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Isolation and molecular identification of cellulolytic bacteria from Dig Rostam hot spring and study of their cellulase activity

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Abstract: Cellulose is the main structural component of lignocellulosic wastes that can be converted to sugars and biofuels by cellulase. Due to wide applications of this enzyme in various industries around the world, cellulase is considered as the third industrial enzyme. The ability of thermophilic bacteria in the production of heat-stable cellulases has made them valuable tools in biotechnology. The aim of this study was isolation and molecular identification of cellulolytic thermophile bacteria from Dig Rostam hot spring and investigating their cellulase activity. Samples were taken from water and sediments of this hot spring, and cellulolytic bacteria were enriched in media containing cellulose as the only carbon source. The bacteria were incubated at 60°C, and single colonies were then isolated on solid media. Congo red assay was used as a quick test for the qualitative screening of cellulase activity. According to these qualitative results, four colonies named CDB1, CDB2, CDB3, and CDB4 were isolated, and their growth curve and some other characteristics were determined by biochemical assays. Moreover, endoglucanase, exoglucanase, and FPase activities of the isolates were investigated quantitatively. Results indicated that CDB1 exhibited the highest endoglucanase (0.096 U/mL) and exoglucanase (0.156 U/mL) activities among other isolates. 16S rDNA partial sequencing indicated that CDB1 had 99% similarity to the genus *Anoxybacillus*, and the other isolates showed the highest similarity to the genus *Geobacillus*. The cellulase gene of CDB1 isolate with the highest cellulase activity was also cloned, and its sequence is reported for the first time. Further studies on this thermophilic enzyme might be useful for industrial applications.

Introduction

Cellulases as the third important group of enzymes are used in various industries and biotechnological applications such as pulp and paper, textile, animal feed, agriculture, fuel and organic chemical synthesis (Sukumaran *et al.*, 2005; Sreena and Sebastian, 2018; Imran *et al.*, 2018; Bhagia *et al.*, 2018). Cellulases are required for efficient bioconversion and saccharification of lignocellulose (Nigam, 2013). Lignocellulose is the most abundant, natural and renewable resource that can be converted to numerous products in bio-industry on a commercial scale (Demain *et al.*, 2005). It constitutes 60% of the woody plants and non-woody plants cell walls and consists of lignin, hemicellulose, and cellulose (Sajith *et al.*, 2016; Mmango-Kaseke *et al.*, 2016). For hydrolysis of cellulosic materials, three different types of cellulases synergistically work (Nigam, 2013), including endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.176; EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Hmad and Gargouri, 2017; Prasanna *et al.*, 2016). The endoglucanase randomly hydrolyzes α-1,4 bonds from free-reducing and non-reducing ends of the cellulose chain, and the exoglucanase usually liberates cellobiose units in alternative reactions. Finally, the cellobiose is converted to glucose by β-glucosidase activity (Kim and Ku, 2018; Imran *et al.*, 2018).

Several microorganisms including some species of bacteria (*Clostridium*, *Cellulomonas*, *Bacillus*, *Pseudomonas*, *Fibribacter*, *Ruminococcus*, *Butyrivibrio*, etc.), fungi (*Aspergillus*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Neurospora*, *Penicillium*, etc.,) and actinomycetes (*Thermomonospora*, *Thermoactinomyces*, etc.) (Sajith *et al.*, 2016; Sreena and Sebastian, 2018; Khatiwada *et al.*, 2016) produce cellulases during their growth on cellulosic materials. They utilize lignocellulosic biomass as a carbon and energy source for their growth (Sajith *et al.*, 2016; Davies and Henrissat, 1995; Juturu and Wu, 2014).

