Screening of Bacteria Producing Acid-stable and Thermostable Endo-1, 4-β-Glucanase from Hot Springs in the North and Northwest of Iran

Hassan Diba¹, Jafar Hemmat*¹, Mohsen Vaez¹, Mohammad Ali Amoozegar²

¹ Biotechnology Department, Iranian Research Organization for Science and Technology (IROST), P.O. Box 33535111, Tehran, Iran.
² Extremophiles Laboratory, Department of Microbiology, Faculty of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran, Iran.

Abstract

Endo-1,4-β-glucanase is one of three cellulolytic enzymes in a triplet catalytic system that are required for extracellular cellulose hydrolysis. Thermostable endo-1,4-β-glucanase is important and is included in a considerable portion of industrial enzymes. Hot springs are extreme niches which are important sources of the bacteria producing thermostable enzymes. Accordingly in this study, sampling was performed from four hot springs in the north and northwest of Iran and the screening and identification of acid-stable and thermostable of endo1,4-β-glucanase producing bacteria was investigated. Endo1,4-β-glucanase activity of these isolated strains were determined by qualitative Congo-Red staining as well as quantitative Carboxymethyl cellulose/Dinitrosalicylic acid methods as indicators of cellulase production. Three isolates out of twelve initially selected bacteria showed noticeable endo 1,4-β-glucanase activity, including Paenibacillus sp. ASH4, Bacillus sp. AGH1 and Bacillus sp. AG2 with 90%, 77% and 45% residual activity at pH 4, 60°C after three hours. Molecular identification of the bacteria was carried out using 16S rDNA partial sequencing, in which two isolates were shown to belong to Bacillus sp. and one to Paenibacillus sp.. The results showed that the isolated acidothermotolerant Bacillus spp. and Paenibacillus sp. had the capability to produce suitable acid-stable and thermostable endoglucanase. These isolates may also be considered as candidates for the production of other thermostable metabolites with additional biotechnological applications.

Keywords:
Acidostability
Cellulase
Hot spring
Thermostability

1. Introduction

Currently most cellulase enzymes are derived from fungi due to their high activity, but some factors have shown that bacteria have the potential for a considerable higher growth rate than fungi leading to more enzyme production. Enzymes derived from bacteria have a higher thermal resistance than fungi and are more convenient for genetic manipulations (Guar & Tiwari, 2015). Moreover, valuable information and access to new gene resources can be obtained in the fields of protein and gene engineering by studying the stability pattern of extremozymes from thermophilic or thermo-
tolerant bacteria (Dumorne et al., 2017). Some of these bacteria are Bacillus spp., Cellulomonas spp., Rumminococcus spp., Alteromonas spp. and Activibrio spp. (Guar & Tiwari, 2015). Cellulases are amongst the most prevalent and indispensable enzymes for industrial applications. These enzymes are applicable in bioconversion industries such as cellulose fermentation to ethanol and other organic acids (Bhardwaj et al., 2017; Kuhad et al., 2011), food industries including refining food and vegetable juices, oil extraction from seeds and fruits, nectar production, fruit nutrition, improving malt production from barley, and carotenoid extraction for pigment production (Singh et al., 2016; Sukumaran et al., 2005). Another areas of great interest is industries engaged in renewable energy sources using agricultural residues. To be able to use these substrates, their lignocellulosic chains must be broken by pretreatment into three major compounds of cellulose, hemicellulose and lignin (Prasetyo et al., 2010) to provide substrates for other enzymes.

Hydrolysis of cellulose to a fermentable substrate requires a triplet catalytic system of extracellular enzymes including endo-1, 4-β-glucanase (EC 3.2.1.4), exo-1, 4-β-glucanase (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Among cellulosic enzymes, endo-1, 4-β-glucanase, which randomly attacks the internal parts of the cellulosic chain and produces new oligosaccharide chains with different lengths, is known as a viscosity reducing agent (Lentzen & Schwarz, 2006).

Temperature has a significant effect on viscosity reduction and increasing the rate of the reaction. On the other hand, cellulase enzymes are generally more stable at pH 4 to 6, so many industrial processes are carried out at high temperatures and preferably at an acidic pH for this group of enzymes (Irfan et al., 2017). Such enzymes (extremozymes) from extremophile microorganisms are among the metabolites currently of great interest to researchers for use in novel applications in biotechnology. Accordingly, in this research, isolation, screening and molecular identification of the bacteria with capability to produce acid-stable and thermostable endo-1,4-β-glucanase, from some hot springs in the north and northwest of Iran were investigated.

