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# Screening and Molecular Characterization of Cellulase Producing Thermophilic Bacteria Isolated from an Egyptian Hot Spring in Ras-Sedr

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## ABSTRACT

This research was carried out to screen and characterize the rmophilic bacteria that have the ability to produce cellulase enzyme. Bacterial strains were isolated from the sediment samples collected from Ras-Sedr's hot spring located in Ras-Sedr, Egypt. For cellulolytic activity testing, Carboxymethylcellulose (CMC) agar was used as growth medium. Seven bacterial isolates showed variable zones of CMC clearance. Evaluation of cellulase enzyme thermal activity was carried out at varying temperatures from 35°C to 85°C and the impact of pH on cellulase activity was evaluated at different pH levels of 5-9. Molecular identification for the isolates also conducted based on 16S rRNA gene sequencing. At 55°C and pH 7, all the seven bacterial isolates showed their optimum growth conditions. Strains were gram positive, have rod shape cells, spore forming and motile with the ability to produce thermostable cellulase. The results of the phylogenetic analysis reveal a high degree of sequence similarity between the isolated bacteria involved in this research and *Geobacillus* sp. These thermophiles have the ability to produce useful thermostable cellulase enzyme of industrial significance, which in turn indicates that Ras-Sedr's hot spring considered a prospective source of economically significant microorganisms which needs more studies through further researches.

**Keywords**

Hot Springs - Thermophilic Bacteria- Cellulase - 16S rRNA.

## 1. INTRODUCTION

Since the development of the polymerase chain reaction, the enzymes of extreme thermophiles have been of significant concern in biotechnology [1]. It is not surprising, given the usefulness of thermostable enzymes in molecular biological laboratory techniques, that they were also suggested as strong resources for industrial catalysis [30]**.** The challenge of thermal inactivation of the enzyme is commonly reported in

industrial applications where enzymes are applied [26]. For industrial uses, therefore, enzyme stability is a key factor.

Thermophilic bacteria are major sources of thermostable enzymes, and their suitable habitats are typically geothermal conditions such as hot springs [20]**.** Hot springs are described as natural thermal spots generated by groundwater with geothermal heating. Geothermal springs are often highly energetic environments not only because temperatures can reach the boiling point, but also because their waters are often charged with elevated sulfur levels. They contain microorganism populations that may be a source of significant bioactive compounds such as thermostable enzymes [24]**.** Because of their broad applications, cellulases have attracted a lot of interest. Together with agriculture and other research purposes, cellulases are critical in fruit, beverage and wine, textile, paper and pulp industries [5, 12]. In this work, an effort was made to screen bacterial isolates from hot spring of Ras- Sedr that produce cellulase enzyme. Cellulase characterization based on pH and temperature was performed then followed by molecular characterization of the isolates based on16s rRNA.

## 2. MATERIALS AND METHODS

### 2.1 Source of Microorganisms

Sediment samples from the hot spring were gathered. Hot spring located in Egypt, Ras -Sedr, Abo Swira, El Mahager road (**Figure 1)**. Sediment samples were collected during summer season (May-June) in 1000 ml bottles of sterile Pyrex using sterile glass flasks and were maintained on ice until processing. Upon arrival at the University of Suez Canal, all samples were immediately stored at 4°C; sediment samples were subjected within 24 h to chemical analysis and cellulase screening.

### 2.2 Cellulolytic Activity Assessment:

For cellulolytic activity testing, Carboxymethylcellulose (CMC) agar according to [3] was used as growth medium. Cultivation medium containing: 0.1 g of calcium chloride dehydrate ($CaCl_2.2H_2O$), 5.0 g of sodium chloride, and 10.0 g of peptone, 2.0 g of CMC and 20.0 g of agar. With the addition of 1M NaOH, the pH of medium was set to 7.0. First, sediment sample were enriched by adding 90 ml of 0.9% NaCl saline solution to 10 gm sediments in 250 ml Erlenmeyer flask with shaking at 300 rpm for 1 h at 55 °C. Then, one ml of sediment extract was poured to the sterile Petri- dishes. Triplicate set of sample was used and the plates were wrapped with parafilm and kept for 24-48 h of incubation at 55° C in clean plastic bags. The resulting colonies were streaked on CMC plates and incubated for 24-48 h at 55° C. Gram's iodine was used as indicator and the development of halo zones proved positive activity of CMC hydrolysis.

