microorganisms

In the present study thermophilic microorganisms were isolated from thermal hot water springs of Himachal Pradesh and were studied for their ability to produce industrially important enzymes such as amylase, cellulase and xylanase enzymes.

A total of 101 microbial strains have been isolated from different sources. Amongst these 101 isolates, only 2 were fungal isolates and rest 99 were bacterial. All the isolates were aerobic in nature and grew at or above 50 ^{0}C within a pH range of 5 to 11. Morphologically, the isolates showed great variation in the color, shape and texture of the colonies (Plate 2). The color of the colonies varied from transparent, white, dirty white, pale white, and yellow to pink. Out of all the isolates, more than 60% were rods and rest were either coccus or coccobacillus. Gram staining revealed that only 4 isolates were gram negative while all others were gram positive.

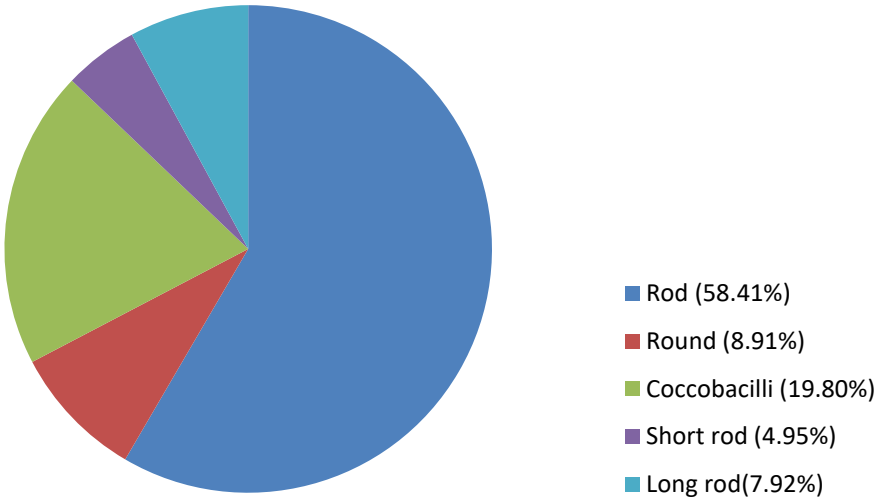


Plate 2: An overview of morphological diversity of bacterial isolates from hot springs of Northern Himalayas.

Various thermophilic bacteria have been isolated from different hot water springs throughout the world, like *Rhodothermus marinus* isolated from hot springs of Iceland [30, 31].

Two alkali tolerant thermophilic bacterial strains were isolated by continuous cultivation from samples collected near Bulgarian hot springs by [32]. Similarly Touzel *et al*., (2000) [33] isolated an aerobic, thermophilic, xylanolytic spore forming bacterium, XETP from farm soil situated underneath a manure heap in northern France. It grows at temperature range of 63 ^{0}C and pH range of 6.5-8.5. Various amylolytic bacteria have also been isolated *viz. Bacillus coagulans* [34], *Bacillus stearothermophilus* [35]. Sunna & Hashwa (1990) [36] isolated an obligately aerobic, rod shaped, gram negative, non spore forming bacterium from soil of Jordan on starch nutrient agar.

Screening of bacterial isolates based on their industrially important enzyme production potential

All the isolates were screened for their amylase, xylanase and cellulase production potential based on qualitative and quantitative tests. The results obtained are:

Amylolytic bacteria

Morphologically, the amylolytic isolates obtained from the hot springs of northern Himalayas have been found mostly to be rods (Plate 3). Around 97% of the strains were gram positive and rest 3% were gram negative.

