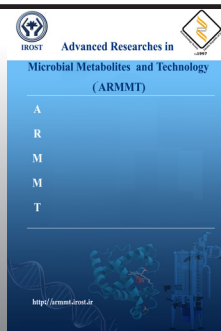




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Screening of Bacteria Producing Acid-stable and Thermostable Endo-1,4- β -Glucanase from Hot Springs in the North and Northwest of Iran

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.

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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 endo-1,4- β -glucanase producing bacteria was investigated. Endo-1,4- 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 acid-thermotolerant *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.

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-

*Corresponding author. Tel: + 982156276020

E-mail address: j.hemmat@gmail.com

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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 1. Geographic Coordinates of the Sampling Areas

Region	Province	Latitude (N)	Longitude (E)	Elevation (m)	pH	Temperature (°C)
Ghahvesouee	Ardabil	38°9'12.75"	48°04'17.67"	1690	6	48
Ghotoorsouee	Ardabil	38°20'13.00"	47°51'35.00"	2571	3	42*
Shabil	Ardabil	38°19'48.37"	47°50'37.09"	2667	6	48
Ramsar	Mazandaran	36°54'9.00"	50°39'26.15"	33	8	41

* 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 plus 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 ($\mu\text{mol min}^{-1} \text{mL}^{-1}$ (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'-AGAGTTTGATCCTGGCTCAG-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'-GGCTAACTACGTGCCAGCAG-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 Ghahvesouee springs (AG_1 , AG_2), two isolates from the Ghotoorsouee springs (AGh_1 , AGh_2), four isolates from the Shabil springs (ASh_1 - ASh_4), and four isolates from the Ramsar (NR_1 - NR_4) 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 AG_2 , AGh_2 and ASh_4 isolates, respectively, these are approximately similar to the industrial spot in C3. Among these three isolates ASh_4 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 ASh_1 isolate had $0.96 \mu\text{mol min}^{-1}$ 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 AG_2 , AGh_1 , AGh_2 , ASh_1 , ASh_4 , NR_1 and NR_3 (Fig. 2).

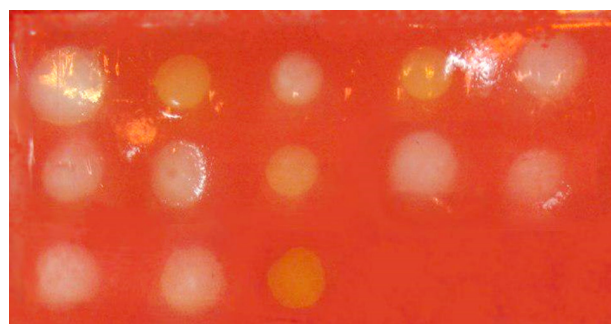


Figure 1. Congo-Red staining for screening of the isolated bacteria.

Isolates abbreviations:

Spot	A1	B1	C1	D1	E1
Sample	AG_1	AG_2	AGh_1	AGh_2	ASh_1
Spot	A2	B2	C2	D2	E2
Sample	ASh_2	ASh_3	ASh_4	NR_1	NR_2
Spot	A3	B3	C3	D3	E3
Sample	NR_3	NR_4	Control*		

* Industrial Enzyme (Control)

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

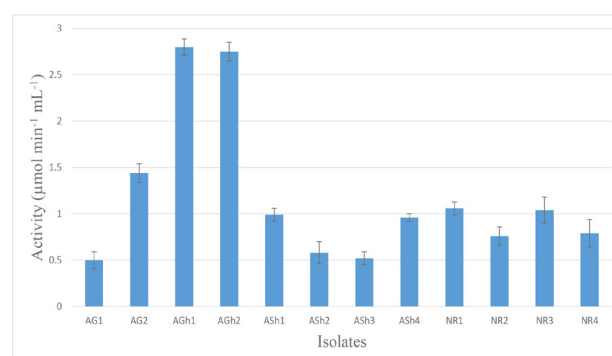


Figure 2. Endo-1,4- β -D-glucanase activity of supernatants of the isolates.

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 AGh₂ 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.

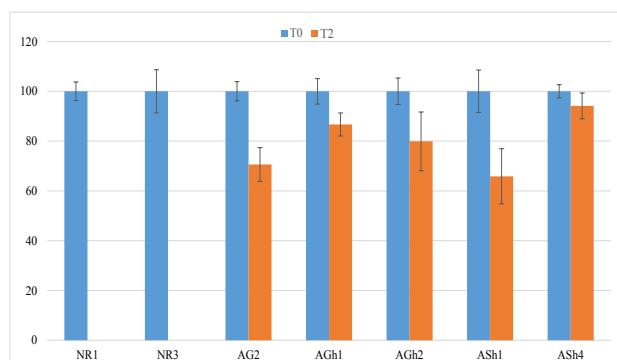


Figure 3. Thermostability of endo-1,4- β -glucanase from NR₁, NR₃, AG₂, AGh₁, AGh₂, ASh₁, and ASh₄ at 60°C and pH 4 after 2h (T0= primary activity, T2= residual Activity after 2 hours).