The cellulolytic potentials of thermophilic bacterial enzymes are more efficient compared with fungi cellulases (Liang *et al.*, 2010b) due to the following advantages: (1) these cellulases remain active at extreme conditions, such as higher temperatures and prolonged reactions (Irwin *et al.*,

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2003), (2) the probability of contamination can be decreased (Demain *et al.*, 2005), (3) bacteria have a shorter division time, and they are capable to grow in inexpensive carbon and nitrogen sources, (4) they can produce large amounts of enzymes, and (5) genetic manipulation of bacteria is more feasible (Li *et al.*, 2008; Sreena and Sebastian, 2018). In this regard, various thermophilic bacteria, including *Clostridium thermocellum* (Freier *et al.*, 1988), *Thermoanaerobacterium ethanolicus* (Wiegel and Ljungdahl, 1981), *Geobacillus stearothermophilus* (Nazina *et al.*, 2001), *Anoxybacillus sp. 527* (Liang *et al.*, 2010a), *Anoxybacillus pushchinoensis A8* (Kacagan *et al.*, 2008), *Bacillus licheniformis* (Balsam *et al.*, 2017), *Geobacillus* sp. HTA426 (Potprommanee *et al.*, 2017), etc., have been investigated (Liang *et al.*, 2010a). Recognizing thermostable enzymes of bacteria can be an important source for next-generation biofuel production especially from the natural environments (Liang *et al.*, 2010a; Wooa *et al.*, 2014; Imran *et al.*, 2018; Pachauri *et al.*, 2017) like compost pile, forestry or agricultural wastes, ruminants feces (Doi, 2008; Imran *et al.*, 2018; Khatiwada *et al.*, 2016), and hot springs (Kazue *et al.*, 2006; Doi, 2008; Potprommanee *et al.*, 2017; Balsam *et al.*, 2017).

Furthermore, use of specific bacteria like *Escherichia coli* provides more advantages for industrial applications. There are different strains of *E. coli* with specific mutations that can easily be manipulated for inducible expression of specific enzymes. *E. coli* cultivation does not require high temperatures, it has a shorter lag phase, and more importantly, purification of thermophilic enzymes from *E. coli* is less tedious (Jia *et al.*, 2016; Pandey *et al.*, 2014).

In this study, four thermophilic and cellulolytic bacteria were isolated and identified from Dig Rostam hot spring, a rich source of thermophilic microorganisms, situated in the southeast of Iran. The cellulase activities of these isolates were investigated and additionally, the cellulase gene of the most active strain was cloned and sequenced.

Materials and Methods

Isolation and screening of thermostable cellulase producing bacteria

Water and sediments were collected from Dig Rostam hot spring, Kerman, Iran (N32° 16' 40", E57° 30' 33.4"), with temperature ranges between 52-73°C and pH 6-7 from 10-20 cm depths. Cellulolytic bacteria were enriched in a modified BM7 medium, containing 1.5 g/L KH_2PO_4, 2.9 g/L K_2HPO_4, 2.1 g/L urea, 6.0 g/L yeast extract, 0.5 g/L cysteine hydrochloride, 0.5 mg/L $MgCl_2.6H_2O$ and 0.0075 mg/L $CaCl_2.2H_2O$, pH 7.0 (Tachaapaikoon *et al.*, 2012). All reagents were purchased from Merck, Darmstadt, Hesse, Germany. The medium was supplemented with 1% microcrystalline cellulose powder (Sigma-Aldrich, Munich, Germany). The bacteria were incubated at 60°C and sub-cultured in this medium three times to test their ability for utilizing microcrystalline cellulose as the sole carbon source. Single colonies were then inoculated into another medium containing 1.36 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 2 g/L $MnO_4.7H_2O$, 10 mg/L Fe_2SO_4, 2 g/L NaCl, 1 g/L yeast extract, and 0.3% microcrystalline cellulose powder (Acharya and Chaudhary, 2012). Primary identification of colonies was carried out by morphological characteristics, Gram staining, spore formation, and some biochemical assays such as catalase, malonate, KIA (Kliger's Iron Agar), urea, motility and esculin tests (Bittona and Dutka, 1983). Growth curves of the isolates were also plotted during 8-11 days of culture. Purified colonies were sub-cultured on solid media containing 1% carboxymethyl cellulose (CMC) at 60°C for 48 h, and the Congo red assay was used as a qualitative method for screening CMCase activity (Teather and Wood, 1982; Pachauri *et al.*, 2018; Kim and Ku, 2018), and the diameters of clear zones were measured. *Bacillus subtilis* (PTCC 1720) was used as a control in these experiments.

