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Cellulolytic, amylolytic and xylanolytic potential of thermophilic isolates of Surajkund hot spring

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A total of 41 isolates were obtained from various samples (soil, mud, and water) of Surajkund hot spring, Jharkhand, at three different isolation temperatures of 50°C, 60°C, and 70°C. However, our interest was in the thermophilic strains that were isolated at 60°C and 70°C. Four isolates at 70°C (BITSNS038, BITSNS039, BITSNS040, BITSNS041) are the producers of thermozymes, namely amylase, xylanase, and cellulase, respectively. The highlights of the present study also showed that three out of four isolates demonstrated all three enzymatic activities, i.e. amylolytic, xylanolytic and cellulolytic on agar plate assay conditions at 70°C. One of the isolates, BITSNS038, was further chosen for phenotypic characterization as well as 16S rRNA gene sequencing and was affiliated to *Geobacillus icigianus.* The presence of *Geobacillus icigianus* was reported first time from hot spring, Surajkund, which showed amylolytic index of 1.58, xylanolytic index of 1.5 and cellulolytic index of 2.3 based on plate assay, and amylase activity of 0.81 U/mL, xylanase activity of 0.72 U/mL and very less cellulase activity of 0.15 U/mL after 24 h of growth in submerged conditions. One isolate at 60°C BITSNS024 was found to exhibit maximum amylase activity with an enzymatic index value of 3.5 and was identified as *Anoxybacillus gonensis*.

Keywords. Amylase; cellulose; hot spring; thermozymes; xylanase

1. Introduction

Microorganisms which flourish in extreme habitats are termed as 'extremophiles' and they produce extremozymes that are functional under intense conditions (Van Den Burg 2003). The adaptations of thermophiles to these intense habitats contribute to their high genomic and sophisticated metabolic flexibility, and their thermostable enzyme proteins are considered suitable for both industrial and environmental applications (DeCastro *et al.* 2016). Thermozymes perform bioprocess at elevated temperatures which result in reduced risk of contamination, improved use of raw materials (Loperena *et al.* 2012) and controlled process optimization under stringent conditions (Kurosawa 2013), and thus act as versatile tools for the development and improvement of a range of industrial and biotechnological processes. Moreover, there is a decrease in viscosity, increase in the diffusion coefficient of organic compounds (Panosyan 2017), bulk mixing, better substrate solubility, increased mass transfer rate leading to better yield and productivity in terms of bio-catalyzing reactions leads to favorable equilibrium displacement in endothermic reactions and thus thermozymes offers robust catalyst alternatives for sustainable environmental process (Haki and Rakshit 2003).

The majority of existing industrial enzymes have a hydrolytic mode of action, and primarily used for the degradation of various natural substances. Various hydrolases, like amylases, cellulases, xylanases and proteases, find applications in industrial processes, namely starch, textile, detergent, baking industries, organic wastewater treatment, bioethanol production, paper and food industry, respectively (Madhavan *et al*. 2017; Paul *et al*. 2017; Acer *et al*. 2016; Ghosh *et al.* 2019; Pandey *et al*. 2019). Recently, the use of a number of hydrolytic microbial enzymes is being used as an alternative to chemical hydrolysis of different raw material in food processing and pharmaceutical industries (Awasthi *et al*. 2018). For the industrial purpose, the enzymes obtained from thermophilic microorganisms have attracted a great deal of interest among industrial and research communities (Hosseini and Aziz 2013). Thermozymes from thermotolerant and thermophilic microorganisms are of special concern due to their high enzymatic activity under a wide range of physicochemical conditions, their high thermal stability and also the commercial production of these enzymes is simple and economically feasible (Cheng and Chang 2011; Bala and Singh 2018). The microbes producing amylases, cellulases and xylanases are able to actively hydrolyze the complex glycosidic bonds which are present in lignocellulosic organic waste polymers. Maximum

hydrolytic degradation of the agricultural or food wastes containing more than 60% starchy and cellulolytic wastes can be achieved by the combined action of amylases and cellulases.

Thermostable α-amylases are more attractive in biotechnological processes since they are stable over a temperature of 60°C allowing enhanced enzymatic reaction. Tanyildizi *et al*. (2006) also described that bacterial strains *Bacillus amyloliquefaciens* and *Bacillus licheniformis* NH1 were producers of thermotolerant amylases. Due to their wide application range, the amylases represent 25% of the world enzyme market (Naili *et al*. 2016). In recent years, the investigation of new amylase producing microorganisms has been of great interest particularly considering the thermostability for varied applications. Similarly, thermostable cellulases and xylanases have diverse applications in degradation of organic wastes, hydrolysis of raw materials, improving digestibility of chemical feedstock, paper and bio-bleaching of pulp, etc. (Bohra *et al.* 2019; Kaur *et al.* 2010; Bin *et al.* 2012; Bhalla *et al.* 2015). In the context of this background, the present work was focused towards isolation and characterization of thermophilic bacteria from hot spring of Surajkund and evaluation of different enzyme producing capability.

2. Materials and methods

2.1 *Sample (soil, mud, water) collection*

The sampling location as shown in figure 1a did not include any wildlife area nor is surrounded by forest belt that encompasses endangered species or exotic flora. Field measurements and sample collections were carried out in March, 2017. The physiochemical parameters such as temperature and pH were recorded on site using thermometer and pH strip (Hi-Media) and it was observed at 85°C and 7.4 respectively suggesting that it is a neutral hot spring. Water, soil and mud samples were collected from hot spring Surajkund (24°08′58″N, 85°38′44″E), Jharkhand, India as shown in figure 1b and c. Collected samples were transported to the laboratory without temperature control and stored at 4°C until further processing.

2.2 *Characterization of soil and mud samples*

1g (wet weight) of soil and mud sample was taken and dried on a blotting paper at 45°C. The C, H, N, S (%) content was determined by the elemental analyzer, M/s Elementar, Germany; Vario EL III whereas, other major and minor element distribution was analyzed by Inductively Coupled Plasma, Optical Emission Spectrophotometry (ICP OES; Perkin Elmer, USA; Optical 2100DV).

