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# Evaluation of Arabinofuranosidase and Xylanase Activities of *Geobacillus* spp. Isolated from Some Hot Springs in Turkey

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**Abstract** Some hot springs located in the west of Turkey were investigated with respect to the presence of thermophilic microorganisms. Based on phenotyping characteristics and 16S rRNA gene sequence analysis, 16 of the isolates belonged to the genus *Geobacillus* and grew optimally at about 60°C on nutrient agar. 16S rRNA gene sequence analysis showed that these isolates resembled *Geobacillus* species by ≥97%, but SDS-PAGE profiles of these 16 isolates differ from some of the other species of the genus *Geobacillus*. However, it is also known that analysis of 16S rRNA gene sequences may be insufficient to distinguish between some species. It is proposed that *recN* sequence comparisons could accurately measure genome similarities for the *Geobacillus* genus. Based on *recN* sequence analysis, isolates 11, IT3, and 12 are strains of *G. stearothermophilus*; isolate I4.3 is a strain of *G. thermodenitrificans*; isolates 9.1, IT4.1, and 4.5 are uncertain and it is required to make further analysis. The presence of xylanase and arabinofuranosidase activities, and their optimum temperature and pH were also investigated. These results showed that 7 of the strains have both xylanase and arabinofuranosidase activities, 4 of them has only xylanase, and the remaning 5 strains have neither of these activities. The isolates 9.1, 7.1, and 3.3 have the highest temperature optima (80°C), and 7.2, 9.1, AO4, 9.2, and AO17 have the highest pH optima (pH 8) of xylanase. Isolates 7.2, AO4, AC15, and 12 have optimum arabinofuranosidase activities at 75°C, and only isolate AC15 has the lowest pH of 5.5.

**Keywords:** *Geobacillus* spp., arabinofuranosidase, xylanase, 16S rRNA, *recN*

The genus *Geobacillus* was separated from the genus *Bacillus*, and the type species is *Geobacillus stearothermophilus*. Many kinds of species with thermophilic, acidophilic, alkalophilic, and halophilic properties have been included in this genus [22].

Thermophilic bacilli show optimal growth at temperatures ranging from 45 to 70°C [23, 19] and can be isolated from both thermophilic and mesophilic environments [18]. The importance of these thermophilic bacilli has increased owing to their potential as a source of thermostable enzymes, including proteases, amylases, pullulanases, peroxidase, glucose isomerases, lipases, xylanases, and DNA restriction enzymes. Recently, the interest in xylanase and arabinofuranosidase has markedly increased owing to the potential applications in pulping and bleaching processes, in the food and feed industry, textile processes, the enzymatic saccharification of lignocellulosic materials, and waste treatment. Most of these processes are carried out at high temperatures, so that thermostable enzymes would give an advantage [24]. Over the past decade, there has been a considerable increase in interest in thermophilic endospore-forming bacteria of the genus *Bacillus*, both because of their possible contamination of heated food products and because of their biotechnological importance as sources of thermostable enzymes and other products of industrial interest [8]. Therefore, thermophilic organisms are of special interest as a source of novel thermostable enzymes [2, 4, 17, 29].

16S rRNA analyses are useful for comparing phenotypically close and yet genetically different microorganisms, and it is also known that analysis of 16S rRNA sequences may be insufficient to distinguish between some species [30]. Because of this reason, the certain bacterial systematics require DNA-DNA homology studies, and DNA-DNA hybridization studies have been regarded as the "gold standard" [31]. However, it can be difficult to reproduce hybridization values between laboratories with the necessary precision; hybridization methods often require specialized equipment or the use of radioactive labels. Consequently, systematics have come to rely increasingly on comparison of DNA sequences, especially 16S rRNA gene sequences, as a supplementary or alternative approach [25]. Recently [33], over thirty genes have been identified that can be used in

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bacterial systematic and DNA repair, and the genetic recombination protein (*recN*) gene is one of the strong candidates among these for genome similarity prediction. The *recN* sequence identity scores could predict, with a high degree of accuracy, the whole genome sequence identity shared by two organisms. For the species studied, genome identity scores predicted by *recN* analysis differed from those measured directly in genomic alignments by an average of only 4.4%.