Enzymatic activity assays

Enzyme activity was determined with Filter Paper Assay (FPA) method (Ghose, 1987; Pachauri *et al.*, 2017; Khatiwada *et al.*, 2016) using a specific substrate for each enzyme. Purified colonies were cultured in 200 mL BM7 broth media and incubated at 60°C and cellulase activity was measured every 48 h. Carboxymethyl cellulase, Avicelase, and Filter-paperase activities were determined by measuring the amount of reducing sugar liberated from CMC, Avicel, and Filter paper, respectively, using 3,5-dinitro-salicylic acid (DNS) method (Miller, 1959). The reactions were prepared by mixing 0.5 mL of media containing enzymes with 0.5 mg of each substrate dissolved in 1000 μL 0.05 M citrate buffer (pH 7.0). The mixtures were incubated at 50°C for 60 min. In order to stop the reactions, 3 mL DNS was added. The treated samples were boiled for 10 min, cooled in water for color stabilization, and the optical densities were measured at 540 nm (Unico, Wilnsdorf, Germany) (Potprommanee *et al.*, 2017).

One unit (U) of the enzymatic activity was defined as the amount of enzyme that could hydrolyze the substrate and release 1 μmol glucose within 1 min of reaction (Li *et al.*, 2008). The standard curve of glucose reducing (Khatiwada *et al.*, 2016) was also plotted.

DNA extraction and 16S rDNA amplification

For identification of the four isolates, genomic DNA extraction was performed by the boiling method (Pui *et al.*, 2011; Fatokun *et al.*, 2016). 16S rDNA gene was then amplified by polymerase chain reaction (PCR) using the universal primers 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-GGT TAC CTT GTT GTT ACG ACT T-3' (Li *et al.*, 2008; Liang *et al.*, 2010b). PCR amplification was carried out according to the following protocol: initial denaturation for 5 min at 94°C followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 60.5°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The PCR products were visualized on a 1% agarose gel, sequenced (Macrogen, Seoul, South Korea), and analyzed by NCBI BLASTN. A phylogenetic tree was constructed with Mega4 software based on the 16S rDNA sequences of the strains closer to the isolates.

Cloning of cellulase gene from CDB1

After identification of the most active isolate as *Anoxybacillus* sp., the sequence related to the cellulase gene from *Anoxybacillus flavithermus* was used for designing specific primers (CelF: 5'-ATG GAT TTG CAG TTG TTT C-3' and

CelR: 5'-TTA AGC GTT ATG ACG AAT-3'). The cellulase gene was then cloned in pTZ57R/T vector (Promega, Madison, WI, USA). The resulting plasmid was verified by digestion with *Eco*RI and *Bam*HI enzymes, and the target gene was analyzed by sequencing.

Statistical analysis
All measurements were performed in triplicate. Statistical analyses were carried out using one way and two-way ANOVA in GraphPad Prism (Inc, San Diego, CA, USA) (version 8.0.1) software. Differences at $p < 0.05$ were considered statistically significant.

Results

Isolation and identification of cellulolytic strains
Purified colonies were isolated after enrichment of the samples in broth media containing cellulose as the only carbon source. For verifying the endoglucanase activity of the bacteria, Congo red staining was used. A clear zone around some colonies was formed as a result of CMC decomposition by bacterial enzymes. CDB1 (cellulose degrading bacteria), CDB2, CDB3 and CDB4 isolates were selected with clear zones of 11, 6, 7 and 9 mm, respectively (Fig. 1). The diameter of clear zone for *Bacillus subtilis* was 6 mm. The isolated strains were Gram-positive, rod shaped and were able to produce spores. More details about the properties of these isolates are shown in Tab. 1.