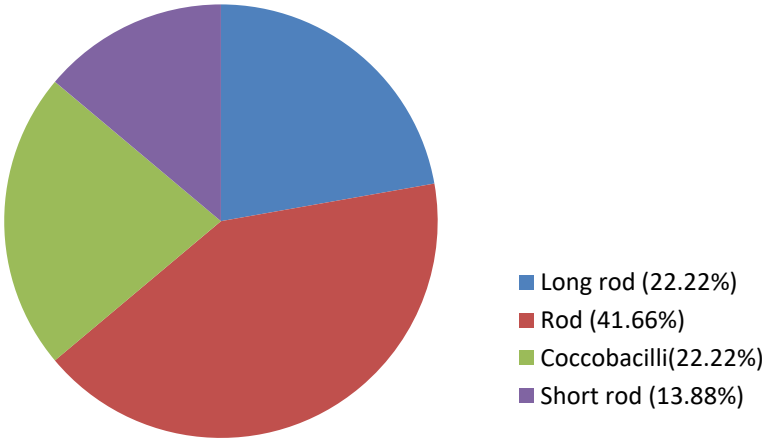


Plate 3: Morphological diversity of amylolytic bacterial isolates from hot springs of northern Himalayas

The strains showed amylase production in the range of 0.077 IU/ml to 29.77 IU/ml. Among all of them, isolate NM9 showed highest amylase production of 29.77 IU/ml after optimization of environmental and culture conditions such as substrate concentration, temperature, incubation time, pH, carbon source etc. Besides producing high titres of enzyme, it has been found to be an acidophilic thermophile growing and showing activity at 50 ^{0}C and pH 4.5. Efficient amylase producers have been given in table 2. There are only a few reports regarding the thermostable amylase producer to be an acidophile. Acidophilic amylase producers are highly desirable in food industry for the liquefaction of starch to produce various starch sweeteners. *Bacillus acidocaldarius* has been reported to produce amylase at low pH of 3.5 [37].

Table 2: Morphological and cultural characteristics of amylolytic bacteria isolated from hot water springs of northern Himalayas

Sr. No.	Isolate No.	Gram Staining & Morphology	Amylase activity (IU/ ml)	Tentative identification
1.	NM (E)	G (+) Rods	10.156	*Bacillus sp.*
2.	NM (G)*	G (+) Rods	23.75	*Bacillus sp.*
3.	NM (R)	G (+) Short rods	10.93	*Bacillus sp.*
4.	NM (S)	G (+) Rods	10.54	*Bacillus sp.*
5.	NM (T)	G (+) Rods	11.39	*Bacillus sp.*
6.	NM 9*	G (+) Rods	29.77	*Bacillus sp.*

Isolation at 50^0 C, *Strains selected for molecular characterization based upon higher enzyme production

Another strain NM (G) showed maximum amylase production of 23.75 IU/ml. It has been found to be an alkalophilic thermophile showing activity at a temperature of 50 ^{0}C and pH 8.5. There are only a limited number of microorganisms which are known to grow at high temperature and high pH. The alkalophilic thermophiles are much desired in the detergent and chemical industry. This potent novel amylase producer can be of a great value to the industry. Highest enzyme producing strain (NM 9) was selected for molecular characterization.

Xylanolytic bacteria

Xylanolytic isolates showed a great diversity morphologically, ranging from rods to coccobacilli and then to round organisms (Plate 4). Around 97% of the strains have been found to be gram positive and rest 3% gram negative.

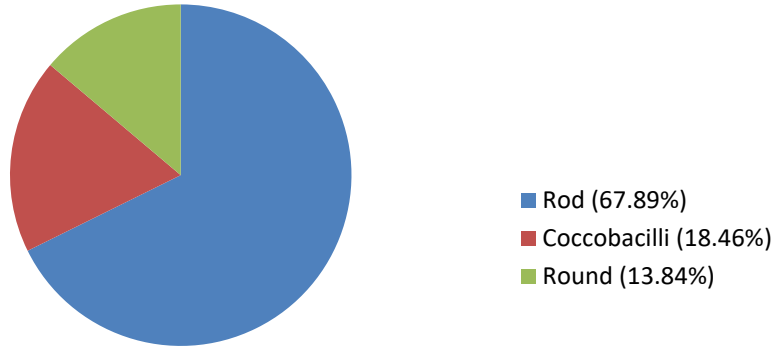


Plate 4: Morphological diversity of xylanolytic bacterial isolates from hot springs of northern Himalayas

Isolated strains have shown the range of xylanase production from 2.100 IU/ ml to 24.600 IU/ ml. N_1 strain isolated from the hot springs of Manikaran showed the appreciable extracellular xylanase production i.e. 52.3 IU/ml (Table 3) in the basal salt medium after 3 days at pH 9, temperature 50 °C, inoculum size 12.5%, amino acid- tryptophan carbon source - xylose, nitrogen source- $(NH_4)_2HPO_4$ and additive- Tween 20. Optimization of different factors i.e. media, incubation time, pH, temperature, inoculum size, amino acids, carbon sources, nitrogen sources and additives for the production of extracellular xylanase from hyperxylanolytic bacterial strain N_1 was carried out under the submerged fermentation.