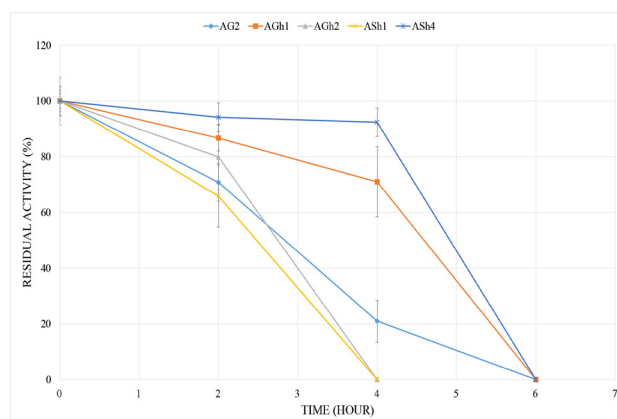


Figure 4. Thermostability of endo-1,4- β -glucanase of selected isolates at pH 4 and 60°C during 6 hours with 3 replications. .

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 *Xanthamonas* sp. EC102 with an optimum temperature and pH of 70°C and 5.5, respectively. The endoglucanase produced by *Isobactericola 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 ASh₄. 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 AGh₁ (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).

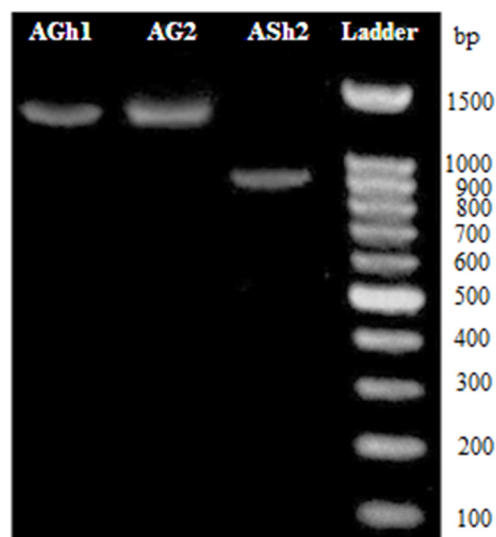


Figure 5. PCR bands of partial 16S rDNA amplification for ASH₄, AG₂, and AGh₁ on 1.7% agarose gel with SYBR safe stain. The 1400 bp bands are related to AG₂ and AGh₁ and 900bp for ASH₄.

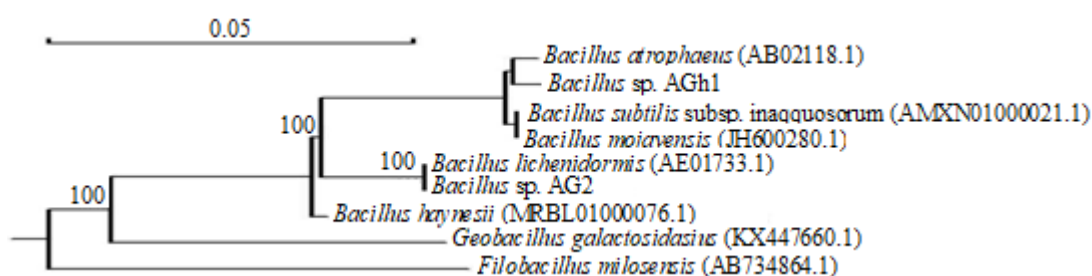


Figure 6. Maximum likelihood of phylogeny of *Bacillus* spp. (AGh₁ and AG₂ isolates) based on 16S rDNA sequences. Numbers above branches indicate percentage of trees in which the associated taxa clustered together with 1000 replicates. Other closely related bacterial species are *Geobacillus galactosidasius*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

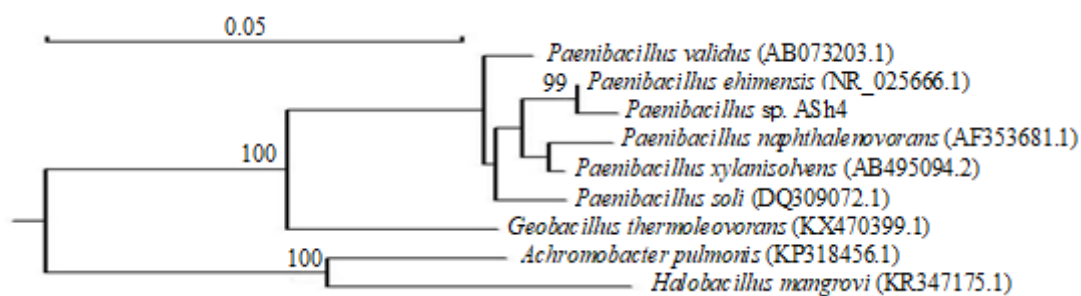


Figure 7. Maximum likelihood of phylogeny of *Paenibacillus* sp. ASH₄ based on 16S rDNA sequences. Numbers above branches indicate percentage of trees in which the associated taxa clustered together with 1000 replicates. Other closely related bacterial species are *Geobacillus thermoleovorans* as well as *Halobacillus mangrovei* 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-thermotolerant *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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