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# SCREENING & ISOLATION OF EXTRACELLULAR THERMO-STABLE ENZYMES PRODUCED BY BACTERIAL ISOLATES FROM SALBARDI HOT SPRING REGION

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**Abstract:**

A wide range of thermo-stable enzymes is extracted from thermophilic bacteria from hot springs which are utilized in industries and in research. Proteases are the important range of thermo-stable enzymes which constitutes 59% of the global market share of industrial enzymes. The present study is based on Salbardi hot spring in central India which is known for its medically important hot spring water and its different chemical composition. This study is the first microbiological phylogenetic characterization of soil and water samples from salbardi hot spring region. The study reveals the presence of wide range of thermo-tolerant organisms specifically *firmicutes, bacilli* which are able to give optimum growth at a higher temperature range of 55-80°C., these bacteria are the major source of economically important thermo-stable enzymes. Protease production at 60°C was shown in this study also its stability from 50-90°C was checked at different incubation time representing maximum enzyme activity 178.95-216.84 U/ml. Salbardi hot spring is proved to be a wide source for thermo-stable bacteria as well as thermo-stable enzymes.

**Introduction:**

Enzymes are the backbone of any catabolic and anabolic reactions, there is always a need and search for stable and high yield enzymes. Enzyme Technology based industries have shown booming business in various technological areas. Thermo-stable enzymes have phenomenal importance in the research & development and industrial utilization. The actual study of thermophiles and how their proteins are able to function was started early in 1960 by Brock and his colleagues but from past two decades, thermo-stable enzymes and microorganisms have been topics for much research.[1] The major sources of thermo-stable enzymes are thermophilic bacteria from hydrothermal vents. The advantage of using these enzymes is that they are stable at high temperature.[2] High-temperature stability of these enzymes in any enzymatic reaction allows higher substrate concentrations, lower viscosity; fewer risk of contamination and often higher reaction rates at elevated temperatures.[3]

Microorganisms have the ability to be cultured in large scale fermentation in lesser time with abundant desire product which makes attractive sources for protease and other enzymes. Gene manipulation is easy in microorganisms than in plants and animals for improvement of the enzyme performance.[4] In totality, protease constitutes 59% of the global market share of industrial enzymes, which was exceeded $ 2.9 Billion by 2012.[5] A wide range of proteases has been commercially used in detergents, leather, food and pharmaceutical industries. Protease has been a group of enzymes whose main catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids.[6,7]

Geothermal springs including hot springs are substantially higher in temperature than the air temperature of the surrounding region and it has been a rich source of thermo-tolerant microorganisms. Salbardi thermal spring is situated near Maru River, in central India and discharges through the Deccan volcanic and in particular, Salbardi falls along the Satpura fault where DFB (Deccan flood basalt) are in tectonic contact with the sedimentary Archean formations of the `Gondwana Supergroup'.[8] Salbardi hot spring has the base temperature of $66^0$c. The springs flow into a small stone cistern which was formerly divided into compartments for the hot and cold water. Now they are mixed due to the human interference and development. This thermal spring water is Na-Cl-$SO_4$ type against the Ca-Mg-$HCO_3$ type regional groundwater. Heat source appears to be normal earth temperature gradient indicated reservoir temperature is 110 ±10°C.[9,10] The present study focuses on the water and soil sampling from Salbardi hot spring region for the screening and isolation of thermo-stable bacteria for economically important extracellular thermo-stable enzymes.

**Materials and Methods:**

**Isolation and Identification:**

Soil and water samples collected from salbardi hot spring region, samples were collected from hot spring including other geological points showing different latitude and longitude which is shown in Table-1, in

different weather conditions. Samples were processed in the laboratory within 24 hrs after collection, for isolation Luria-Bertani (LB) broth was used and for the thermo-tolerant organisms, selective media was used. Serial dilution and enrichment culture techniques were used to isolate bacteria at different incubation temperatures from mesophilic to the thermophilic range.[11]

**Screening of Thermo-Stable Enzyme Producing Microorganisms Using Solid Medium:**

After subsequent sub-culturing at a higher temperature (50-80°C), 11 isolates selected and were subjected to study growth kinetics also analyzed for sugar fermentation and hydrolysis of lipid and protein. Apart from that isolates were also tested for production of thermo-tolerant enzymes i.e., Chitinase, Amylase, Lipase, Cellulases, Glucanases and Protease enzyme production on Chitin Agar (1%), Starch Agar (1%), Olive oil hydrolysis Agar, Cellulose Congo-red Agar, Cellulose Agar (1%) and Skim milk Agar respectively at 60°C. Enzyme production was detected by the clear zone around the growth of bacterial isolate.