An isolation of extremophilic bacterial isolate N_1 from hot water springs of Manikaran (H.P.) is a major contribution of the present study. Its thermoalkalophilic nature is a highly desirable attribute which advocates its use for commercial application. It was thus selected for further molecular identification.

Table 3: Morphological and cultural characteristic of xylanolytic bacteria isolated from hot water springs of northern Himalayas

Sr. no.	Isolate no.	Gram staining and Morphology	Xylanase activity IU/ml	Tentative identification
1	T5	G (+) Rod	10.200	*Bacillus sp.*
2	T8	G (+) Round	15.600	*Micrococcus sp.*
3	T17	G (+) Rod	10.500	*Bacillus sp.*
4	T18	G (+) Rod	13.200	*Bacillus sp.*
5	T20	G (+) Round	10.000	*Micrococcus sp.*
8	T23	G (+) Rod	10.2	*Bacillus sp.*
9	T24	G (+) Rod	12	*Bacillus sp.*
10	M1	G (+) Round	17.200	*Micrococcus sp.*
11	M4	G (+) Rod	13.200	*Bacillus sp.*
12	M10	G (-) Round	21.000	*Micrococcus sp.*
13	M11	G (+) Coccobacillus	15.500	*Micrococcus sp.*
14	M12	G (+) Rod	21.000	*Bacillus sp.*
15	M13	G (+) Rod	23.000	*Bacillus sp.*
16	M14	G (+) Rod	15.200	*Bacillus sp.*
17	M16	G (+) Rod	10.000	*Bacillus sp.*
18	M17	G (-) Rod	13.200	*Bacillus sp.*
19	M18	G (+) Rod	11.000	*Bacillus sp.*
20	N1*	G (+) Cocococbacilli	24.600	*Micrococcus sp.*
21	N2	G (+) Coccobacillli	21.000	*Micrococcus sp.*
22	N3	G (+) Rod	15.100	*Bacillus sp.*
23	N4	G (+) Rod	13.000	*Bacillus sp.*
24	N5	G (+) Coccobacillii	14.200	*Micrococcus sp.*
25	N9	G (+) Rod	11.300	*Bacillus sp.*
26	V4	G (+) Rod	13.040	*Bacillus sp.*
27	V9	G (+) Rod	14.200	*Bacillus sp.*
28	V10	G (+) Coccobacilli	21.100	*Micrococcus sp.*
29	V11	G (+) Rod	10.000	*Bacillus sp.*

Isolation at 50^0C, *Strain selected for molecular identification based upon highest enzyme production