### 2.3 Morphological & Biochemical Test:

Cell colony morphology and Gram reaction were investigated according to the method described by [10]**.** Spore formation was examined according to the method described by [6]. Some biochemical and physiological tests were performed using the method of [9]**.**

### 2.4 Assessment of isolates growth pattern:

The temperature range for growth was determined using a protocol described by [28]. Inoculated in 50 ml

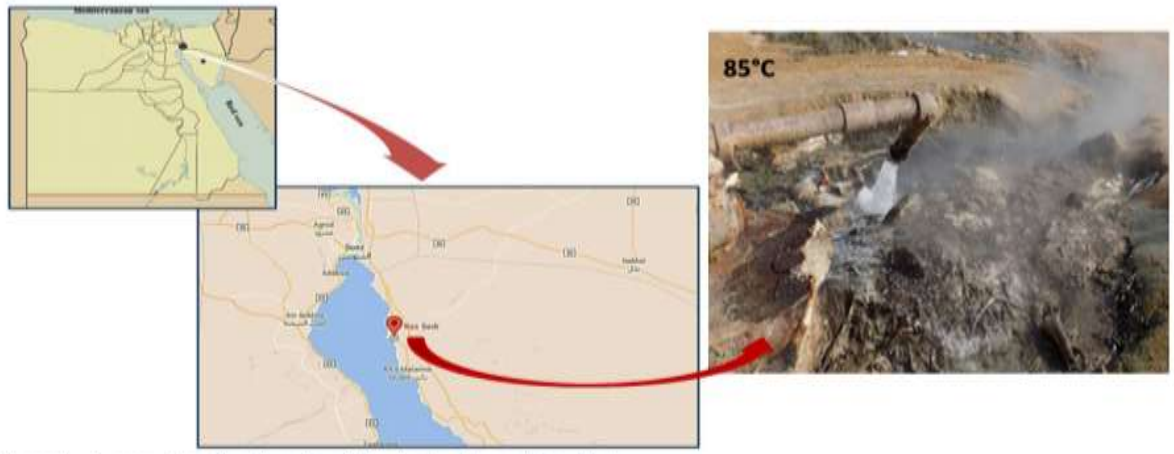


Figure1: A map showing the site of the hot spring of Ras-Sedr.

nutrient broth (NB) media, the isolates were incubated for 24 hours. Quantities of broth cultures were adapted to obtain similar optical density (OD) at 600 nm with the addition of sterile water. In 100 ml NB media, a volume of 0.1 ml from each bacterial isolates was incubated at 182 rpm at 35°C, 45°C, 55°C as well as 65°C separately in the shaker. For each culture, three replicate flasks of 100 ml capacity were maintained. In a spectrum of 600 nm, the OD of isolates was obtained by measuring around 3 ml of isolates at intervals of 4 hours. In the same manner, growth pH dependency was tested at pH levels between 5 and 9. Acetate buffer (for pH 5.0), sodium phosphate buffer (for pH 6.0 and 7.0) and Tris Buffer (for pH 8.0 and 9.0) were the used buffers at concentration 0.1 M to evaluate the optimum pH.

**2.5 Isolation of extracellular cellulase enzyme:**

In order to obtaining the crude cellulase, the isolate with the maximum hydrolysis zone was grown in an environmental shaker with the addition of 100 ml of CMC broth at 170 rpm and 55 °C. At 10,000 rpm CMC broth was centrifuged for 10 min at 4°C. Cell debris was collected as pellets and the supernatant which contains the crude cellulase enzyme was collected. Supernatants were then filtered using pore size membrane 0.45µm at 4°C, a method described by [23]. The resulting crude cellulase enzyme was used to assess the effect of temperature and pH on the activity of the enzyme.

**2.6 Effect of Temperature and pH on the Activity of Cellulase:**

The relative activity of cellulase at different pH and temperatures was measured by DNS (3, 5-dinitrosalicylic acid) method by measuring the quantity of reducing sugar produced during hydrolysis [15].The optimum temperature for the activity of the crude cellulase enzyme was determined by incubating the enzyme separately at different temperatures ranging from 35°C to 85°C for 30 min. One hundred microliters of crude cellulase was added to one hundred milliliter of CMC (1 %) as a substrate of the enzyme on Tris-HCl 50 mM (pH 7). Finally, the optical density was measured at 540 nm. For the assessment of enzyme stability under various degrees of temperatures, the crude cellulase enzyme was kept for an hour separately at temperatures ranging from 35°C to 85°C for an hour and the optical density was measured at 540 nm.