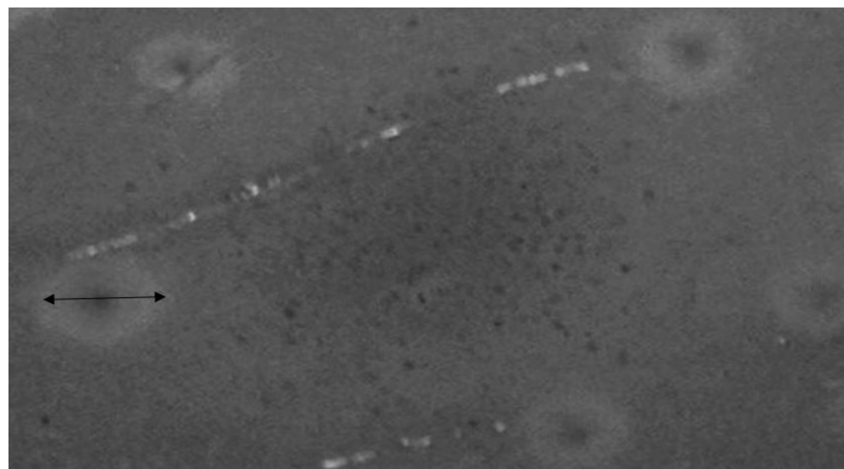

FIGURE 1. Screening for endoglucanase producing bacteria using the Congo red assay, after colony selection and culturing for 48 h, the clear haloes appeared around the cellulolytic colonies.

TABLE 1

Morphological and biochemical characteristics of isolated strains

Characteristic	CDB1	CDB2	CDB3	CDB4
Gram staining	+	+	+	+
Catalase	+	-	+	+
Citrate	-	-	-	-
Malonate	-	+	-	-
KIA	-	+	+	+
Urea	-	-	-	-
Motility	+	-	-	-
Esculin	-	-	-	-
CMC	+	+	+	+
Cellulose	+	+	+	+
Spore formation	+	+	+	+
Growth condition	aerobic	aerobic	aerobic	aerobic
Cell shape	rod	rod	rod	rod

CDB, cellulose degrading bacteria; CMC, carboxymethyl cellulose; KIA, Kliger's Iron Agar.

Enzyme assays

Tab. 2 shows maximum endoglucanase, exoglucanase and FPase activities of the four isolates as measured according to the FPA method. All isolates were able to produce cellulolytic enzymes in varying degrees during the ten days of investigation (Fig. 2).

TABLE 2

Maximum enzymatic activity of four isolates was measured by FPA method

Enzyme activity (U/mL) / Isolates	Maximum endoglucanase activity	Maximum exoglucanase activity	Maximum FPase activity
CDB1	0.096 ± 0.0016	0.158 ± 0.0044	0.085 ± 0.0012
CDB2	0.078 ± 0.0004	0.148 ± 0.0024	0.081 ± 0.0008
CDB3	0.080 ± 0.0005	0.113 ± 0.0047	0.087 ± 0.0008
CDB4	0.084 ± 0.0008	0.118 ± 0.0050	0.084 ± 0.0008
B. subtilis	0.081 ± 0.0005	0.133 ± 0.0064	0.081 ± 0.0011

Values are means of triplicate ± standard deviation.