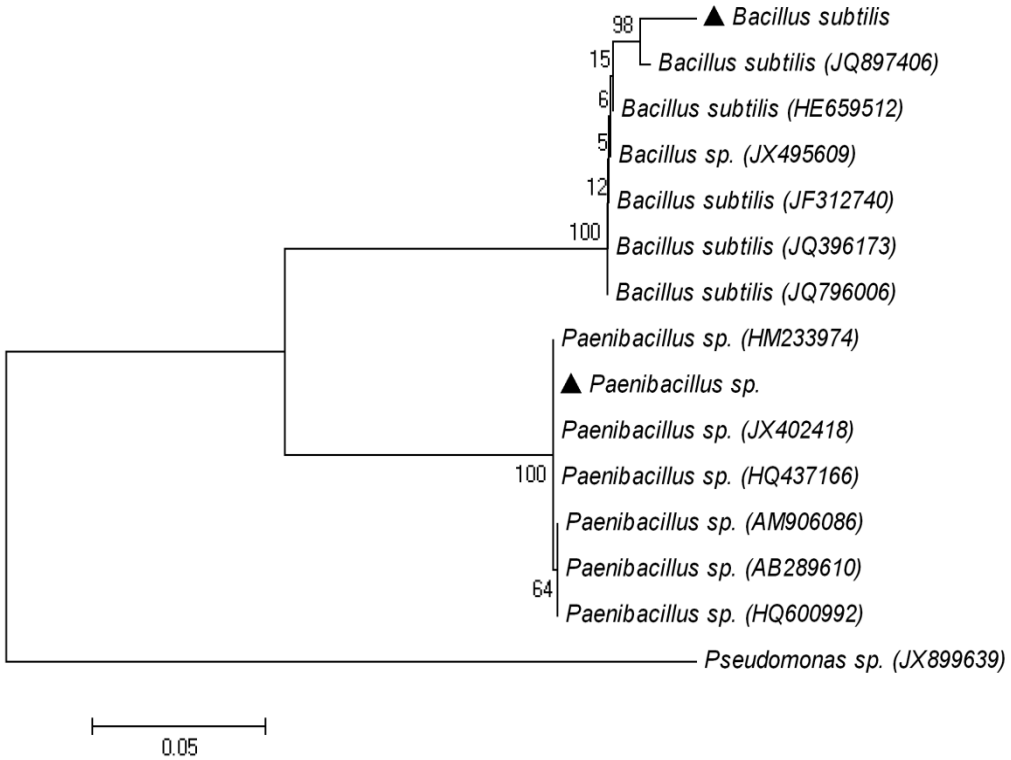
A thermoalkalophilic xylanase was isolated from *Enterobacter* spp. with significant activity. Maximum xylanase activity was observed in isolate BGCC#259 (*E. cloacae*) i.e. 0.056 IU/ml at 24h [38]. Maximum production of xylanase was observed in culture incubated at 50^0C, pH 7 for 12 h with cell density of 1.78×10^9 by *Bacillus* sp. AQ-1 under submerged fermentation [39]. Similarly maximum xylanase production 125 IU/ml was recorded in the stationary phase (36h) of culture by *Bacilllus subtilis* isolated from estuarine environment by optimization of culture conditions [40].

Identification of Screened Bacterial Isolates

The best enzyme producing strains were identified morphologically as well as at genomic level. Amylolytic bacterial isolates NM(G) and NM9 were gram positive, aerobic, non-motile rods occurring singly. Isolate NM(G) was spore forming whereas NM 9 was a non-sporulating strain. NM(G) showed growth well at a temperature range of 10-55 °C, pH range of 5-10 and sodium chloride concentration of 2.5 to 10%. On the other hand, Strain No.9 grew well in the termperature range of 15-65 °C, pH range of 4-11 and sodium chloride concentration of 2.5-8.5 %. Another bacterial strain N_1 showing xylanolytic properties was found to be a gram positive, coccobacillus, it showed growth at a temperature range of 30-50 °C and a pH range of 4-10.

The identification of selected bacterial isolates based on the highest enzyme production potential at genomic level was done by using 16S rRNA PCR.

Genomic DNA was isolated from N1 by using protocol of Genei which resulted in decent amount of DNA, good quality and quantity. The isolated genomic DNA (50-100ng) was used in PCR to amplify small units of rrs (16S rRNA) using specific primer (16S 1375 U: GCAAGT CGAGCGGACAGATGGG AGC and 16S 1375 D: AACTCTCGTGGTGTGA CGGGC GGT). Expected size (in 1375 bp) amplification product (Plate 5) in the isolates was obtained in PCR. The PCR product (1500 bp) was purified from contaminating product by electroelution of the gel slice containing the excited desired fragment with Qiaquick gel extraction kit. The elution was carried out in 300 µl of nuclease free water. The purified and amplified gel fraction was used for sequencing and sequencing was performed in ABI automated sequencer using 16SF primer. After using BLASTN tool xylanolytic isolate N_1 was identified as *Paenibacillus* sp. (≅98% homology) (Plate 6) The amylolytic strain NM (G) was identified as *Bacillus megaterium* and NM9 was found out to be *Bacillus subtilis* (Plate 7) and laboratory of CABI Bioscience UK centre (Egham), UK International Mycological Institute). The phylogenetic tree prepared with 16S rRNA gene sequences of *Paenibacillus sp.* and *Bacillus subtilis* is shown in Figure 1.