The assessment of the pH optimum for the enzyme activity was done by incubating the enzyme for 30 min at a temperature of 55°C in pH in between 5.0 to 9.0. One hundred microliters of cellulase was added to one hundred milliliter of CMC (1 %) as the substrate of the enzyme with the addition of various buffers, separately. 50 mM of each of acetate, phosphate, Tris-HCl and glycine buffer was used to obtain pH 5, pH 6-7, pH 8 and pH 9 respectively. The assessment of enzyme stability under various degrees of pH was determined by mixing one hundred microliters of crude cellulase separately with the previously mentioned buffers for an hour at 55°C; finally the optical density was measured at 540 nm.

Each experiment was done in triplicate and the mean value for each experiment was calculated.

**2.7 DNA extraction, PCR amplification & molecular identification:**

Genomic DNA was extracted according to protocol described by [21] with slight modifications. 1 ml of an overnight culture was transferred to an Eppendorf tube; cells were harvested and resuspended in 400µl saline-EDTA. Cells were incubated at 60°C for 15 min after the addition of 10 µl of lysozyme (0.1 mg / ml). Eventually, cells were lysed by adding 10 µl of proteinase K (1.0% w/v) and 10 µl of SDS (25%). Using equal amounts of phenol, phenol-chloroform, and crude DNA was extracted. Nucleic acids were precipitated with isopropyl alcohol washed with 70% ethanol, dried and dissolved TE buffer. Spectrophometrically, the concentration of DNA was determined. For 16S rRNA identification, the universal bacterial primers 27F (5'-AGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') was used for PCR. With the addition of fifty microliter of the reaction combination, the PCR was carried out with the next circumstances the former denaturation was performed at 95 °C for 4 minutes followed by denaturation at 94 °C for 30 seconds then the annealing stage at 55 °C for 30 second and the extension at 72 °C for 90 seconds finally the extension at 72 °C for 7 minutes. PCR product (50 ng/µl) of each isolate was used to prepare the samples which were delivered to MacroGen Company in Korea following their specifications. The sequence similarity search was conducted with Basic Local Alignment Search Tool (BLAST) of the National Center of Biotechnology Information (NCBI) on the following site http:/blast.ncbi.nlm.nih.gov/Blast.cgi to estimate the degree of similarity for the seven isolates in the Genebank. In the BLAST database tracking program, the phylogenetic tree was constructed using the 16S-rRNA sequence of other bacteria using MEGA Molecular Evolutionary Genetics Analysis software version 10. https://www.megasoftware.net/

## 3. RESULTS AND DISCUSSION

Samples of the sediment were obtained from hot spring in egypt, ras sedr, abo swira, el mahager road. The temperature of the sediment in the site recorded to be 85°c and 7.09 for ph. The texture of the sediments were sandy loamy and the concentration of the organic matter was 2.615% with different anions, cations and heavy metals composition listed in (Table 1).

The quantity of cellulose consumed by soil microbes as a main source of carbon depends on abiotic conditions such as organic matter, ph and soil temperature. The sediment analysis showed that the organic matter content in sediment sample was 2.625%. According to [32] cellulose is the principal mass of organic matter that is degraded by soil microorganisms completely or partially. Another factor can affect the activity of cellulase is ph of the sample. In this study, the ph was 7.09; [33] detected high cellulase activity and stability in the range of neutral and alkaline phs. The main action of cellulase enzyme is deconstruction of cellulose, which has highly ordered structure, and it represents the most important challenges in biotechnology. It worth mentioning that, [34] discussed the positive effect of anions/cations in deconstruction of cellulose polymers. He found that anions/cations controlling the dissolution process which is an important step before conversion of cellulose. In this study, chemical analysis of sediments showed the presence of anions such as $hco_3^-$- $so_4^{2-}$ - $co_3^{2-}$ and cations such as $mg^{2+}$-$na^+$ - $k^+$.

A maximum of twenty organisms those have the ability to grow on the agar plates of the cmc were isolated, but only seven thermophillic bacteria have been successfully isolated with the ability to use carboxymethylcellulase (cmc) as their primary source of carbon. Isolates were gram positive, have rod shape cells, spore forming and motile bacteria with the ability to produce acid from glucose, fructose, maltose and mannose. Other differentiations in phenotypic characterizations illustrated on Table 2

Table (1). Analysis of sediment sample.