2. Materials and methods

2.1. Sampling

Samplings were done from four hot springs, including Ghahvesouee, Ghotoorsouee, Shabil, and Ramsar, to isolate bacteria. The details of the sampling points are given in Table 1.

The microbial cultures for isolation and purification of bacteria were done in a modified Mandels medium (Mandels & Weber, 1969). Growth conditions were at the same temperature

<table>
<thead>
<tr>
<th>Region</th>
<th>Province</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
<th>Elevation (m)</th>
<th>pH</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghahvesouee</td>
<td>Ardabil</td>
<td>38°9&quot;12.75&quot;</td>
<td>48°04&quot;17.67&quot;</td>
<td>1690</td>
<td>6</td>
<td>48</td>
</tr>
<tr>
<td>Ghotoorsouee</td>
<td>Ardabil</td>
<td>38°20&quot;13.00&quot;</td>
<td>47°51&quot;35.00&quot;</td>
<td>2571</td>
<td>3</td>
<td>42°</td>
</tr>
<tr>
<td>Shabil</td>
<td>Ardabil</td>
<td>38°19&quot;48.37&quot;</td>
<td>47°50&quot;37.09&quot;</td>
<td>2667</td>
<td>6</td>
<td>48</td>
</tr>
<tr>
<td>Ramsar</td>
<td>Mazandaran</td>
<td>36°54&quot;9.00&quot;</td>
<td>50°39&quot;26.15&quot;</td>
<td>33</td>
<td>8</td>
<td>41</td>
</tr>
</tbody>
</table>

* Temperature was 36°C at the sampling point.
and pH of the sampling points. Slant cultures and glycerol-containing stocks from the single colonies were also prepared.

2.2. Primary Screening

The broth culture media of isolates were centrifuged after 24 h and the endo-1, 4-β-D-glucanase activity of the supernatant was measured by qualitative Congo-Red followed by quantitative Carboxymethyl cellulose/ Dinitrosalicylic acid (CMC/DNS) (Ghose, 1987) methods. Comparison of the transparency and size of halo zones around the bacterial colonies were used as the criteria for initial screening of isolates and selection of candidate strains. The positive control in this study includes 25 mg mL\(^{-1}\) of industrial enzyme, in which activity was determined to be at 60°C and similar pH conditions to the sampling locations by the Congo-Red staining method. In the Congo-Red staining method, 1% w/v CMC-Na with 1% w/v agar at pH 7 were mixed and poured into the Petri-plates, to which 0.025 mL of supernatant of each of the isolates was added as droplets and incubated at 45°C for 30 min. Then a solution of 0.1% (w/v) Congo-Red stain was poured on the plate surfaces and washed with 1M NaCl after 10 min (de-Oliveira et al., 2016). The halo zones were then measured by caliper and compared to each other.

For quantitative measurement of endo-1, 4-β-D-glucanase activity, the CMC/DNS method was used (Ghose, 1987). The medium was prepared by addition - (w/v) CMC to 0.05 M acetate buffer, adjusted to pH 4 (423.5 mL of 0.1 M Acetic acid and 76.5 mL of 0.1 M Sodium acetate trihydrate). For spectrophotometer calibration and negative control, buffer solution and supernatants were used, respectively.

2.2.1. Calculation of Endo-1,4-β-D-glucanase activity

The regression line of reduced sugar concentrations against their OD absorption at 540 nm was used as a standard curve. The supernatant reaction took place with CMC-Na as the substrate (Equation number 1). The amount of enzyme solution required for producing 0.5 mg dextrose was calculated. After calculating the amount of glucose released from the CMC by enzymatic reaction, the international unit of enzyme activity per mL (µmol min\(^{-1}\) mL\(^{-1}\)(IU)) on a CMC-Na substrate was determined (Ghose, 1987). The experiments were performed three times and the mean of the results was calculated using formula [1]. Error bars were inserted by Microsoft Office Excel 2016 software.

\[
[1] \quad 0.6052(\text{OD}) + 0.0273 \times 48
\]

2.3. Thermostability and pH stability of Endo-1,4-β-D-glucanase of the isolates

Thermostability and pH stability assay of endo-1,4-β-D-glucanase produced by the isolates were carried out simultaneously at 60°C in a buffer with pH 4 (423.5 mL of 0.1 M Acetic acid and 76.5 mL of 0.1 M Sodium acetate trihydrate) using the CMC/DNS method. The enzymes activities were determined every 2h. The isolates that kept their activity until the fourth hour were selected as top strains.