**Extraction of Protease:**

Total 6 isolated thermo-tolerant strains with maximum clear zone of protease activity were inoculated in 50 ml of protease specific medium broth containing (g/L) glucose, 5.0; peptone, 7.5; ($MgSO_4.7H_2O$, 5.0; $KH_2PO_4$, 5.0; and $FeSO_4.7H_2O$, 0. 1, pH-7.0 and were cultured in a rotary shaker (180 rpm) at 55°C for 3 days. After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies.[12]

**Protease Assay:**

Protease activity was measured by using casein as a substrate. A mixture of 400 µl casein solution (2% w/v in 50 mM Phosphate buffer pH 7.0) and 100 µl extracted enzyme was added in each tube and incubated for 10 min at 50°C. The reaction terminated by addition of 1ml trichloroacetic acid (TCA) (10% v/v). The mixture allowed to centrifuge at 14,000 g for 20 min and 1 ml supernatant was removed carefully. Tyrosine/tryptophan content was determined by using Lowery method. The blank was prepared by adding 1ml of TCA before addition of an enzyme. One unit of protease activity (U) is defined as the amount of enzyme that hydrolyzed casein to liberate one µmole tyrosine per min under the above assay condition.[13]

**Determination of Total Protein Content:**

The total protein contents of the samples were determined according to the method described by Lowry (Lowry et al, 1951); the protein standard used was BSA (Bovine Serum Albumin) (Merck). The absorbance of the samples was measured spectrophotometrically at 660 nm using UV/Visible spectrophotometer (Perkin Elmer).[14]

**Thermal Stability:**

To check the thermal stability of the enzyme it was incubated at different temperatures i.e., $50^0$c, $60^0$c, $70^0$c, $80^0$c and $90^0$c with the substrate for different incubation time 10 min and 20 min. using 50mM phosphate buffer of pH 7.[15]

**Phylogenetic Analysis by 16S rRNA Gene Sequencing:**

Total 2 isolates showing maximum protease activity were selected for genomic DNA isolation by using modified CTAB protocol followed by PCR amplification using primers: 16S F primer: 5' AGA GTT TGA TCC TGG CTC AG 3', 16S R primer: 5' AAG GAG GTG ATC CAG CCG CA 3'

The amplified PCR products were separated on 1.2 % agarose gel along with 500 bp of DNA ladder (NEB, Beverly, MA). The DNA sequencing was done using 50 ng PCR products having 8 µl of ready reaction mix (BDT v 3.0, Applied Biosystems, Foster City, CA) and 5 pMol of the forward primer. The sequences were checked against the microbial nucleotide databases using BLASTN search algorithms. Obtain 16S rRNA gene sequences were used to perform the multiple sequence alignment by CLUSTALW program. CLUSTALW performs multiple sequence alignment by accepting the closely linked sequences in the FASTA format file for the phylogenetic analysis. .DND file obtained from CLUSTAL alignment was used for the phylogram built up by using the MEGA5 software. [16, 17,18,19]

**Results:**

**Salbardi Hot Spring Water Chemical Characteristics:**

The chemical analysis of water samples shows that the chemical characteristic of hot spring water is different from other groundwater sources of salbardi hot spring region. In spring water concentration of sulfate was found higher i.e. 103 mg/kg and water is Na-Cl-$SO_4$ type against the Ca-Mg-$HCO_3$ type regional groundwater and also approximately same concentration and water type recorded in the study of Ball J. W., 1991 and Pitale U.L., 1980.[20,21]