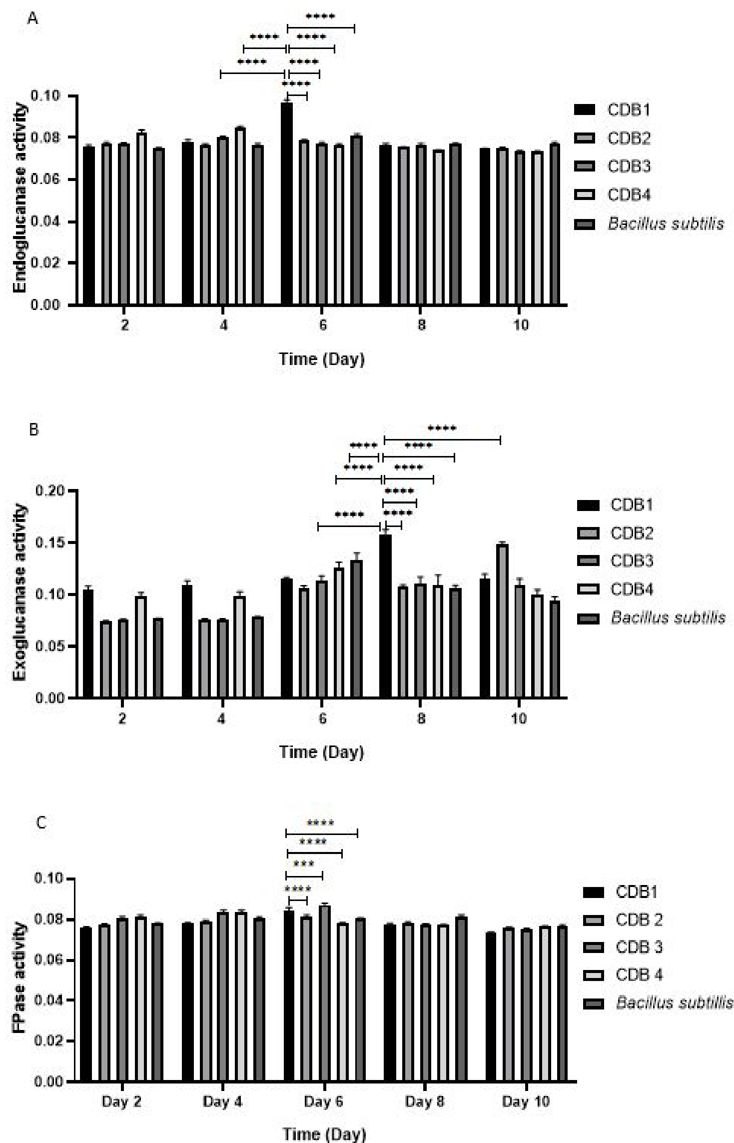

FIGURE 2. (A) Endoglucanase, **(B)** exoglucanase, and **(C)** FPase activities of the four isolates by the FPA method during the ten days of investigation. Values are mean ± SD from three independent experiments. Statistical analysis indicated a significant difference between the maximum cellulolytic activity of CDB1 and other three isolates and control (****p < 0.0001 and ***p = 0.0005).

CDB1 showed maximum endoglucanase and exoglucanase activities on days 6 and 8, respectively, which were significantly higher compared to the other three isolates and *B. subtilis* in similar time points. Similarly, maximum endoglucanase and exoglucanase activities of CDB1 were significantly different in comparison to maximum activities of the three isolates and the control ($p < 0.0001$) (Figs. 2(A) and 2(B)).

Furthermore, the maximum FPase activity of CDB1 was significantly different compared to CDB2, CDB4, and *B. subtilis* in sixth day ($p < 0.0001$), however CDB3 showed more FPase activity compared to CDB1 in the same duration ($p = 0.0005$) (Fig. 2(C)). Therefore, more investigations on CDB1 isolate might be advantageous.

Bioinformatics and phylogenetic analysis of 16S rDNA sequencing

Genomic DNA was successfully extracted from the four isolates, and PCR was carried out to amplify a 1500 bp fragment of the 16S rDNA gene (Fig. 3). PCR products were sequenced, and similarity search using Basic Local Alignment Search Tool revealed that CDB1 had maximum homology with *Anoxybacillus* while the other three isolates were more similar to genus *Geobacillus*. 16S rDNA partial sequences related to CDB1, CDB2, CDB3, and CDB4 isolates were submitted to GenBank with accession numbers KC914388, KC914389, KC914390, and KF990497, respectively. The phylogenetic tree of four isolates and related species is depicted in Fig. 4.