2.3 *Isolation, culturing and phenotypic characterization of thermophilic bacteria*

The samples (soil, mud, water) collected were processed for the enumeration and isolation of culturable moderate thermophilic (50°C–60°C) and thermophilic (70°C) bacteria. It was carried out by standard serial dilution and spread plate technique as described in (Kumar *et al.* 2014) by employing two different media. Nutrient Agar (Hi-Media) and Tryptone Soybean Agar/ Soyabean Casein Digest Agar (Hi-Media) at three different temperatures viz 50°C, 60°C, and 70°C. To avoid desiccation of agar plates, it was wrapped in autoclavable sterile bags and incubated. After incubation for 24 h, the plates were observed for different morphotypes. The colonies were picked on the basis of morphological difference and were continuously streaked on the same medium to obtain a pure culture. Based on enzyme secretion, the most suitable isolate was characterized for phenotypic, biochemical property and 16S rRNA analysis by Microbial Type of Culture Collection, Institute of Microbial Technology, Chandigarh, India.

2.4 *Assessment of hydrolytic enzyme activity using primary screening*

For the assessment of different enzyme activity, pure isolates were allowed to grow on specific agar medium plates (qualitative) and incubated at their respective temperatures.

2.4.1 *Xylanase activity:* Xylan Congo Red Agar Plate Assay was used for the detection of xylanase producing isolates with slight modification as described by Nagar *et al.* (2012). The composition of the medium in g/100 mL: Peptone 0.5, Yeast Extract 0.2, $MgSO_4$ 0.05, NaCl 0.05, $CaCl_2$ 0.01, Xylan 0.5, Agar 2.5%, pH 7. The congo red dye (0.01% w/v) was incorporated in the medium itself which eliminated the step of destaining with 1M NaCl solution. The pure isolates were spot inoculated on xylan congo red agar plates and were incubated at 70°C for 24–48 h to allow enough growth of the isolate for the secretion of xylanase. The formation of a clear zone of hydrolysis indicated xylan degradation and the enzymatic index was calculated for the positive isolates as described by (Florencio *et al.* 2012).

2.4.2 *Amylase activity:* Starch Agar Plate Assay (Vaikundamoorthy *et al.* 2018) was used for the detection of amylase producing isolates. The media composition (Hi-Media) for amylase consisted of g/L; Beef Extract 3.0, Starch Soluble 10.0, and 2.5% agar, pH 7.5 ± 0.2. The plates were spot inoculated with the pure bacterial isolates and incubated for 24–72 h to allow the secretion of amylase. After the growth on plates, the agar medium was overflown with Grams Iodine for 10 min. The excess Grams Iodine solution was then discarded and the formation of halo zones indicated

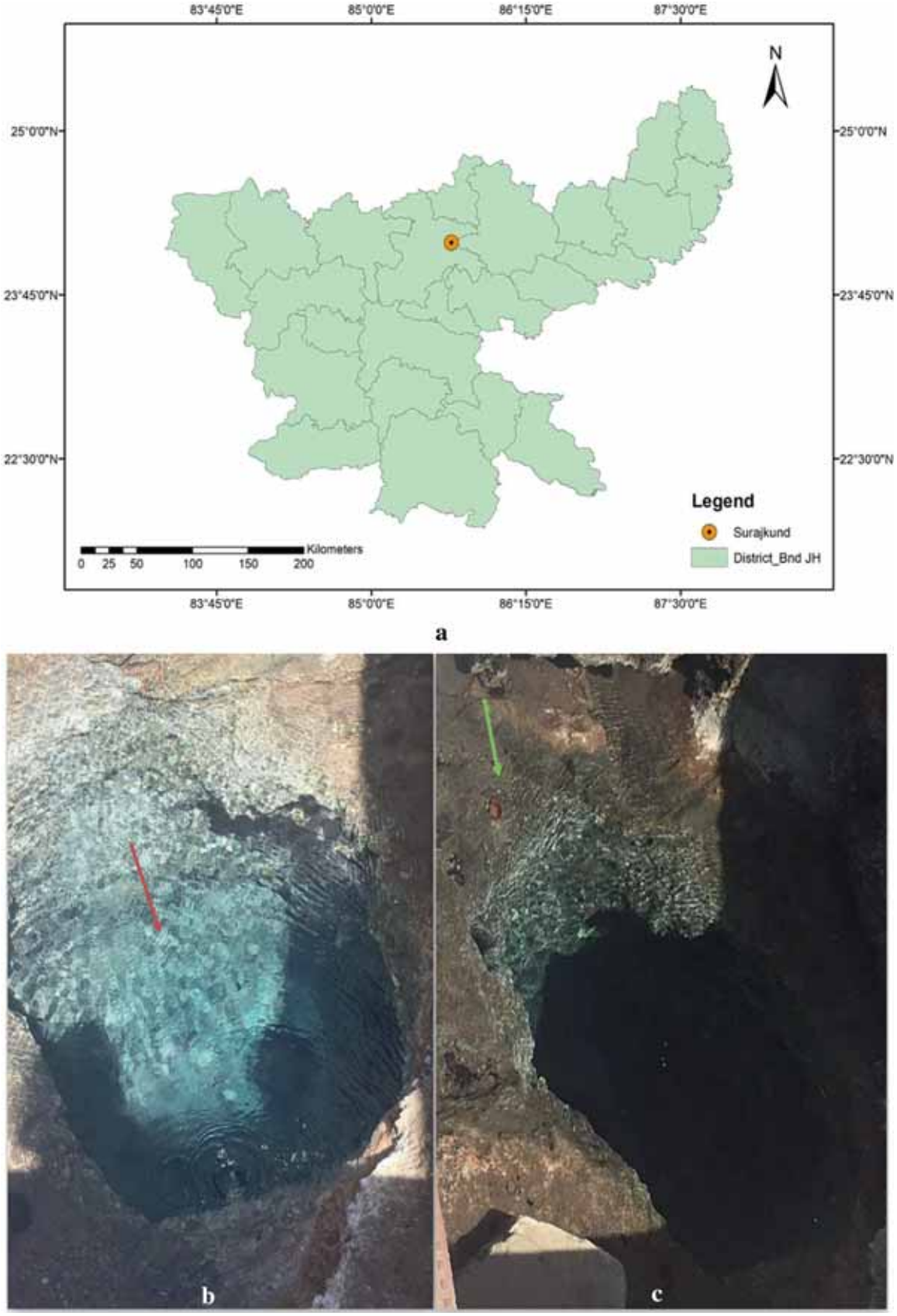

Figure 1. (**a**) Location of Surajkund Geothermal Area and the Hot Spring and (**b**) Hot spring of Surajkund. the red arrow indicates the sampling site (**c**) The surrounding soil and sediment area indicated as green arrow.

starch degradation and the enzymatic index for each positive isolate was further calculated.