| EC $dSm^{-1}$ | pH | Organic matter % | Sand % | Silt % | Clay % | Texture |
|---|---|---|---|---|---|---|
| 5.15 | 7.09 | 2.615 | 66.0 | 24.0 | 10.0 | Sandy Loam |

| Cations $meql^{-1}$ | | | Anions $meql^{-1}$ | | |
|---|---|---|---|---|---|
| $Mg^{2+}$ | $Na^{+}$ | $K^{+}$ | $HCO_3^-$ | $SO_4^{2-}$ | $CO_3^{2-}$ |
| 8.60 | 18.5 | 1.50 | 1.50 | 18.0 | 0.00 |

| Cu ppm | Zn ppm | Co ppm | Pb ppm | Fe ppm |
|---|---|---|---|---|
| ND | 11.360 | ND | ND | 3796 |

**(Table 2): Phenotypic characterization of isolated strains.**

| Characteristic | Strain code | | | | | | |
|---|---|---|---|---|---|---|---|
| | HRS1 | HRS12 | HRS13 | HRS14 | HRS5 | HRS6 | HRS7 |
| Pigmentation | off-white | creamy | off-white | off-white | beige | creamy | creamy |
| Colony size (mm) | 1.5 | 1 | 1 | 2 | 1.5 | 1.5 | 2 |
| Colony elevation | flat | flat | flat | convex | raised | convex | raised |
| Colony appearance | shiny | shiny | shiny | shiny | shiny | shiny | shiny |
| Cell shape | rod | rod | rod | rod | rod | rod | rod |
| Spore formation | + | + | + | + | + | + | + |
| Motile | + | + | + | + | + | + | + |
| Gram reaction | + | + | + | + | + | + | + |
| Casein hydrolysis | - | - | - | + | - | - | + |
| Acid from glucose | + | + | + | + | + | + | + |
| Acid from fructose | + | + | + | + | + | + | + |
| Acid from maltose | + | + | + | + | + | + | + |
| Acid from ribose | - | + | + | - | - | - | + |
| Acid from mannose | + | + | + | + | + | + | + |
| Nitrate reduction | - | - | - | - | + | + | + |
| Voges–Proskauer | - | + | - | + | + | + | - |
| Catalase | + | + | - | + | + | + | + |
| Oxidaes | - | - | + | - | - | - | + |

Such isolates developed cmc clearance variable zones in the presence of iodine solution staining figure 2. [14,18] performed a similar experiment for screening and identification of cellulase producing bacteria, which found the iodine solution to be quick and effective in detecting many positive cellulase producing bacteria from broad range of samples. [17] also used cmc agar plates and iodine solution staining to evaluate cellulase producing bacteria from hot spring.

The temperature range for growth was determined by incubating the isolates separately in 35°C, 45°C, 55°C as well as 65°C. All of the isolates showed the longest lag phase when grown at 35°C as well as the lowest optical density values **Figure 3.** When the isolates were grown on 45°C **Figure 4** the values of OD became higher in comparison with the OD values when isolates were grown in 35°C. Isolates ' growth characteristics are found to be different at 55°C, isolates showed the shortest lag phase and the highest optical density values on 55°C **Figure 5**. The OD values started to reduce again when the isolates were grown in 65°C **Figure 6**.

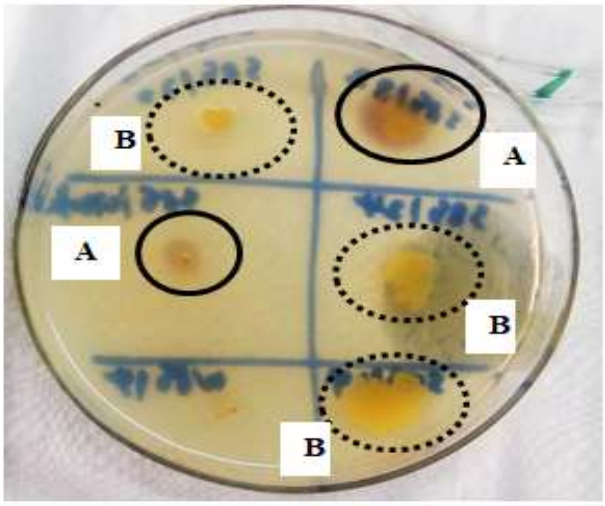


**Fig. 2. A.** No halo zones showed (-ve cellulase), the circle showed that the colony has the blue color of iodine indicator. **B**. Zones of hydrolysis shown in the plate (+ve cellulase). The circle showed that the colony has clear color (no iodine`s blue color showed).

