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

SAREH HAJIABADI1; MANSOUR MASHREGHI1; AHMAD REZA BAHRAMI1,*; KIARASH GHAZVINI2; MARYAM M. MATIN1,2,*

1 Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
2 Novel Diagnostics and Therapeutics Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran
3 Microbiology Research Center & Department of Microbiology and Virology, Ghaem Medical Center, Mashhad University of Medical Sciences, Mashhad, Iran

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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 cellulose 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 cellulose 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.,

*Address correspondence to: Maryam M. Matin, matin@um.ac.ir

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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 therophilic 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., 2017), 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 cellulose, 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, Wilsdorf, 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.

Materials and Methods

Isolation and screening of thermotolerant 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 KH2PO4, 2.9 g/L KH2PO4, 2.1 g/L urea, 6.0 g/L yeast extract, 0.5 g/L cysteine hydrochloride, 0.5 mg/L MgCl2.6H2O and 0.0075 mg/L CaCl2.2H2O, 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 KH2PO4, 1 g/L (NH4)2SO4, 2 g/L MnO4-7H2O, 10 mg/L Fe2SO4, 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.

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., 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., 2016). 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 65.5°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**

| 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).

| 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).](image-url)
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.](image)

![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.](image)
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 KM555226. The comparison of this sequence with cellulase from *A. flavitherm us* showed 88% similarity.

Discussion

In recent years cellulase enzymes have had remarkable shows 88%

1. **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). Among bacteria, thermophilic species are more useful in biotechnological and industrial applications.

2. 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.

3. 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.

4. *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, Gehel 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 (Gehel 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 therophilic 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.

References

Acharya S, Chaudhary A (2012). Alkaline cellulase produced by a newly isolated thermophilic Aneurinibacillus thermoacervoribacillus WBS2 from hot spring. African Journal of Microbiology Research 6: 5453-5458.

Balsam TM, Daghistani HI, Jauani A, Abdellatif S, Kennes C (2017). Isolation and characterization of thermophilic bacteria from Jordanian hot Springs: Bacillus licheniformis and Thermomonas hydrothermalis isolates as potential producers of thermostable enzymes. International Journal of Microbiology 2017: 6943952.

Bhagia S, Dhiri R, Kumar R, Wyman CE (2018) Deactivation of cellulase at the air-liquid interface is the main cause of incomplete cellulose conversion at low enzyme loadings. Scientific Reports 8: 1350-1362.

Bittona G, Dutka BJ (1983). Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment: a review. Critical Reviews in Environmental Control 13: 51-67.

Davies G, Henriottat B (1995). Structures and mechanisms of glycosyl hydrolases. Structure 3: 853-859.

Demain, AL, Newcomb M, Wu JH (2005). Cellulase, clostridia, and ethanol. Microbiology and Molecular Biology Reviews 69: 124-154.

Doi R (2008). Cellulases of mesophilic microorganisms: cellulolase and nocardiozyme producers. Annals of the New York Academy of Sciences 1125: 267-279.

Fatokun EN, Nwodo U, Okoh AI (2016). Classical optimization of cellulase and xylanase production by a marine Streptomyces species. Applied Sciences 6: 286-300.

Fia G, Giovani G, Rosi I (2005). Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. Journal of Applied Microbiology 99: 509-517.

Freier D, Mothershed C, Wiegel J (1988). Characterization of Clostridium thermocellum Jw20. Applied and Environmental Microbiology 54: 204-211.

Ghose TK (1987) Measurement of cellulase activities. Pure and Applied Chemistry 59: 257-268.

Gohel HR, Contractor CN, Ghosh SK, Braganza VJ (2014). Comparative study of various staining techniques for determination of extracellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. International Journal of Current Microbiology and Applied Sciences 3: 261-266.

Gupta P, Samant K, Sahu A (2012). Isolation of cellulase-degrading bacteria and determination of their cellulosytic potential. International Journal of Medical Microbiology 2012: 578925.

Hankin L, Anagnostakis SL (1977). Solid media containing Carboxymethylcellulose to detect Cx cellulase activity of microorganisms. Journal of General Microbiology 98: 109-115.

Hatami S, Alikhani HA, Besharati H, Salehrastin N, Afrousseh M, Yazdani Jahromi Z (2008). Investigation on aerobic cellulolytic bacteria in some of north forest and farming soils. American-Eurasian Journal of Agricultural & Environmental Sciences 3: 713-716.

Hebbale D, Bhargavi R, Ramachandra TV (2019). Saccharification of macroalgal polysaccharides through prioritized cellulase producing bacteria. Heliyon 5: e01372.

Hmad IB, Gargouri A (2017). Neutral and alkaline cellulases: production, engineering, and applications. Journal of Basic Microbiology 57: 653-658.

Imran M, Anwar Z, Irshad M, Javaid Asad M, Ashfaq H (2018). Cellulase production from species of fungi and bacteria from agricultural wastes and its utilization in industry: a review. Advances in Enzyme Research 4: 44-55.

Irwin D, Leathers TD, Greene RV, Wilson DB (2003). Corn fiber hydrolysis by Thermobifida fusca extracellular enzymes. Applied Microbiology and Biotechnology 61: 352-358.

Jia X, Chen J, Lin C, Lin X (2016). Cloning, expression, and characterization of a novel therophilic monofunctional catalase from Geobacillus sp. CHB1. BioMed Research International 2016: 7535604.

Juturu V, Wu JC (2014). Microbial cellulases: engineering, production and applications. Renewable and Sustainable Energy Reviews 33: 188-203.

Kacagan M, Canakci S, Sandalli C, Colak D, Belduz A (2008). Characterization of xylanase from a thermophilic strain of Anoxybacillus pushchinensis A8. Biologia 63: 599-606.

Kasana R, Salwan R, Dhar H, Dutt S, Gulati A (2008). A rapid and
nov. spec. nov., a new, extreme thermophilic, anaerobic bacterium. *Archives of Microbiology* **128**: 343-348.

Wooa HL, Hazena TC, Simmons LA, DeAngelis KM (2014). Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils. *Systematic and Applied Microbiology* **37**: 60-67.