2.4.3 *Cellulase activity:* The screening of cellulase-producing bacteria was conducted in medium containing g/100 mL: carboxymethylcellulose sodium salt (CMC) 1.0, Agar 2.5, and pH 7.0 (Kasana *et al.* 2008). The isolates were transferred on to Carboxymethylcellulose (CMC) agar plate medium and incubated for 24–48 h. For the detection of cellulase enzyme expression, 1 mL of Grams Iodine solution was added to the CMC plate after completion of growth for 30 minutes and excess iodine was removed. The strains forming clear halo zones on CMC media were selected as cellulase producing strains.

2.5 *Assessment of hydrolytic enzyme production using secondary screening*

The strain was cultivated in minimal media supplemented by 1% starch, 1% CMC and 0.5% xylan for the production of amylase, cellulase and xylanase respectively. The amylase, cellulase and xylanase activities were analyzed using cell-free culture supernatant of isolate BITSNS038, all over its growth period (24 h) using starch, CMC and xylan as substrate according to Nelson Somogyi method (Nelson 1944). The assay reaction mixture was incubated for different time periods at 70°C temperature to optimize suitable incubation period for enzyme activities and thus the assay was carried out accordingly as described by Narang and Satyanarayana (2001).

2.6 *16S rRNA gene sequencing and phylogenetic analysis of isolate BITSNS038 and BITSNS024*

The partial 16S rRNA gene sequence obtained was compared with other sequences available in the EZBioCloud database for evaluation of specific microbe. The isolate was identified to species level on the basis of 16S rRNA gene sequence similarity of $\geq$ 98% with the sequences in the database. Sequence alignment and construction of the phylogenetic tree were performed using the neighbor-joining (NJ) method (Saitou and Nei 1987; Kumar *et al.* 2016) in MEGA7 software. The ENDMEMO software was used for calculation of G+C content of the identified bacterium from the 16S rRNA sequence.

2.7 *Scanning electron microscopy (SEM) analysis*

The isolate BITSNS038 and BITSNS024 were also subjected to SEM (JSM-6390 LV, Jeol Japan) analysis for studying cell morphology according to the methodology described by Kaláb *et al.* (2008). The washed cell pellet of 24 h old culture of the BITSNS038 was fixed in 2.5% glutaraldehyde and incubated at 4°C overnight. The dehydration of cells was carried out using a different concentration of ethanol and suspended in PBS Buffer for overnight.

3. Results

3.1 *Study of microbial abundance in relation to sample elemental chemistry*

A total of 41 bacterial isolates were obtained from hot spring Surajkund as shown in table 1. From the pattern observed, it was found that, with the increase in temperature of incubation, the number of isolates decreased. Twenty-two isolates (BITSNS001-BITSNS022) were obtained at 50°C, 15 isolates (BITSNS023-BITSNS037) at 60°C which further decreased to 4 (BITSNS038-BITSNS041) at 70°C. It was also evident from table 1 that only 6 isolates were obtained from a water sample at 50°C whereas no isolate was found at high temperatures from the water sample. A maximum number of 28 isolates was obtained from a mud sample, followed by 7 isolates from a soil sample.

The elemental analysis of both soil and mud was carried out to find the reason for the microbial richness from a particular sample source and the factors contributing to the same (table 2). The results of elemental analysis of both soil and mud shown in table 2 revealed that there is indeed specific variation between mud and soil sample elements. The mud sample contains 4 times higher carbon content i.e. 1.831% as compared to 0.459% in soil. Sulfur content was

Table 1. Number of isolates from different samples at different isolation temperature

Sl. No.	Isolation Temperature (°C)	Soil	Mud	Water
1.	50	2	14	6
2.	60	5	10	-
3.	70	-	4	-

Table 2. Elemental analysis profile of soil and mud samples

Sl. No.	Elements	Soil	Mud
1.	Sulphur	0.514%	0.621%
2.	Carbon	0.459%	1.831%
3.	Hydrogen	0.046%	0.056%
4.	Nitrogen	0%	0%
5.	Calcium	20.2ppm	23.11 ppm
6.	Ferrous	11.38 ppm	13.69 ppm
7.	Magnesium	4.825 ppm	7.885 ppm
8.	Manganese	0.41 ppm	0.524 ppm
9.	Zinc	0.072 ppm	0.517 ppm
10.	Chromium	0.033 ppm	0.038 ppm
11.	Nickel	0.047 ppm	0.028 ppm

Table 3. Thermophilic isolates with amylolytic, xylanolytic and cellulolytic activity

Sl. No.	Strain	Source	Amylase	Xylanase	Cellulase
1.	BITSNS038 (70°C)	Mud	+++	+++	+++
2.	BITSNS039 (70°C)	Mud	+++	-	++
3.	BITSNS040 (70°C)	Mud	+++	+++	+++
4.	BITSNS041 (70°C)	Mud	+++	+++	+++
5.	BITSNS024 (60°C)	Soil	+++	-	++

+++ indicates optimum activity, ++ indicates good activity, - indicates no activity