2.4. Strains Identification

The genomic DNA of the candidate isolates was extracted by a gram positive genome extraction kit, MBST Company, Tehran, Iran. The forward and reverse primers including 27F (5’-AGAGTTTGTACCTGGCTCAG-3’) and 1429R (5’-ACGGYTACCTTGTTACGACTT-3’) were used to partially sequence the 16S rRNA gene. A band of 1400 bp was obtained after each PCR. Internal primers, F416 (5’-GGCTAATACGCTGTAGCCAGCGA-3’), were also designed for complementary sequences after the first sequencing. The reaction was
carried out in 50 µL volumes using PCR Master Mix, Sinaclon Company, Tehran, Iran. PCR conditions included initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C for 40s), annealing (65°C for 45s) and extension (72°C for 45s) and a final extension step at 72°C for 10 min. The PCR products were visualized on 1.7% agarose gel using SYBR Safe DNA Gel Stain, and the samples were purified using a PCR product purification kit and sequenced by different primers by a commercial sequencing facility, Macrogen Inc., Seoul, S. Korea. A phylogenetic tree was made using the results of DNAMAN software.

**3. Results and discussion**

**3.1. Cellulase hydrolyzing bacteria diversity**

According to the results, twelve bacteria were isolated from four hot springs: two isolates from the Ghahvesouyee springs (AG₁, AG₂), two isolates from the Ghotoorsouyee springs (AGh₁, AGh₂), four isolates from the Shabil springs (ASh₁-ASh₄), and four isolates from the Ramsar (NR₁-NR₄) springs. Then, their glycerol stocks were prepared from a single colony of each isolate and maintained at -70°C.

**3.2. Primary Screening**

The results showed that the bacterial cellulose production in all the initial screening was achieved by a comparison between the halo zones from supernatants of each isolate to the one from an industrial enzyme as a positive control. As it is shown in Fig. 1, the spots B₁, D₁ and C₂ belong to AG₂, AGh₂, and ASh₁ isolates, respectively, these are approximately similar to the industrial spot in C₃. Among these three isolates ASh₁ showed less activity using the quantitative CMC/DNS method than two others, so the activity of this strain was considered for selecting other strains in additional screening steps. The ASh₁ isolate had 0.96 µmol min⁻¹ activity as a minimum; so the isolates having more activity than this amount were selected for additional screening. At this stage, 7 isolates were selected for additional screening including AG₁, AGh₁, AGh₂, ASh₁, ASh₄, NR₁, and NR₃ (Fig. 2).

![Figure 1. Congo-Red staining for screening of the isolated bacteria.](image)

**Isolates abbreviations:**

<table>
<thead>
<tr>
<th>Spot</th>
<th>A1</th>
<th>B1</th>
<th>C1</th>
<th>D1</th>
<th>E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>AG₁</td>
<td>AG₂</td>
<td>AGh₁</td>
<td>AGh₂</td>
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<thead>
<tr>
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<th>C2</th>
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<td>ASh₂</td>
<td>ASh₄</td>
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<table>
<thead>
<tr>
<th>Spot</th>
<th>A3</th>
<th>B3</th>
<th>C3</th>
<th>D3</th>
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<tr>
<td>Sample</td>
<td>NR₃</td>
<td>NR₄</td>
<td>Control*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Industrial Enzyme (Control)

Abbreviations Guide: AG: Ghahvesouyee; AGh: Ghotoorsouyee; ASh: Shabil; NR: Ramsar

![Figure 2. Endo-1,4-β-D-glucanase activity of supernatants of the isolates.](image)
3.3. Thermostability and pH stability assay

After 2 h treatment at 60°C with pH 4, the thermo stability and pH stability of endo-1, 4-β-glucanase were determined. Although the residual activity of NR1 and NR3 were very low and deactivated completely after 2h, the residual activity of AG2, AGh1, AGh2, ASh1 and ASh4, were 94.15%, 93.45%, 86.69%, 78.89% and 70.83%, respectively (Fig. 3); therefore, the thermo stability and pH stability of endo-1, 4-β-glucanase activity in these strains were also measured after 6 h of treatment. The evaluation of the thermo stability of endo-1,4- β-glucanase at 60°C showed that the enzymes of ASh1 and AGh deactivated after 4h of treatment; however, ASh4, AGh1 and AG2 had 92.93%, 71% and 21% of residual activity, respectively (Fig. 4). Therefore, the ASh4, AG2 and AGh1 isolates were selected as top isolates and identified at the genus level by molecular methods.