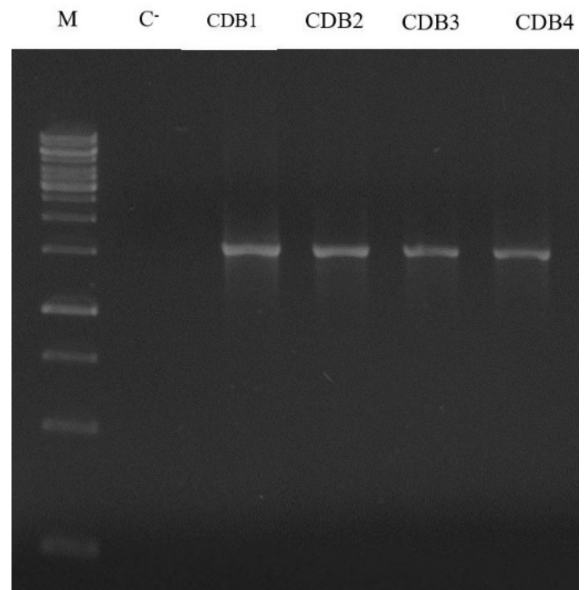

FIGURE 3. Amplification products of 16S rDNA gene from four isolates are verified by gel electrophoresis. M: 1 kb DNA ladder, C⁻: negative control, PCR products related to CDB1, CDB2, CDB3, and CDB4.

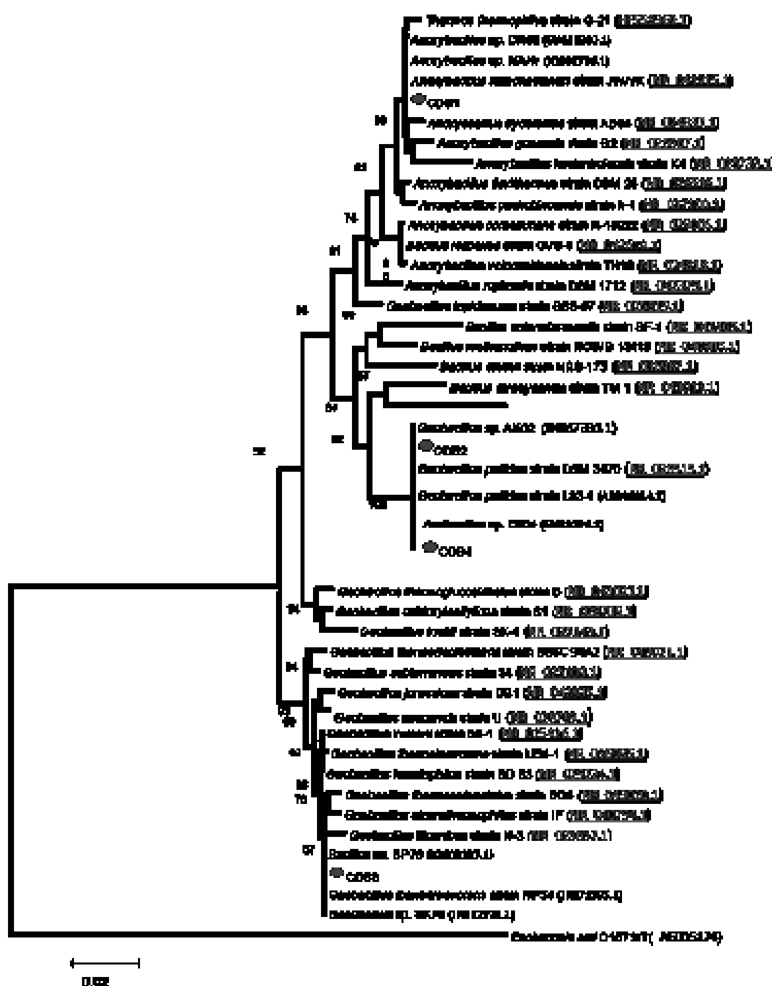

FIGURE 4. Phylogenetic tree based on 16S rDNA sequences of the four isolates, the tree was constructed using Mega4 software, CDB1 most closely related to genus *Anoxybacillus*, whereas CDB2, CDB3, and CDB4 were most closely related to genus *Geobacillus*. *Escherichia coli* (O157-H7) was selected as out-group to root the tree.