Table 4. Enzymatic index (EI) of five different isolates

		Amylase activity			Xylanase activity			Cellulase activity		
Sl. No.	Strains	Halo colonies Mean Φ_c^*	Halo Hydrolysis Mean Φ_h^{**}	EI	Halo colonies Mean Φ_c^*	Halo Hydrolysis Mean Φ_h^{**}	EI	Halo colonies Mean Φ_c^*	Halo Hydrolysis Mean Φ_h^{**}	EI
1.	BITSNS038 (70°C)	1.9	3.0	1.58	1.0	1.5	1.5	0.9	2.1	2.3
2.	BITSNS039 (70°C)	1.8	3.0	1.60	-ve	-ve	-ve	0.6	1.2	2.0
3.	BITSNS040 (70°C)	2.0	3.5	1.75	1.0	1.4	1.4	0.9	1.9	2.1
4.	BITSNS041 (70°C)	2.0	3.4	1.70	1.0	1.4	1.4	0.9	1.9	2.1
5.	BITSNS024 (60°C)	1	3.5	3.50	-	-	-	1	2	2

reported as the second abundant element in all the samples without any difference followed by hydrogen. Nitrogen content was negligible in both cases. It was revealed from table 2 that both mud and soil sample were richer in calcium being 23.11 ppm and 20.2 ppm, followed by iron as second abundant metal 13.69 ppm and 11.38 ppm respectively. Magnesium was third in dominance as 7.88 ppm in mud which was twice the concentration present in soil (4.82 ppm). Zinc was observed to have been present sevenfold higher in case of mud relative to soil whereas Chromium and Nickel were not present in a significant amount. This supported the fact that a maximum number of isolates were obtained from mud rather than soil and water irrespective of different isolation temperatures as shown in table 1. High microbial abundance and diversity in terms of morphotypes from mud may be attributed to high carbon content, as well as the presence of macro and microelements which play a crucial role in biological processes ranging from cellular function to whole organism performance (Warne 2014).

3.2 *Activity of enzymes using primary screening*

The cultures which were isolated from mud and soil sample of hot spring at 70°C and 60°C were tested for amylolytic, xylanolytic and cellulolytic enzymes and shown in table 3.

The other parameter for calculating the enzyme activity of five isolates was the measurement of the enzymatic index (EI) by the given formula:

$$\text{EI} = \frac{\text{diameter of hydrolysis zone}}{\text{diameter of colony}}$$

The isolates with hydrolysis zone >1.0 cm are considered significant (Gaur and Tiwari 2015) because it reflects the amylolytic, xylanolytic and cellulolytic potential. The greater is the halo zone, the higher is the EI value. The results of the EI values of all the five bacteria are summarized in table 4.

The observation of table 4 revealed that all 70°C isolates showed very good enzymatic activity i.e. amylolytic, xylanolytic and cellulolytic activity respectively. The enzymatic index falls in the range 1.4–2.3, in which BITSNS038 had the highest enzymatic index of 1.5 in case of xylanase and 2.3 in case of cellulase. BITSNS024, the 60°C isolate however exhibited highest amylolytic activity with an EI value of 3.5. Among four isolates of 70°C, three isolates BITSNS038, BITSNS040, BITSNS041 produced amylase, xylanase and cellulase activity and one isolate namely BITSNS039 exhibited amylase and cellulase activity as shown in figure 2 (plate assay method). BITSNS024, on the other hand, exhibited only amylolytic and cellulolytic activity as shown in figure 2.

3.3 *Activity of enzymes using secondary screening*

BITSNS038 was further selected for enzyme production in a liquid medium which showed maximum activity for amylase