These results indicated that these isolates showed different growth behaviors depending on the temperature during their development. [25] Reported that thermophilic bacteria are categorized as facultative and obligate according to growth temperatures which can varied from 45 °C to 80 °C. High temperatures are required for the growth of obligate thermophiles, while facultative thermophiles could survive at both low (less than 50 °C) and high temperatures. [28] Also conducted a study on the growth curve of thermophilic Bacillus sp. The growth was measured at75°C and the optimal temperature for growth was at 60°C.

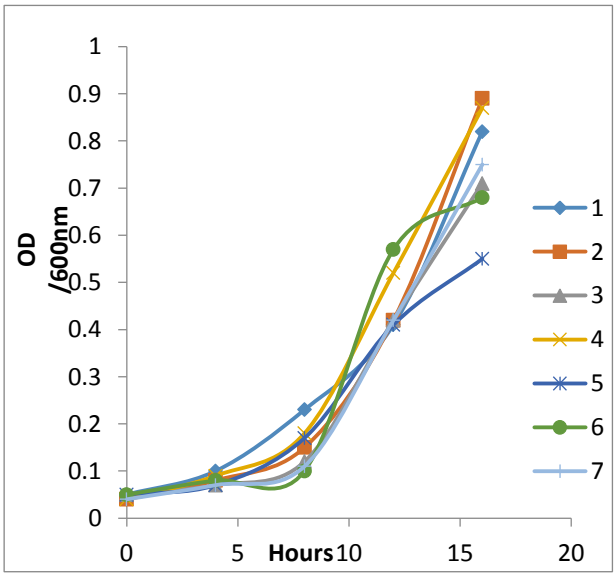


Fig.3. The growth curve of bacteria (35°C)

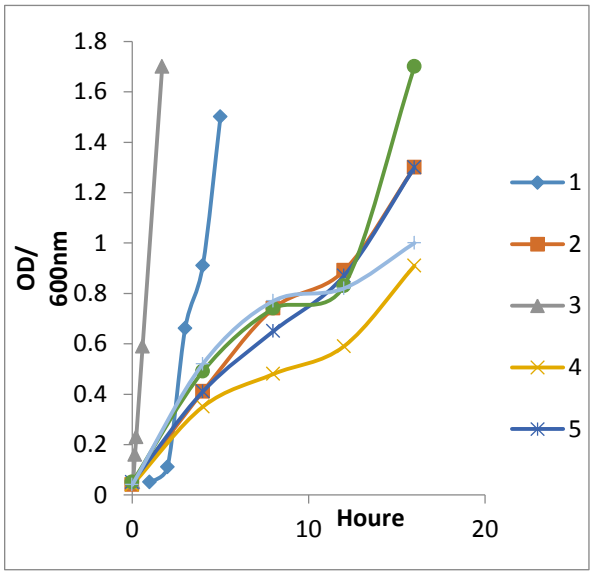


Fig.4. The growth curve of bacteria (45°C)

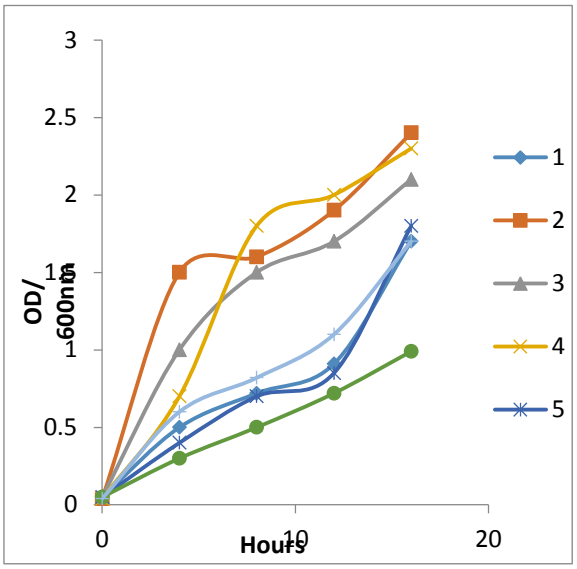


Fig.5. The growth curve of bacteria (55°C)