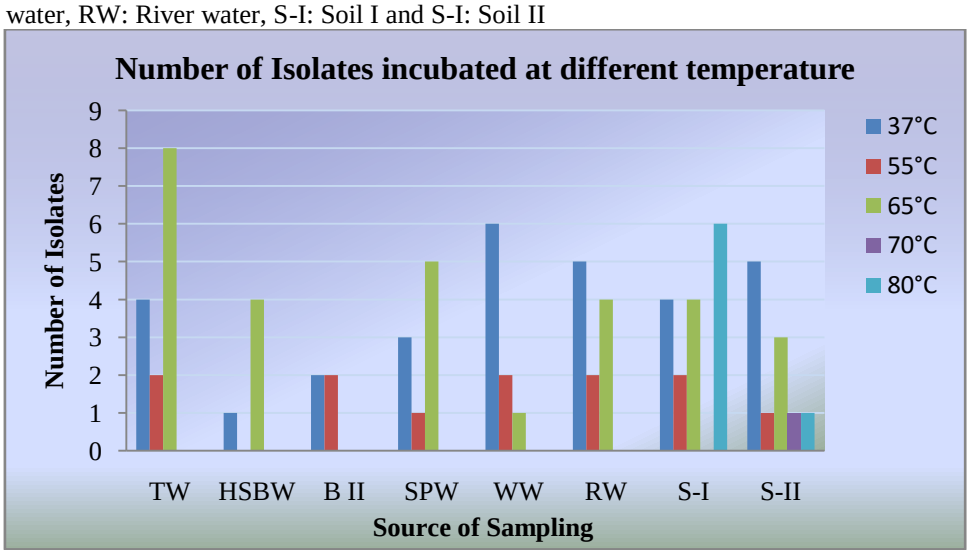
**Isolation of Thermo-Tolerant Bacterial Isolates:**

Total 79 bacterial isolates were isolated from water and soil samples at various incubation temperatures. Out of 79 bacterial isolates, 30 were isolated at an incubation temperature of $37^0$C which is the optimum temperature for the most of the bacteria's whereas, 12 isolates at $55^0$C, 29 isolates at $65^0$C, 7 isolates at $80^0$C and 1 bacterial isolate at $70^0$C. The numbers of isolates from the different source at different incubation temperatures are given in Table-1.

Table 1: Number of isolates at various incubation temperatures from different sources

| Source of Sampling | Latitude | longitude | Number of isolates incubated at different temperature | | | | |
|---|---|---|---|---|---|---|---|
| | | | 37°C | 55°C | 65°C | 70°C | 80°C |
| TW | 21° 25' 11.2" N | 78° 00' 41.1" E | 4 | 2 | 8 | 0 | 0 |
| HSBW | 21° 25' 11.8" N | 78° 00' 40.5" E | 1 | 0 | 4 | 0 | 0 |
| B II | 21° 25' 11.9" N | 78° 00' 40.6" E | 2 | 2 | 0 | 0 | 0 |
| SPW | 21° 25' 11.9" N | 78° 00' 41.4" E | 3 | 1 | 5 | 0 | 0 |
| WW | 21° 25' 11.7" N | 78° 00' 40.6" E | 6 | 2 | 1 | 0 | 0 |
| RW | 21° 25' 12.0" N | 78° 00' 39.5" E | 5 | 2 | 4 | 0 | 0 |
| S-I | 21° 25' 11.6" N | 78° 00' 40.3" E | 4 | 2 | 4 | 0 | 6 |
| S-II | 21° 25' 11.7" N | 78° 00' 41.5" E | 5 | 1 | 3 | 1 | 1 |

Note: TW: Hot spring Tank water, HSBW: Hot Spring Bore water, BII: Bore II water, SPW: Small pond water, WW: Well water, RW: River water, S-I: Soil I and S-I: Soil II




Graph 1: Number of Isolates incubated at Different Temperature from Salbardi region

**Selection of the Thermo-Tolerant Bacterial Isolates:**

Criteria for selection of thermo-tolerant bacterial isolates were that isolates should sustain the optimum growth for five subsequent sub-culturing at a higher temperature. On the basis of repeating sub-culturing on Luria-Bertani (LB) agar at the thermophilic range, 11 isolates (1TW2, 1SI2, 1tTW1, 1tTW2, 2HSBW1, 2HSBW2, 2HSBW3, 2SI3, 2SII, 1SI1A, and 1SI2a) was found to be showing a stable growth.