▲ represent the native isolates *Bacillus subtilis* NM 9 and *Paenibacillus* N_1.

Figure 1: Neighbour joining tree of bacterial isolates based on 16S rRNA sequences technique

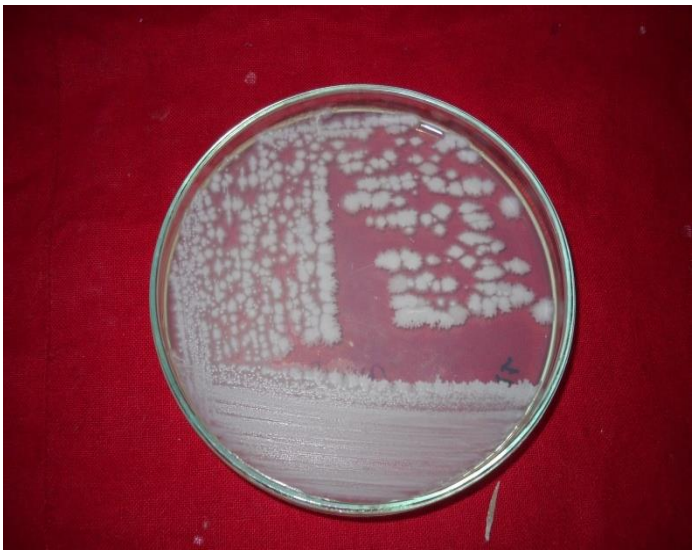


Plate 6: *Paenibacillus* sp.N1 isolated from hot spring of Manikaran

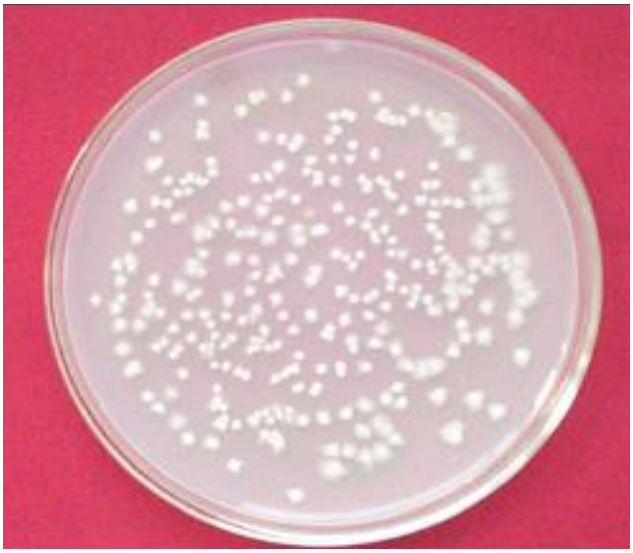


Plate 7: *Bacillus subtilis* NM9 isolated from hot spring of Manikaran

Fungal isolates

Among the thermophilic microorganisms isolated from the hot springs of Himachal Pradesh, two were fungal isolates. Those fungal isolates were capable of producing cellulase and xylanase. Their morphological and cultural characteristics have been shown in Table 5. Fungus F_1, light brown in color having rough surface and septate hyphae had been isolated from Manikaran whereas F_2 fungus having septate and white colored hyphae were isolated from hot springs of Tattapani.

Table 5: Morphological and cultural characterization of fungi isolated from the hot springs of Himachal Pradesh

Fungal isolate	**Source**	**Mycelium**	**Spore**		
			Colour	**Texture**	**Tentative Identification**
F_1	Manikaran	Short hyphae	Light brown	Rough	*Myceliopthora* sp.
F_2	Tattapani	Short hyphae	White	Smooth	*Determomyces* sp.