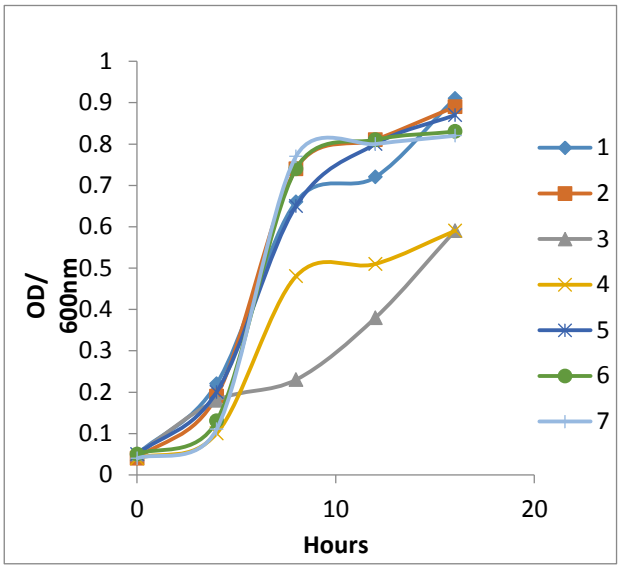


Fig.6. The growth curve of bacteria (65°C)

The Isolates had a broad pH range for growth from 5.0 to 9.0 with an optimum of 7.0 **Figure 7.** [8] Also conducted a research discussing the effect of pH on the growth of thermophilic Bacillus species. They reported that the optimum pH for growth was 7.0.

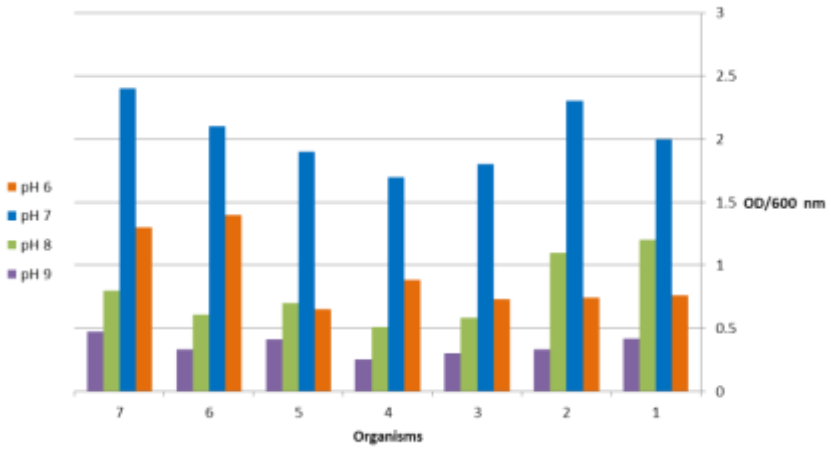


Fig.7. Bacterial growth at different pH values.

Stability of the enzymes is a key factor when considering whether the operation of biocatalysis will be effective commercially. Enzymes lose part of their function when they undergo heat or intense pH action [35]. Stability of an enzyme is defined by its ability to maintain its effective structural conformation against destructive conditions, such as rise in temperature [36].

The impact of temperature on both stability and activity of cellulase enzyme was studied in the current work at temperatures range from 35°C to 85°C (**Figure 8**). The crude cellulase activity showed the highest value at 55°C with 100% of the enzyme relative activity. The activity started to decline gradually at temperatures higher than 55°C until reached to 60% at 85°C. In a similar study conducted by [11], they reported that the maximum cellulase activity in *Bacillus* sp. was at 40°C. Furthermore, different

thermophilic bacteria was reported to be producer of thermostable cellulase which does not possess activity for prolonged periods at elevated temperatures (as high as 60 °C) [13].

Cellulase enzyme kept 80% of its stability at 55°C and then stability started to decline gradually until reached to 40% at 85°C possibly due to thermal denaturation. On the other hand, researches on thermostability of cellulase enzyme obtained from *Geobacillus thermoleovorans T4*, reported that with 80 % of its stability, the crude cellulase's maximum operation was reached at 65°C. Cellulase stability decreased immediately even with a minor temperature increase above 65°C, indicating that the enzyme was extremely sensitive to temperatures above 65°C [29].