**Screening of Isolates for Thermo-stable Enzymes:**

All the 11 gram-positive rod-shaped thermo-tolerant isolates were tested for phenotypic and biochemical characterization. Almost all the isolates had given positive sugar fermentation test by producing acids from glucose and mannitol except 2SI3 and 1SI2a showing a negative result for glucose fermentation and 1tTW1, 2HSBW3 and 1SI2a showing no acid formation for mannitol. Hydrolysis of lipid and protein for all 11 bacterial isolates was done and growth kinetics of bacterial isolates were studied at different temperatures i.e., $37^0$C, $45^0$C, $55^0$C, $65^0$C, $75^0$C, and $85^0$C at different pH i.e., pH = 4, 7 and 9.2 and by using different sugars dextrose and mannitol. All these 11 bacterial isolates were tested for different thermo-stable enzyme production t 60 $^0$C by microbiological plate method. Table-2 shows the result for enzyme production at 60 $^0$C by different isolates.

Table 2: Thermo-stable enzyme production by bacterial isolates:

| S.No | **Enzyme activity of isolates between 60 $^0$C** | | | | | | |
|---|---|---|---|---|---|---|---|
| | Isolate No | Chitinase Test | Amylase Test | Lipase Test | Cellulase Test | Endo & Exo-Glucanases Test | Protease Test |
| 1. | 1TW2 | + | (+) | (-) | (+) | (+) | ++ |
| 2. | 1SI2 | (+) | - | ++ | - | (+) | ++ |
| 3. | 1tTW1 | - | - | (+) | + | - | + |
| 4. | 1tTW2 | - | - | - | + | - | + |
| 5. | 2HSBW1 | - | - | (+) | (+) | - | ++ |
| 6. | 2HSBW2 | - | - | (+) | ++ | - | +++ |
| 7. | 2HSBW3 | (+) | - | - | ++ | - | + |
| 8. | 2SI3 | + | (+) | - | ++ | - | +++ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 9. | 2SII | + | (+) | - | (+) | - | + |
| 10. | 1SI1A | - | - | - | - | - | ++ |
| 11. | 1SI2a | - | ++ | (-) | + | ++ | - |

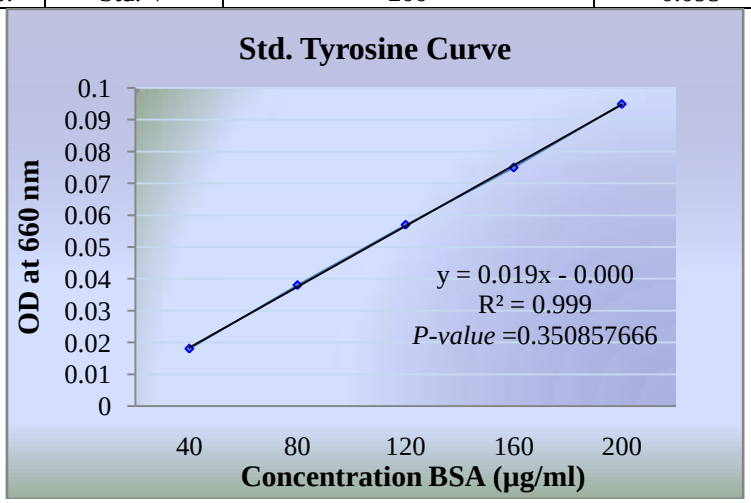
Note: '++'= Large zone, '+' = Clear zone, '(+)' = Light zone/small zone, (-) = slight zone but not clear - = No zone of enzyme activity.

**Standard Graph Tyrosine:**

Standard Tyrosine curve was obtained by Lawry method at 660 nm by using standard bovine serum albumin (BSA). Table-3 shows OD of different concentrations at 660 nm by UV – Visible spectrophotometer.