Cloning of the cellulase gene and its sequence analysis
The putative cellulase gene was amplified by CelF and CelR primers from CDB1 genomic DNA. For further characterization of the cellulase gene, the sequence was cloned into pTZ57R/T vector. The sequencing results indicated that the cloned sequence contained an open reading frame (ORF) that started with an ATG start codon and terminated with a TAA stop codon. This ORF consists of 1068 bp and is submitted to GenBank under the accession number of KM555226. The comparison of this sequence with cellulase from *A. flavithermus* showed 88% similarity.

Discussion

In recent years cellulase enzymes have had remarkable applications in different industries (Rawat and Tewari, 2012; Bhagia *et al*., 2018; Hmad *et al*., 2017). Bacteria present an attractive potential for cellulase production due to their rapid growth rate, extreme habitat diversity, and robust enzymatic systems (Maki *et al*., 2009; Sreena and Sebastian, 2018). Among bacteria, thermophilic species are more useful in biotechnological and industrial applications.

Thermophilic bacteria have been isolated from various sources including soil of gold mine (Rastogi *et al*., 2009), soil of forests and agriculture regions (Hatami *et al*., 2008; Khatiwada *et al*., 2016), hog wastes (Liang *et al*., 2010b), marine (Hebbale *et al*., 2019) and hot springs (Li *et al*., 2008; Acharya and Chaudhary, 2012; Potprommanee *et al*., 2017). In this study, we screened and identified cellulose-degrading bacteria from Dig Rostam hot spring for the first time.

Enrichment for the screening of cellulose-degrading bacteria can be performed in media containing microcrystalline cellulose as the sole carbon source and can be pursued by 16S rDNA analysis for identification of isolates (Rastogi *et al*., 2009; Tachaapaikoon *et al*., 2012; Potprommanee *et al*., 2017). So, the water and sediment samples were enriched in BM7 media containing cellulose, followed by determination of cellulase activity and identification of the isolates using biochemical and molecular techniques.

Congo red, hexadecyltrimethylammonium bromide, and Gram's iodine are usually used for qualitative screening of bacteria with endoglucanase activity (Hankin and Anagnostakis, 1977; Kasana *et al*., 2008; Gupta *et al*., 2012; Pachauri *et al*., 2018; Kim and Ku, 2018, Hebbale *et al*., 2019; Rahikainen *et al*., 2019). For example, Gohel *et al.* (2014) screened five cellulose-degrading colonies from garden soil. Staining with Congo red showed that the diameter range of the five isolates was 8.5-17 mm (Gohel *et al*., 2014). Similarly, in 2018, the cellulase gene from *Bacillus licheniformis* ATCC 14580 was cloned, over-expressed, and surface displayed in recombinant *E. coli* using an ice-nucleation protein (INP). The hallow zone diameters using Congo red staining of wild type *B. licheniformis*, *E. coli*, and INP-cellulase recombinant cells were 0.5, 0, and 22 mm, respectively (Kim and Ku, 2018). The maximum diameter of the clear zone in this study was 11 mm in CDB1 as a thermophilic bacterium. However, the utilization of dyes for quantification of cellulase activity is not very accurate due to the weak correlation between enzyme activity and clear zones (Fia *et al*., 2005; Potprommanee *et al*., 2017). As a result, FPA using DNS reagent is more reliable for this purpose.