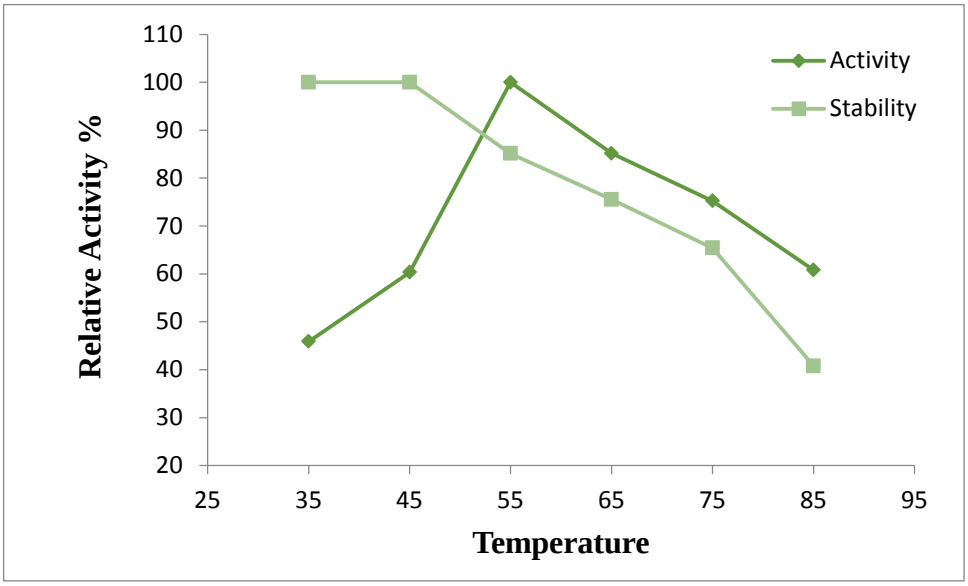


Figure 8. Impact of temperature on cellulase activity & stability

The impact of pH on crude cellulase activity and stability was assessed at pH levels between 5.0 and 9.0 **(Figure 9)**. The crude cellulase showed the highest activity in phosphate buffer with100 % at neutral pH 7.0, while the minimum enzyme activity was measured at acid pH 5.0 with 50% of the enzyme activity. Similar results were observed by [29, 4] who reported the maximum activity of cellulase obtained from *Geobacillus* sp. T1 at pH 7.0. Another similar study was conducted by [11] where the optimum cellulolytic activity of *Bacillus* sp. was at pH 7. On the other hand [16] reported that *Geobacillus* sp. 70PC53 expressed an optimal cellulase activity at pH 5.0.

The crude cellulase kept 90% of its stability when incubated for an hour in pH 7 at 55°C and stability declined to be 50% and 60% at pH 5 and 9 respectively. Similar results was discussed by [37] who reported that the maximum cellulase stability of *Bacillus.* sp. DL3 at pH 7. On the other hand [38] reported the maximum cellulase stability of *Bacillus* sp. HSC7 to be at pH 5.0.

PCR of genomic DNA amplification of the seven isolates revealed a ~1500 bp fragment amplification result using universal 16S rRNA primers (forward 27F and reverse 1492R primers). On the basis of BLAST analysis, the thermophilic strains were all found to be similar to those from different species of genus *Geobacillus* and closely related with the species *Geobacillus thermodenitrificans, Geobacillus*

*stearothermophilus and Geobacillus jurassicus* with similarities ≥ 97% to known strains **(Table 3)**. Neighbor-joining evolutionary distance tree based on 16S rRNA gene sequences for isolated strains and selected reference sequences was constructed **(Figure 10)**

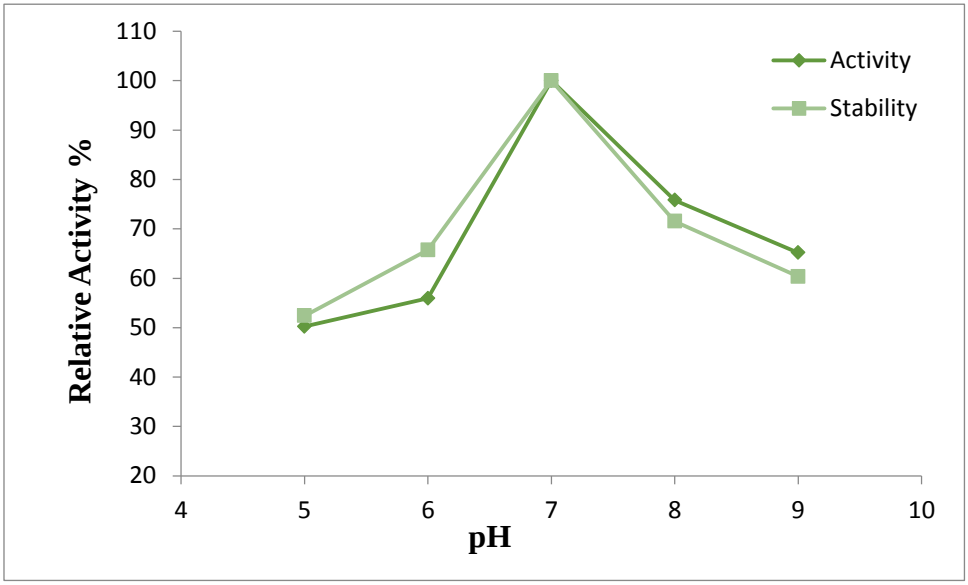


Figure.9. Impact of pH on cellulase activity and stability.