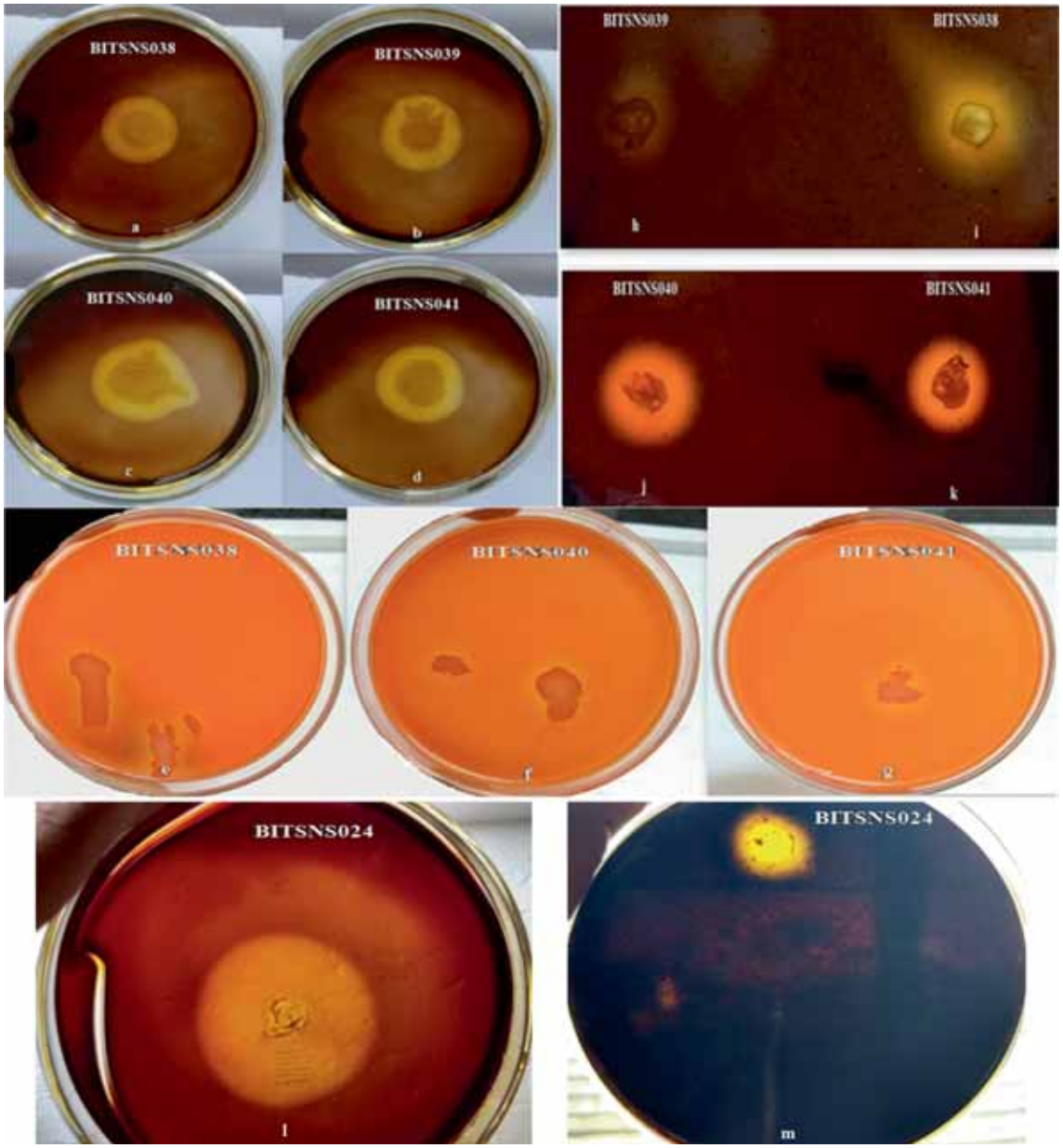

Figure 2. Plate assay for amylase (**a–d**), xylanase (**e–g**) and cellulase (**h–k**) activity of 70°C isolates. Plate assay for amylase (**l**) and cellulase (**m**) activity of 60°C isolate (BITSNS024).

as 0.81 U/mL followed by xylanase as 0.72 U/mL and cellulase as 0.15 U/mL respectively at the end of 24 h of growth (figure 3). Thus, it was revealed that this particular strain is able to produce all three enzymes in the liquid medium.

3.4 *Optimization of the incubation period for enzyme activity*

The optimization of the incubation period of the reaction mixture for all the three enzymes produced by isolate BITSNS038 is shown in figure 4. The observation of the figure revealed that amylase activity reached optimal at 30 min of incubation; however, 15 min of incubation period was suitable for xylanase and cellulase activity.

3.5 *Phenotypic and molecular characterization of the isolate BITSNS038 and BITSNS024*

The isolates BITSNS038 and BITSNS024 with considerable enzymatic activity were chosen for phenotypic and molecular characterization. The *Geobacillus icigianus* (BITSNS038) had a circular configuration, rhizoidal margin, raised elevation, cream pigmentation, opaque density. It was gram-positive, spore-forming, non-motile, and was able to grow at a

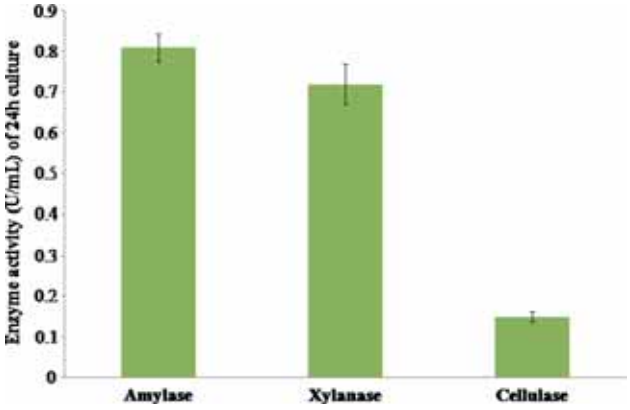

Figure 3. Amylase, xylanase and cellulase activity at 24 h grown culture of BITSNS038.

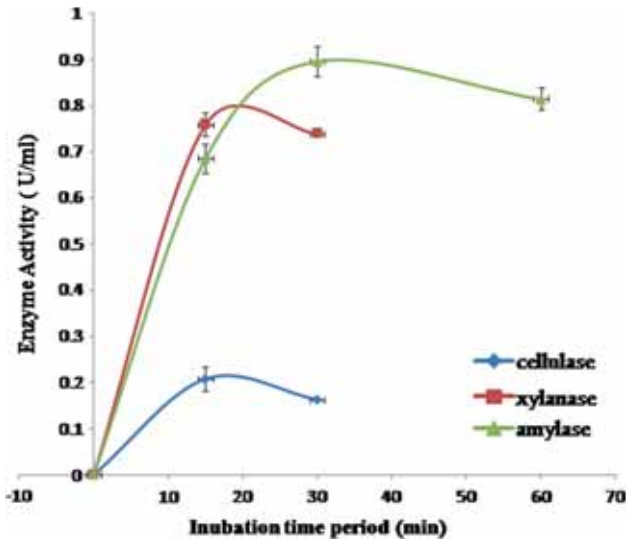

Figure 4. Optimization of the incubation time period for enzymatic activity by BITSNS038.

temperature range from (50°C–60°C), pH range of 5–9 and NaCl concentration of 2–3%. The isolate showed the ability to hydrolyze casein, gelatin, and esculine. It was methyl red and catalase positive and had glucose, mannitol, and xylose fermentation characteristics. The scanning electron microscopy (SEM) analysis to study the cellular morphology of *Geobacillus icigianus* is shown in figure 5 depicting the cylindrical rod-shaped morphology.

The bacterial isolate BITSNS024 was identified by 16S rRNA gene sequencing as *Anoxybacillus gonensis* and had an irregular configuration, rhizoidal margin, raised elevation, with cream pigment, and opaque density. It had gram-positive and spore-forming characteristics. It was able to grow in a temperature range of (42°C–60°C), pH range of 6–9 and NaCl concentration of 2–5%. It had starch and gelatin hydrolyzing capability and was found positive for citrate utilization, catalase, oxidase. It showed glucose, fructose, mannitol, sucrose and xylose fermentation characteristics. The scanning electron microscopy (SEM) analysis to study the cellular morphology of *Anoxybacillus gonensis* is shown in figure 6 depicting the cylindrical thin rod-shaped morphology.