Ko et al. (2010) detected thermostable endoglucanase in Paenibacillus campinasensis BL11 with an optimum temperature at 60°C. Zafar et al. (2014) detected thermostable endoglucanase in Bacillus subtilis JS2004 with an optimum temperature of 50°C. Pandey et al. (2014) detected thermostable endoglucanase in Bacillus subtilis IARI-SP-1 isolated from an irrigated soil with long-term effluents of paper and pulp mill that had optimum activities at temperatures of 50-60°C. Woo et al. (2014) detected thermostable endoglucanase of Xanthomonas sp. EC102 with an optimum temperature and pH of 70°C and 5.5, respectively. The endoglucanase produced by Isobtericola variabilis subsp. IDAH9 which was isolated from a hot spring in Dehloran, Iran, showed 80% of the residual activity after 1 h of incubation at 65°C (Azizi et al., 2015). Kanchanadumkerng et al. (2017) isolated Paenibacillus sp. M33 from a fresh water swamp forest that had optimum activity at 50°C and pH 5.

3.4. Strain Identification

The partial 16S rDNA of the candidate bacteria were amplified using two primers, 27F and 1429R. These primers were appropriate for AG2 and AGh1, but not for ASh1. So for this strain the universal primers F416 and 1429R were applied to amplify the target gene (Fig. 5). Based on nucleotide BLAST in the EzTaxon (EzBioCloud, 2017) data base, AG2 and AGh1 were identified as Bacillus sp. and ASh4 was identified as Paenibacillus sp. and the relevant sequences were submitted under GenBank accession numbers MG333431, MG333434 and MG365640, respectively.

The phylogeny analysis of AG2, AGh1 and ASh4 isolates is shown in Figs. 6 and 7 using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The number above the branches indicates the percentage of trees in which the associated
taxa clustered together with 1000 replicates using the Maximum Composite Likelihood (MCL) approach. The analysis involved nine partial rDNA sequences including those from closely related species to the isolates, AG₂ and AGh₃, (Fig. 6) and the same number of rDNA sequences for ASh₄ (Fig. 7). Evolutionary analyses were conducted using the DNAMAN software package (Version 5.2.2, Lynnon Biosoft, Canada). AGh₁ and AG₂ belong to the genus Bacillus and are related with species *B. atrophaeus* (AB021181.1) and *B. licheniformis* (AE017333), respectively. Species belonging to genus Bacillus have wide ecological niches ranging from psychrophilic to thermophilic and from acidophilic to alkaliphilic, some strains are salt tolerant and others are halophilic. *Bacillus* spp. are mostly isolated from soil or from environments that may have been contaminated directly or indirectly by soil, but they can also be found in water (Logan and Vos, 2015). ASh₄ belongs to the Genus *Paenibacillus*, which includes more than 80 species with an optimum growth of 28–40°C and pH 7 (Priest, 2015).

![Figure 5.](image1)  
*Figure 5.* PCR bands of partial 16S rDNA amplification for ASh₄, AG₂, and AGh₁ on 1.7% agarose gel with SYBR safe stain. The 1400 bp bands are related to AG₂ and AGh₁ and 900bp for ASh₄.

![Figure 6.](image2)  
*Figure 6.* Maximum likelihood of phylogeny of Bacillus spp. (AGh₁ and AG₂ isolates) based on 16S rDNA sequences. Numbers above branches indicate percentage of trees in which the associated taxa clustered together with 1000 replicates. Other closely related bacterial species are *Geobacillus galactosidasius* and *Filobacillus milosensis* which are used as an outgroup.

![Figure 7.](image3)  
*Figure 7.* Maximum likelihood of phylogeny of Paenibacillus sp. ASh₄ based on 16S rDNA sequences. Numbers above branches indicate percentage of trees in which the associated taxa clustered together with 1000 replicates. Other closely related bacterial species are *Geobacillus thermoleovorans* as well as *Halobacillus mangrove* and *Achromobacter pumonis* as outgroups.
4. Conclusion

In conclusion, twelve bacteria with the ability to hydrolysis and use CMC as a carbon source were isolated from three acidic and one almost neutral hot springs in Iran. The endo-1, 4-β-glucanase of *Paenibacillus* sp. ASh4 as well as the ones from *Bacillus* sp. AG2 and AGh1 showed 40-90% residual activity at pH 4 and 60°C for more than three hours. Among the enzymes derived from the candidate isolates, the most activity belonged to *Paenibacillus* sp. ASh4 with up to 92.39% of its enzyme activity maintained after 4h of treatment. Finally, results showed the isolated acido-thermotolerent *Bacillus* spp. and *Paenibacillus* sp. can produce acid-stable and thermostable endoglucanase with potential industrial application for further thermostable metabolites. Future studies must be considered for other biotechnological applications.

**Conflict of interest**

The authors declare that there is no conflict of interest.

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**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

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**References**