**(Table 3): BLAST analysis of 16S rRNA sequences of the isolated strains.**

| ***Geobacillus* Isolate** | **% Sequence similarity** | **Closest phylogenetic relative** |
|---|---|---|
| **HRS1** | 98.33% | *Geobacillus thermodenitrificans* R-32500 (FN428639) |
| **HRS12** | 97.53% | *Geobacillus thermodenitrificans* R-32615 (FN538995) |
| **HRS13** | 97.75% | *Geobacillus thermodenitrificans* R-32617(FN428661) |
| **HRS14** | 96.66% | *Geobacillus stearothermophilus* A1 (KU248350) |
| **HRS5** | 96.59% | *Geobacillus stearothermophilus* P3 (KU 248348) |
| **HRS6** | 96.95% | *Geobacillus stearothermophilus* IFO 12550 ( NR_040794) |
| **HRS7** | 96.81% | *Geobacillus jurassicus* MK7(MT126376) |

As candidates for industrial application of thermostable hydrolases, the ability of bacilli species should be recognized. The number of thermophilic bacilli species belonging to the *Bacillus* sp., *Geobacillus* sp. and *Anoxybacillus* sp. has been cultured from various geothermal hot springs and identified as thermostable cellulase producers [19, 7]. A study by [27] stated that *Geobacillus toebii* PS4 and *Geobacillus thermoleovorans* PW13 collected from Himachal Pradesh's Tattapani hot spring in India gave optimal 80-90$^{o}$C and 6.0-8.0 pH cellulase activity. The ability to grow at elevated temperatures makes *Geobacillus* sp. attractive agents in the applications of biotechnology. These bacteria could be producers of different enzymes that are considered as thermostable enzymes [29, 2, 22]**.**

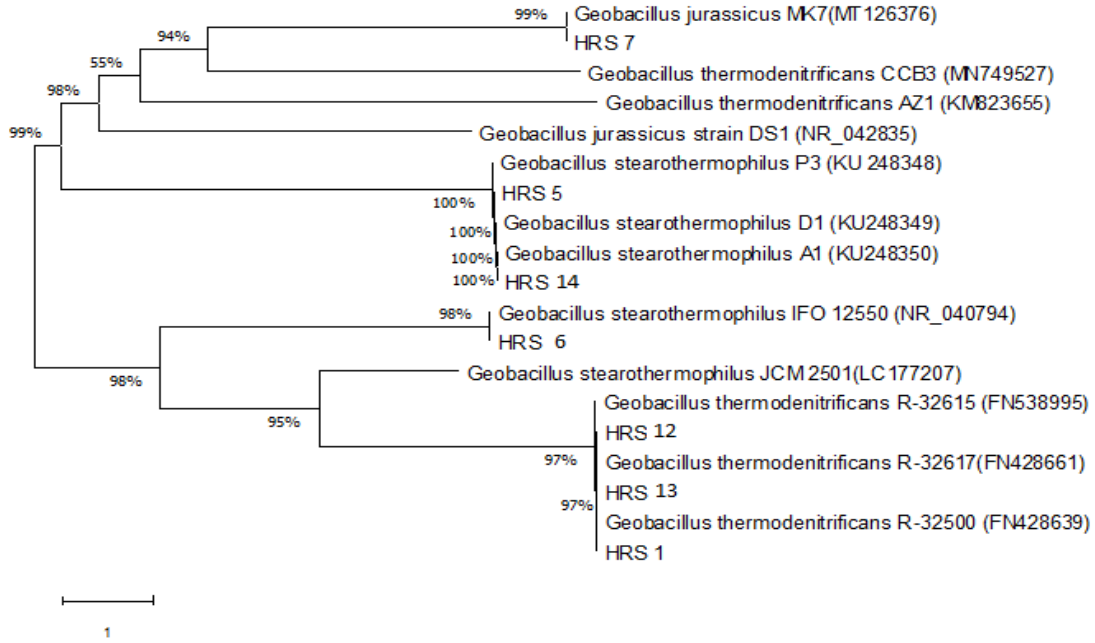


Figure.10. Phylogenetic tree of the identified strains.

## 4. CONCLUSION & RECOMMENDATIONS

The thermophiles which studied through the current work have the ability to produce useful thermostable cellulase of industrial significance. Ras-sedr's hot spring considered a prospective source of economically significant microorganisms which needs more studies through further researches.

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