3.6 *Construction of phylogenetic tree*

The phylogenetic analysis of 16S rRNA gene sequence of the bacterial strain BITSNS038 revealed its 99.92% similarity with *Geobacillus icigianus* strain G1w1 (KF631430) as shown in figure 7. The strain was in the same cluster of the phylogenetic tree (figure 7) with different strains of *Geobacillus* sp. The 16S rRNA partial sequence was submitted to Genbank and the accession number obtained was (MH734196.1).

The phylogenetic analysis of 16S rRNA gene sequences of the bacterial isolate BITSNS024 showed 99.64% similarity with *Anoxybacillus gonensis* strain G2 (CP012152) as shown in figure 8. The sequence was submitted to NCBI Genbank under the accession number (MH973207.1).

3.7 *GC content analysis*

The GC content plays a very important role in defining the thermophilic organisms that enhance their stability and helps in survival under harsh conditions. The GC content of the isolates from hot spring was calculated from the 16S rRNA gene sequence of BITSNS038 (1423bp) and BITSNS024 (1395bp) as 59.66% and 56.846% respectively. The study reports the first time the analysis of GC content from an isolate *Geobacillus icigianus* responsible for secreting three different enzymes.

4. Discussion

Till date, four closely related moderately thermophilic, gram-positive rods (JS1^T, JS5, JS11, and JS15) belonging to genus *Anoxybacillus* from sediment samples of this hot spring have been reported. The strain JS1^T was identified as a novel species of the genus *Anoxybacillus* by polyphasic approach for which *Anoxybacillus suryakundensis* sp. nov. was proposed (Deep *et al.* 2013). Poddar *et al.* (2014) also isolated a novel bacterium at 55°C from the selected location, strain JHK30^T which was identified as genus *Tepidiphilus* for which *Tepidiphilus thermophilus* sp. nov. was proposed. From both above references, it is established that isolation of microorganisms has been reported from hot spring Surajkund previously, but as far as enzyme synthesis is concerned no evidence is available so far from these isolated microorganisms.

This present investigation aims for isolation and characterization of thermophilic bacteria and their potential as enzyme secretion ability from hot spring Surajkund (figure 1). A total of 41 isolates was obtained through culture-dependent approach at a temperature range from 50°C to 70°C having diverse morphotypes which are strikingly

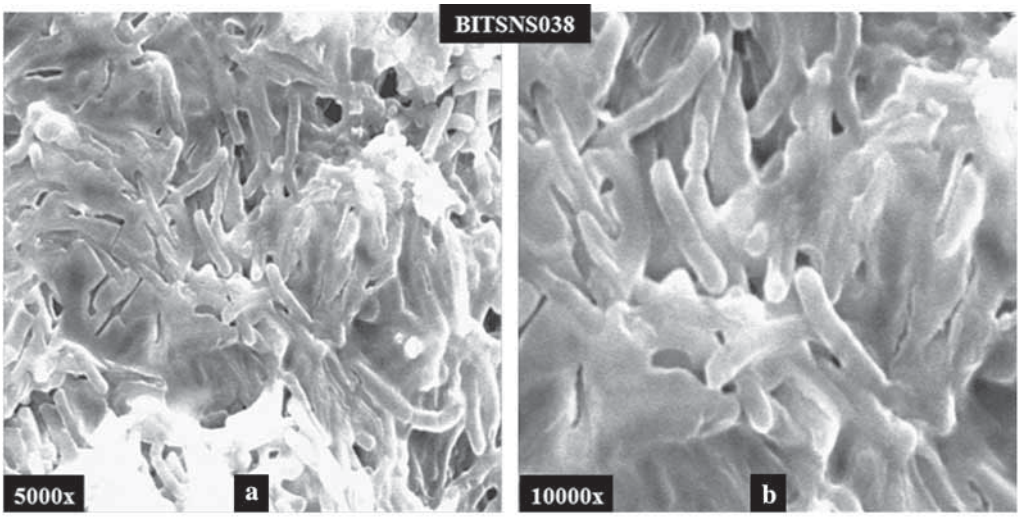

Figure 5. The SEM images of isolate *Geobacillus icigianus* at the magnification 5000X (**a**) and 10000X (**b**) respectively.

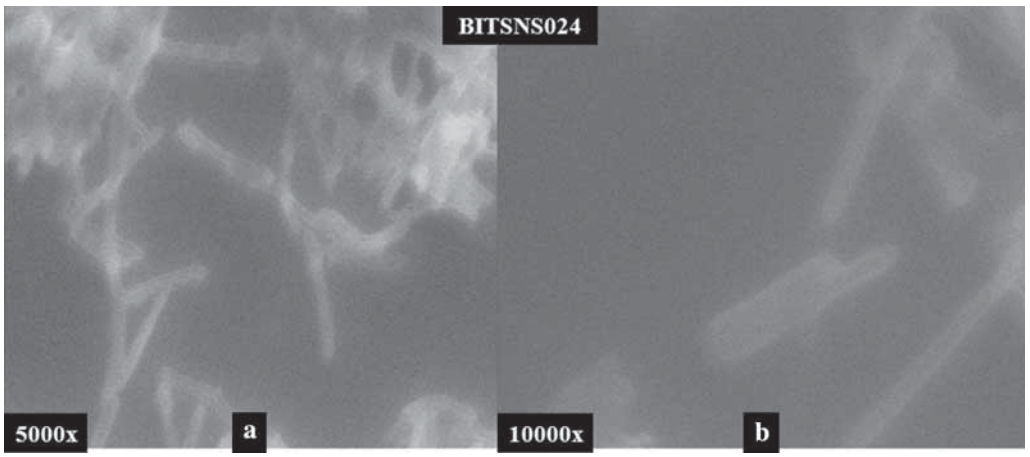

Figure 6. The SEM images of isolate *Anoxybacillus gonensis* at the magnification 5000X (**a**) and 10000X (**b**) respectively.

different from the previously reported studies on Surajkund microflora by Deep *et al.* (2013) and Poddar *et al.* (2014). The number of isolates achieved from mud sample was highest in number because of the abundance of C, H, S, macro- and micronutrients in comparison to water and soil samples supporting the growth of isolated bacteria even at a higher temperature (table 1). Hou *et al.* (2013) have also reported that sediment properties do play a role in microbial richness and diversity as an abundance of growth-supporting nutrients is maximum in sediment.