Table 3: Standard Tyrosine Curve

| S.No | Standard Sol | Concentration of BSA (µg/ml) | O.D. at 660nm |
|---|---|---|---|
| 1. | Std. I | 40 | 0.018 |
| 2. | Std. II | 80 | 0.038 |
| 3. | Std. III | 120 | 0.057 |
| 4. | Std. IV | 160 | 0.075 |
| 5. | Std. V | 200 | 0.095 |




Graph 2: Standard Tyrosine Curve

**Protease Assay:**

Total 6 isolates i.e., 1TW2, 1SI2, 2HSBW1, 2HSBW2, 2SI3 and 1SI1A with a maximum clear zone for protease activity were selected for further quantitative analysis of the enzyme by the standard spectrophotometric method at 660nm. Table–4 and Table–5 shows the measured protease activity at different temperatures with an incubation time of 10 and 20 min. The present study reveals that out of all the six isolates selected the maximum enzyme activity was shown by 2HSBW1 (216.84 U/ml) at 60°C in 10 min incubation time and 1SI2 gives maximum protease activity (187.37 U/ml) at highest temperature 80°C while in prolonged incubation of 20 min maximum protease activity was shown by 2HSBW1 (185.26 U/ml) and at highest temperature by 1SI1A i.e. 60°C (178.95 U/ml). In other studies conducted by Zeba Ansari, et al in 2015 maximum growth OD of 0.69 at 540 nm for incubation temperature of 60°C was found with thermo-stable protease activity of 31.1 U/L was observed at 60°C temperature.[22] Also, Harshada Chandrashekhar Sakpal and Geeta Narayan in 2015 reported thermo-stable alkaline protease production with an enzyme activity of 189.4µM/min/ml from *Bacillus licheniformis* at pH-9 for 72hrs at 55ºC in protease production medium.[23]

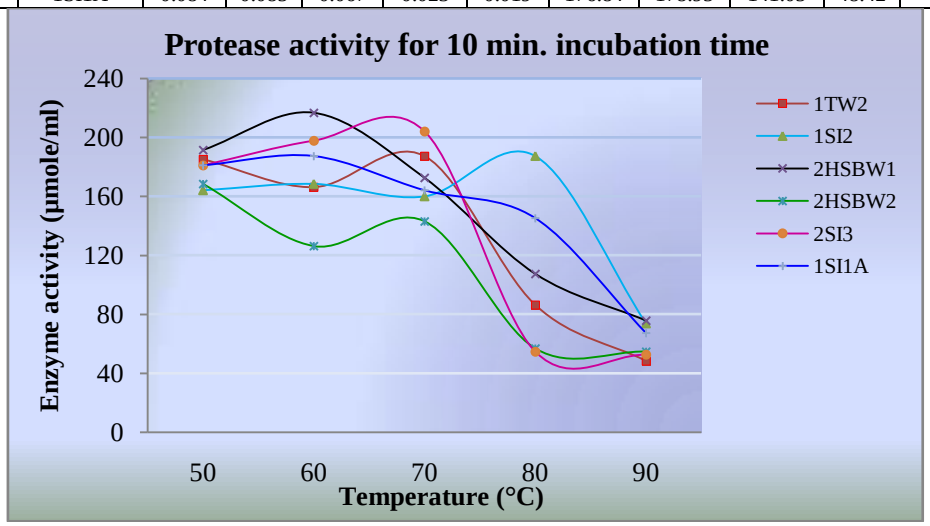
Table 4: Protease activity at 660 nm by UV – Visible spectrophotometer for 10 min enzyme activity

| S.No | Isolate No | Protease activity for 10 min. incubation time | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (OD at 660 nm) | | | | | Enzyme activity (µmole/ml) | | | | |
| | | 50°C | 60°C | 70°C | 80°C | 90°C | 50°C | 60°C | 70°C | 80°C | 90°C |
| 1. | 1TW2 | 0.088 | 0.079 | 0.089 | 0.041 | 0.023 | 185.26 | 166.32 | 187.37 | 86.32 | 48.42 |
| 2. | 1SI2 | 0.078 | 0.080 | 0.076 | 0.089 | 0.035 | 164.21 | 168.42 | 160.0 | 187.37 | 73.68 |
| 3. | 2HSBW1 | 0.091 | 0.103 | 0.082 | 0.051 | 0.036 | 191.58 | 216.84 | 172.63 | 107.37 | 75.79 |
| 4. | 2HSBW2 | 0.080 | 0.060 | 0.068 | 0.027 | 0.026 | 168.42 | 126.32 | 143.16 | 56.84 | 54.74 |
| 5. | 2SI3 | 0.086 | 0.094 | 0.097 | 0.026 | 0.025 | 181.05 | 197.90 | 204.21 | 54.74 | 52.63 |
| 6. | 1SI1A | 0.086 | 0.089 | 0.078 | 0.069 | 0.032 | 181.05 | 187.37 | 164.21 | 145.26 | 67.37 |