Determination of cellulase activity revealed that CDB1, which was identified as *Anoxybacillus* sp., had the highest endoglucanase activity (0.096 U/mL) on the sixth day and in the middle of its stationary phase (data not shown). CDB2, CDB3, and CDB4, which were identified as species of *Geobacillus* genus, also showed their maximum endoglucanase activity in the middle of the stationary phase. The results of the quantitative endoglucanase activity confirmed the results of the Congo red assay. The maximum exoglucanase activity of CDB1 reached 0.158 U/mL on the eighth day and at the end of the stationary phase, but it had approximately the same FPase activity in comparison with other isolates. It should be noted that the decomposition of filter paper as a hard substrate requires a synergy between the enzymes. In 2010, Liang *et al.* (2010b) evaluated the cellulase activity of thermophilic bacteria isolated from pig wastes by the FPA method and using crude enzyme extract. Measuring cellulase activity of their isolate *Anoxybacillus* sp. 527 showed that the maximum CMCase and cellulase activities were 0.04 and 0.02 U/mL, respectively (Liang *et al*., 2010b). So, the CMCase and cellulase activities of CDB1 are 2.4 and 4.25 times higher than related enzymes in *Anoxybacillus* sp. 527.

The maximum CMCase and FPase activities of CDB2, CDB3, and CDB4 were approximately 1.4 and 2 times more than DUSELR7 as a *Geobacillus* sp. isolated from the soil of a gold mine (CMCase 0.058 U/mL and FPase 0.043 U/mL) (Rastogi *et al*., 2009). The CMCase activity of these three isolates is approximately 7 times higher than a *Geobacillus* species grown at 70°C (0.0113 U/mL) by Tai and colleagues (Tai *et al*., 2004). In 2016, Parveen *et al.* isolated cellulase-producing thermophilic bacteria from a hot spring. The isolates, including *Stenotrophomonas maltophilia*, showed the highest cellulase activity (0.43 U/mL) followed by *Bacillus cereus* (0.39 U/mL) and *Bacillus thuringiensis* (0.3 U/mL) (Parveen *et al*., 2016). In order to increase enzymatic activity, different factors can be manipulated such as purification and concentration of the enzyme, optimized culture conditions such as optimum temperature and pH, and also medium composition (Khatiwada *et al*., 2016). For example, the optimization of culture conditions and adding yeast extract and ammonium sulfate led to a two-fold increase in cellulase production in *Geobacillus* sp. (Tai *et al*., 2004). Cellulolytic activity of the four isolates obtained in this study is considered relatively high, and it can still be more improved by manipulation of the mentioned factors. Furthermore, it is important to note that the application of thermophilic enzymes is more desirable than mesophilic types. These thermostable cellulases may simplify the improvement of more efficient and cost-effective forms of saccharification and fermentation processes to convert lignocellulosic biomass into biofuels.

The results of the 16S rDNA sequencing analysis of the isolates showed that CDB1 is 99% similar to genus *Anoxybacillus*. Further studies showed that the members of this genus have similar morphology, are usually motile, and have spherical and oval endospores. The colony shape is different in the 11 known species of this genus, and in *A. kamchatkensis*, it is smooth circular with creamy color (Vos *et al*., 2009). Comparison of the nucleotide sequences of the resulting amplicons from CDB2, CDB3, and CDB4 with sequences in the NCBI database revealed that these isolates

probably belong to genus *Geobacillus*. Members of this genus are rod-shaped, and their cell wall is similar to Gram-positive bacteria, but the Gram staining leads to various results among these species. Members of this genus have spherical endospores, and the shapes of colonies are variable. The optimum temperature for their growth is 37-75°C. Most members of this genus produce catalase but are not able to produce indole (Vos *et al.*, 2009). In general, these properties besides sequencing indicate that these three isolates belong to genus *Geobacillus*.

For more studies on CDB1, as the most active isolate, specific primers were designed to amplify its cellulase gene. Cloning and sequencing of this gene indicated that it was similar to part of the genome reported for *Anoxybacillus kamchatkensis*. Therefore, further characterization of this thermostable enzyme can be performed by the expression of the cellulase gene and measuring its activity in *E. coli* in future studies. In summary, the bacteria isolated in this study are thermophilic and have remarkable cellulolytic activity in comparison with other *Anoxybacillus* and *Geobacillus* species. Isolating more thermophilic species from Dig Rostam hot spring would help to obtain important sources of thermostable enzymes for further biotechnological and industrial applications.

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Conflict of Interest

The authors declare that they have no competing interests.

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