The physiochemical analysis of sample source revealed that the elemental chemistry of hot spring Surajkund is completely different compared to other hot springs as mentioned in table 2. Stout *et al.* (2009) reported 130.99 ppm of Ca, 21.15 ppm of Mg as well as the presence of other elements like Na, Si, B, K. Saxena *et al.* (2017) reported the high concentration of heavy metals and total dissolved solids in the range of 590–880 ppm. Similarly, in other studies as reported by Kumar *et al.* (2004); Hou *et al.* (2013); Kumar *et al.* (2014) also documented high concentration of other metals. As observed from table 2, the mud and soil samples of hot spring Surajkund did not contain heavy metals, silicon, boron and sodium elements.

A number of thermophilic bacteria with thermozyme producing ability have been investigated by several researchers worldwide. Mohammad BT *et al.* (2017) reported the presence of amylase, cellulase, protease and lipase secreting thermophilic bacteria at 55°C from the hot springs of Jordan. Cavello *et al.* (2018) isolated keratinolytic, cellulolytic, xylanolytic, and amylolytic bacteria at 50°C and 65°C from the hot springs of Argentina. Sudan *et al.* 2018 isolated amylase producing *Geobacillus* strain from Manikaran Hot Spring at 75°C. Similar kind of investigations was also carried out by other researchers where xylanase producing

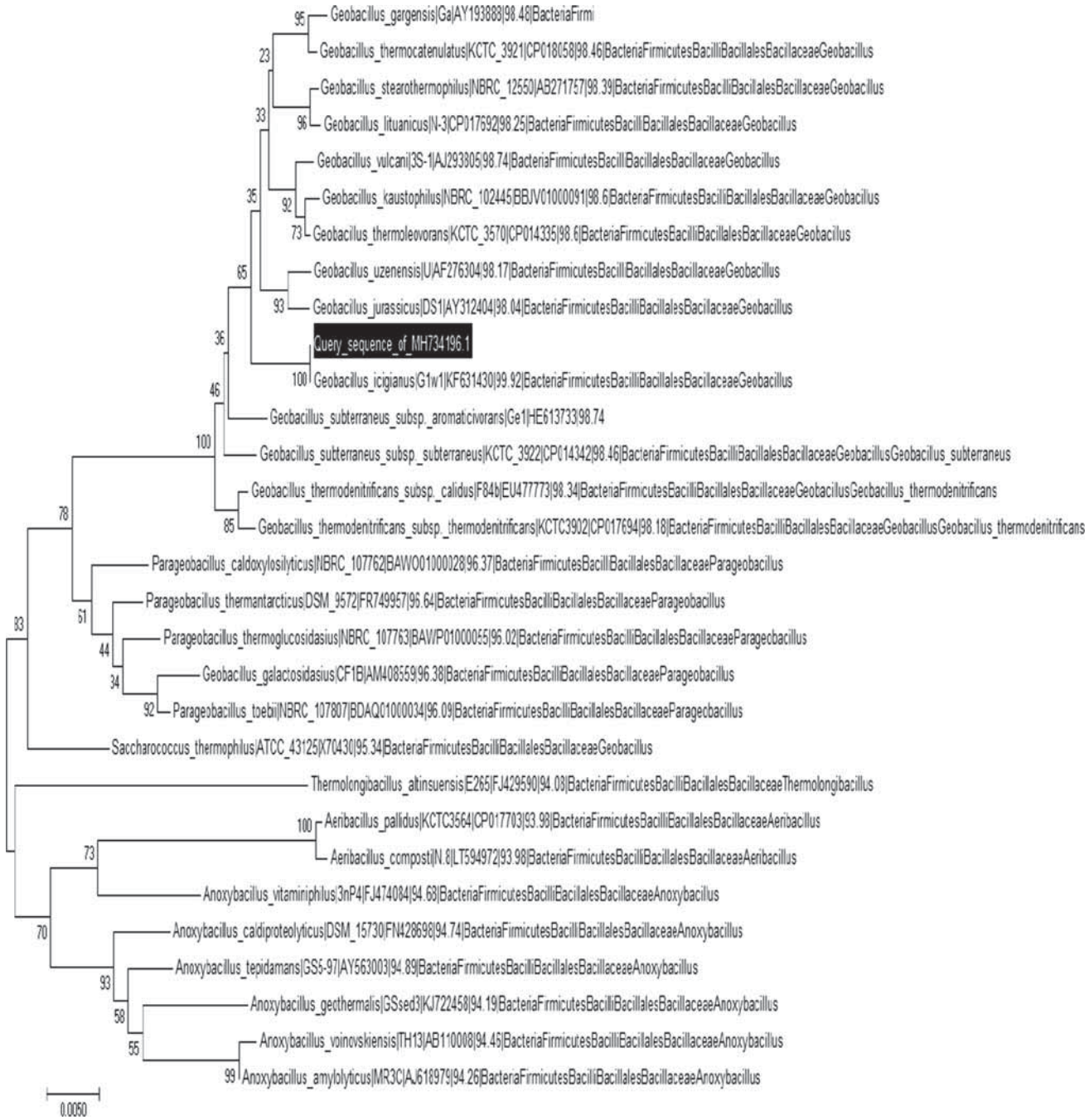

Figure 7. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequence of *Geobacillus icigianus* (MH734196.1). The scale bar indicates 0.005 substitutions per nucleotide position.