The geothermal sites near neutral and alkali thermal springs in Tengchong Rehai National Park were examined by [42] through cultivation-dependent approach to determine the diversity of thermophilic fungi in these environments. The main strain isolated and identified as *Rhizomucor miehei, Chaetomium* sp, *Talaromyces thermophilus, Talaromyces byssochlamydoides, Thermoascus aurantiacus, Miehe var. levisporus, Thermomyces lanuginosus, Scytalidium thermophilum, Malbranchea flava and Coprinopsis* sp., Two species, *T. lanuginosus* and *S. thermophilum* were the dominant species among them, Geothermal soil near Amphitheater Springs in Yellowstone National Park were characterized by high temperatures (up to 70°C), high heavy metal content, low pH values (down to pH 2.7), sparse vegetation and limited organic carbon. Two of these species were thermophilic and six were thermotolerant.

Underground coal mine soil, bird nest materials, vermicompost, cow dung, poultry litter, decomposting pits are prepared from agrowaste, municipal waste and zoo dump materials and industrial waste, were used to isolate 46 thermophilic fungi. Isolates of 446 species belonging to 13 genera on different substrates collected from different places of Andhra Pradesh, belonged to *Humicola lanuginosus* and *Aspergillus fumigates* are found as a thermo-tolerant [43].

Xylanase Activity

Extracellular xylanase synthesis during biodegradation of microwave treated rice straw (*Oryzae sativa*) by fungal isolate F_1 using Vogel's medium as substrate with inoculum size of 10% showed maximum enzyme activity of 14.05 IU/ml (281 U/gds) after standardizing different environmental conditions i.e. microwave pretreatment of biomass, moisture level of 1:6, temperature at 50^0C and incubation time of 5 days.

Cellulase Activity

Production of extracellular cellulase by fungal strain F_1 during biodegradation of pretreated rice straw (*Oryzae sativa*) using Vogel's medium, pH 5.5 with inoculum size of 10% for 8 days was observed. It showed higher cellulase enzyme activity of 43.07 U/gds i.e. FPase (11.311 U/gds) and β-glucosidase (13.500 U/gds) was obtained. Based on the enzyme production potential of the isolate, it was selected for molecular characterization.

SSF systems, which are closer to natural habitat of microbes, prove more efficient in producing certain enzymes and metabolites [33]. The choice of appropriate substrates is of great importance for the successful production of cellulase. The substrate not only serves as the source of carbon but also produce the necessary inducing compounds for the organism [44]. Submerged fermentation (SmF) is being used for industrial production of cellulases but the low enzyme yields and higher production cost are the major problems for industrial applications [45]. It has been reported that solid state fermentation (SSF) is an attractive process to produce cellulase which is economical due to its lower capital investment and lower operation expenses. Production of cellulases by fungi in SSF using agricultural wastes has been reported [41, 46, 47]. It is also to be noted that penetration of fungal hyphae inside the substrate in SSF provides the increased surface area and reduces the chances of contamination due to lesser requirement of water compared to SmF.

Similar studies pertaining to solid state fermentation for cellulase production using lignocellulosic biomass has been enlisted by many authors. Microwave irradiation of lignocellulosic biomass is the latest pretreatment method recently has been applied for production of enzymes and fuels. Microwave technology is supposed to simply three structural polymers along with extraneous components. When the crystalline region is placed between electromagnetic field it acts polarized generating a charge on crystalline interphase.

Cellulase production was carried out in solid state fermentation using waste from the vinegar industry for *Trichoderma koningii* AS3.4262. Optimal FPase of 6.90 IU/ml were obtained after 84h of incubation with media containing vinegar waste, having optimal moisture content of 50%, pH 5.0 and incubation temperature of 30 °C [48].

Molecular Characterization

After molecular characterization on the basis of ITS, the isolate was identified as *Myceliopthora thermophila* SH1 (Plate 8) provided with an accession number of JX124712 by NCBI. The phylogenetic tree was then constructed (Figure 2).