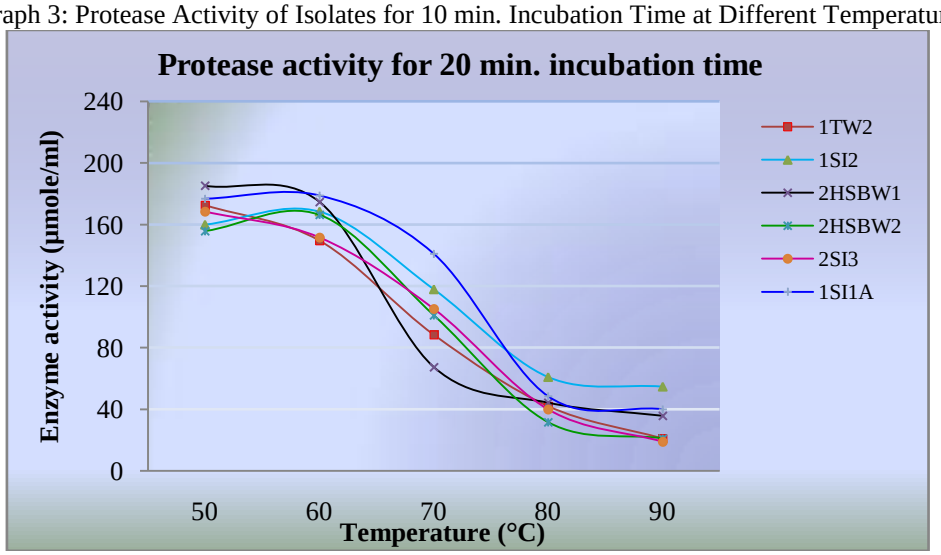
Table 5: Protease activity at 660 nm by UV – Visible spectrophotometer for 20 min enzyme activity

| S.No | Isolate No | Protease activity for 20 min. incubation time | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (OD at 660 nm) | | | | | Enzyme activity (µmole/ml) | | | | |
| | | 50°C | 60°C | 70°C | 80°C | 90°C | 50°C | 60°C | 70°C | 80°C | 90°C |
| 1. | 1TW2 | 0.082 | 0.071 | 0.042 | 0.020 | 0.010 | 172.63 | 149.47 | 88.42 | 42.11 | 21.05 |
| 2. | 1SI2 | 0.076 | 0.080 | 0.056 | 0.029 | 0.026 | 160.0 | 168.42 | 117.89 | 61.05 | 54.74 |
| 3. | 2HSBW1 | 0.088 | 0.083 | 0.032 | 0.021 | 0.017 | 185.26 | 174.74 | 67.37 | 44.21 | 35.79 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4. | 2HSBW2 | 0.074 | 0.079 | 0.048 | 0.015 | 0.010 | 155.79 | 166.32 | 101.05 | 31.58 | 21.05 |
| 5. | 2SI3 | 0.080 | 0.072 | 0.050 | 0.019 | 0.009 | 168.42 | 151.58 | 105.26 | 40.0 | 18.95 |
| 6. | 1SI1A | 0.084 | 0.085 | 0.067 | 0.023 | 0.019 | 176.84 | 178.95 | 141.05 | 48.42 | 40.0 |




Graph 3: Protease Activity of Isolates for 10 min. Incubation Time at Different Temperature




Graph 4: Protease Activity of Isolates for 20 min. Incubation Time at Different Temperature

**Phylogenetic Analysis by 16S rRNA Gene Sequencing:**

Total 2 isolates 2HSBW1 and 2SI3 were selected for the phylogenetic analysis and the criteria for selection were the organisms would be able to produce protease at or above 60°C with a maximum enzyme activity. After the phylogenetic study, bacterial strains were identified and multiple sequence alignment of 2HSBW1 possessed 99% homology with *Geobacillus thermoleovorans* strain CCB_US3_UF5 and 2SI3 possessed 96% homology with *Bacillus subtilis* strain SYH9.