bacteria were isolated from different hot springs (Kaur *et al.* 2010; Bin *et al.* 2012; Kambourova 2018). When hot spring of Surajkund was explored from the thermozymes perspective, it was observed that the four thermophilic bacteria at 70°C showed potential thermozymes secreting ability (figure 2), particularly, the strain BITSNS038 with a considerable enzymatic index for amylase, xylanase and cellulase activity (table 3 and 4). This isolate belonged to the genus *Geobacillus icigianus* (figure 7) which is a significant finding of present study since; this strain has not been reported earlier from this site. The secondary screening result of *Geobacillus* also showed the crude activity of amylase, xylanase and cellulase (0.81 U/mL, 0.72 U/mL and 0.15 U/mL respectively) in liquid culturing conditions at 24 h of growth (figure 3). Cellulase activity was not significant as it was observed in plate assay. Amylase and xylanase activity with crude enzyme was also performed by *Geobacillus* sp. (Verma *et al*. 2018, Sudan *et al*. 2018, Sari *et al*. 2018, Jardine *et al*. 2018) with higher activity in optimized conditions. BITSNS024 emerged as highly amylolytic strain as revealed

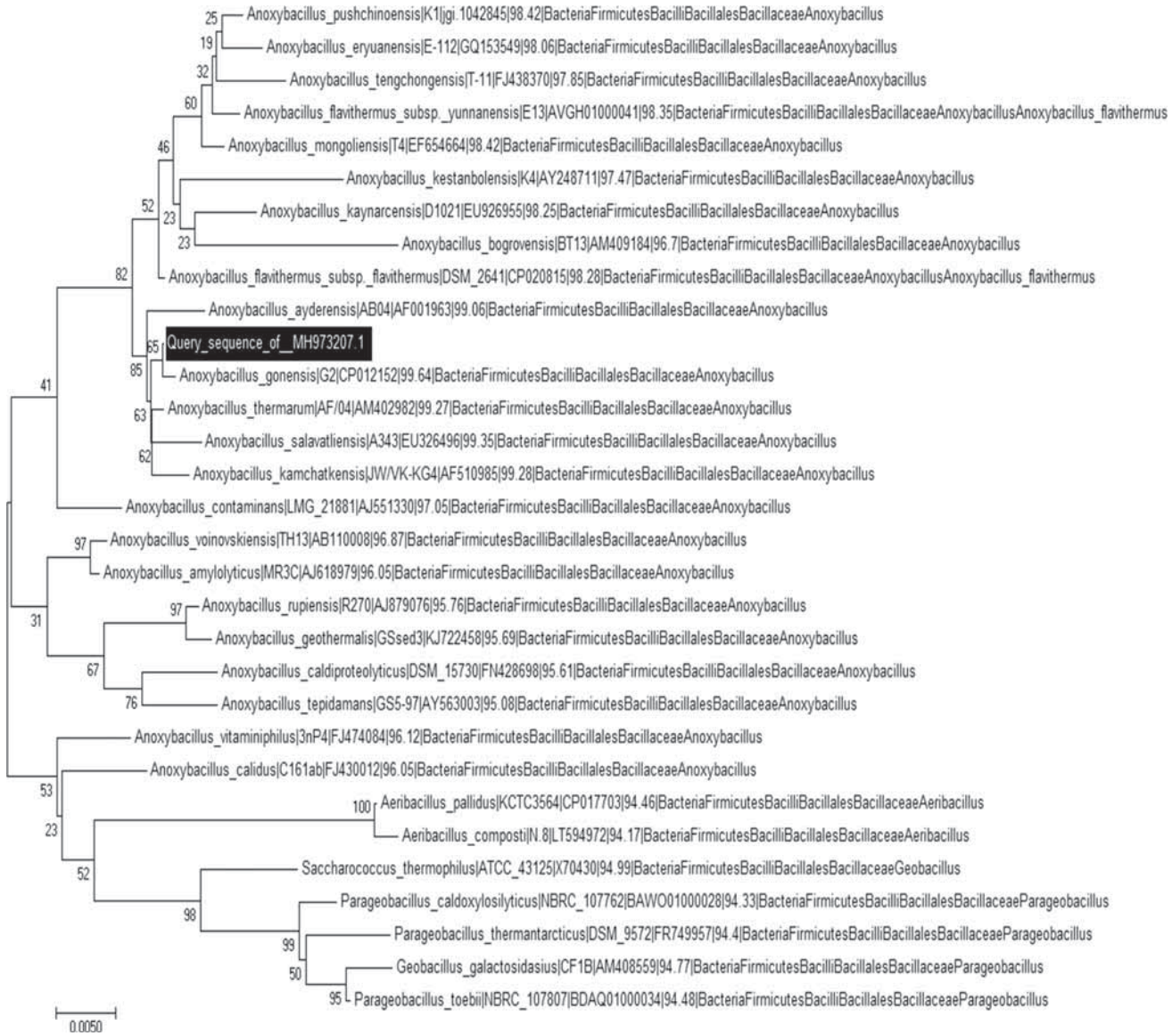

Figure 8. Phylogenetic tree of *Anoxybacillus gonenis* (MH973207.1). The evolutionary history was inferred using the neighbor-joining method. Bar indicates 0.005 substitutions per nucleotide position.

by plate assay (figure 2), with an EI value of 3.5, at 60°C higher than other isolates at 70°C and was identified as *Anoxybacillus gonensis* (figure 8). The presence of different species of *Geobacillus* and *Anoxybacillus* from the sediment and soil samples of other hot springs confirms the thermophilic nature of isolates (Derekova *et al*. 2007; Panda *et al*. 2016). The present study will provide a better understanding of different kinds of microbial abundance from the site responsible for the synthesis of other biological products.

5. Scope for future

Over the last few decades, thermophiles have attracted the attention of researchers in the search for thermostable enzymes and other bio-based products to be used in major sectors of the world economy, particularly considering the energy crisis issues, environmental concerns and alternative cost-effective technologies. Thermozymes finds extensive application in emerging sectors like biorefining, biofuels, food, agricultural, chemical, textile, pharmaceutical, and cosmetic industries. Amylases, cellulases and xylanase, in particular, are essentially required for the disintegration of lignocellulosic biomass. Considering the promising results obtained from the present investigation, the study will be performed for the pretreatment of various agricultural biomass to evaluate the release of sugars, lignin and xylose etc. Further studies needed to investigate the potential of the *Geobacillus icigianus* in submerged conditions via optimization of several bioprocess parameters for enhancement of enzyme production.

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