Plate 8 : *Myceliopthora thermophila* SH1: the novel fungal isolate isolated from hot spring of Northern Himalayas

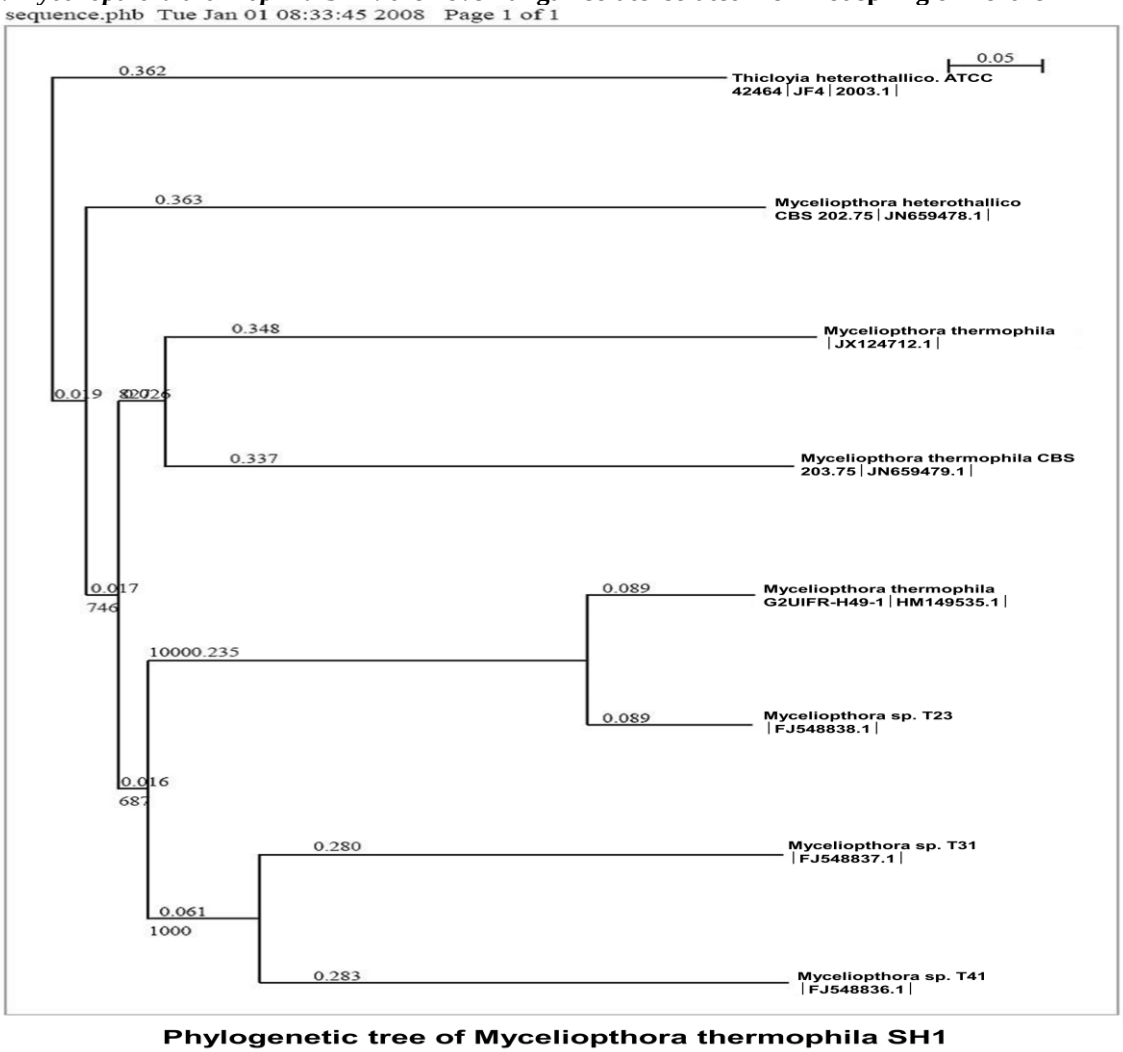


Figure 2: Neighbour joining tree of fungal isolate based on 16S rRNA sequences.

Myceliopthora thermophila SH1 fungal strain is the novel finding and a significant contribution of present study to the industry as it is a thermophilic fungus which has shown high xylanase as well as cellulase production potential. This strain has firstly been reported in literature to show such good enzyme

production potential which is of great importance to industry.

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