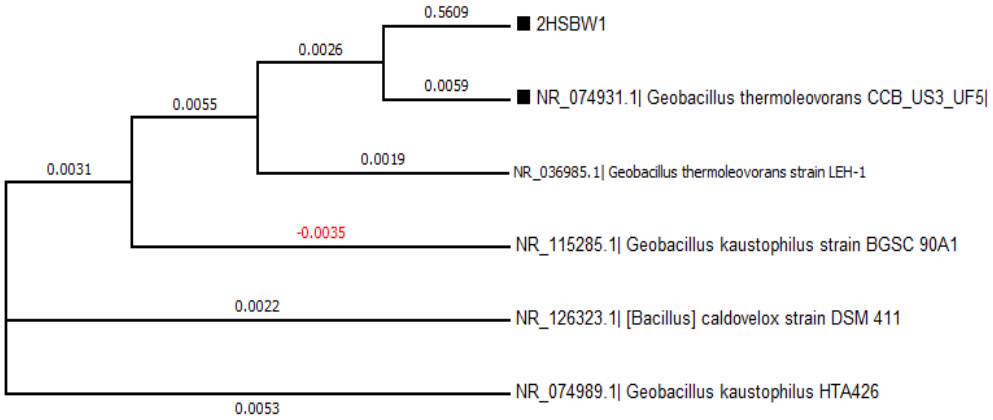



Figure 1: Phylogenetic tree for multiple sequence alignment of 2HSBW1

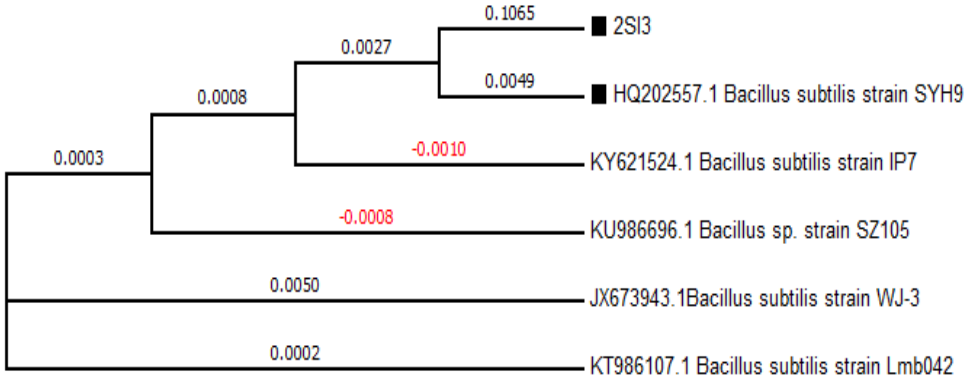



Figure 2: Phylogenetic tree for multiple sequence alignment of 2S13

**Conclusion:**

For the first time, a microbiological study was done for Salbardi hot spring in Central India. The present study shows inhabitance of several thermo-tolerant bacterial species in this geological region. Different strains of *Geobacillus thermoleovorance* (2HSBW1) and *Bacillus subtilis* (2SI3) with registered NCBI accession numbers, LC157908 and LC368287 respectively were found in this phylogenetic study. 2HSBW1 (*Geobacillus thermoleovorance*) and 2SI3 (*Bacillus subtilis*) had shown highest protease activity 216.84 U/ml and 204.21U/ml respectively for 10 min incubation period at 60 and 70°C respectively. It has been concluded that as substrate concentration decreases the stability of enzyme activity at a higher temperature also decreases on the basis of results. Salbardi hot spring is proved to be a wide source for thermos-table bacteria as well as thermo-stable enzymes.

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