The *recN* is superior to the 16S rRNA gene at resolving lower taxa (genus and below), but the 16S rRNA gene is much more useful for resolving higher taxa. At the species-subspecies level, *recN* produced a phylogenetic signal roughly six times stronger than the 16S rRNA gene, with a correspondingly higher percentage of residues showing sequence variation. At the genus level, total phylogenetic signal strength was almost an order of magnitude greater for *recN* than for the 16S rRNA gene. At a phylum level, however, the 16S rRNA gene was clearly superior to *recN*. *recN* likely has a significantly greater resolving power than 16S rRNA for assigning strains to taxa at the genus, species, or subspecies level, but at higher taxa, *recN* might have considerably lower power. For the genus *Geobacillus* (and perhaps for other bacterial taxa as well), *recN* analysis should prove to be a powerful tool for organizing strains into lower taxa. The 16S rRNA gene does contribute enough useful phylogenetic signal even at this level, however, that alignments of concatenated *recN* and 16S rRNA sequences should further enhance the precision and accuracy of species and subspecies assignments.

In this study, a molecular identification study was carried out on some thermophilic *Geobacillus* strains isolated from Dikili-Bergama Kaynarca Hot Spring (İzmir), Omerbeyli Hot Spring (Aydin), Camkoy Camur Hot Spring (Aydin), and Alangullu Hot Spring (Aydin) in Turkey. The thermophilic xylanase and arabinofuranosidase activities of these strains were investigated.

## MATERIALS AND METHODS

### Reference Strains

*Geobacillus stearothermophilus* (ATCC 7953) was obtained from ATCC, and *G. thermoleovorans* (DSM 5366$^T$), *G. subterraneus* (DSM 135522$^T$), *G. thermodenitrificans* (DSM 465$^T$), *G. caldoxylosilyticus* (DSM 12041$^T$), *G. thermocatenulatus* (DSM 730$^T$), and *Bacillus caldotenax* (DSM 406) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Brunschweig, Germany (DSMZ). *G. kaustophilus* (CECT 4264) was kindly provided by A.F. Yenidunya.

### Sampling and Isolation

Sixteen Gram-positive rods were isolated from mud and water samples of Dikili-Bergama Kaynarca Hot spring, and Camkoy Camur, Omerbeyli, and Alangullu Hot springs in the provinces of İzmir and Aydin in Turkey, respectively. The water temperature of these hot springs is between 70–130°C. After collection, mud and water samples were immediately used for enrichment at 70–75°C in both NB and LB containing 1% xylan. One-day-old enrichment cultures were repeatedly subcultured in 10 ml of both NB and LB containing 1% xylan and streaked on NB agar plates and LB agar plates containing 1% xylan in order to obtain separate colonies. Purity of the isolates was assessed by colony morphology and microscopy.

### Phenotypic Studies

Isolates were first Gram stained and examined under light microscopy. The formation of spores was tested by microscopic observations in both liquid cultures and single colonies of the isolates from agar plates at different incubation periods.

### PCR Amplification and Cloning of 16S rRNA Sequence

The 16S rRNA genes were selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of bacterial 16S rRNA genes. The forward primer, UNI16S-L (5'-ATTCTAGAGTTTGATCATGGCTTCA), corresponded to positions 11 to 26 of *Escherichia coli* 16S rRNA, and the reverse primer, UNI16S-R (5'-ATGGTAC-CGTGTGACGGGCGGTGTTGTA), corresponded to the complement of positions 1411 to 1393 of *Escherichia coli* 16S rRNA [10]. PCR reaction conditions were carried out according to Beffa *et al.* [3] and the PCR product was cloned to a pGEM-T vector system.

### SDS-PAGE Analysis

Extraction of proteins from growing cells, measurement of protein concentration in the extracts, electrophoresis, and staining of proteins bands were performed as described previously [6].

### Physiological and Biochemical Characterizations of Isolates

The temperature range for growth was determined by incubating the isolate from 30 to 80°C. The pH dependence of growth was tested in the pH range 5.0–11.0 in nutrient broth medium. Media were adjusted to the initial pH indicated, with either 1 M NaOH or 1 M HCl. Four sets of nutrient broth were prepared containing 1, 2, 3, 4, 5, and 7% of NaCl, respectively. The growth of isolate at different salt concentrations was tested using NB as organic substrate and a control broth without any NaCl supplementation. Catalase and oxidase were detected by the method of Cowan and Steel [12]. Conventional biochemical tests were determined using the identification card (ID-BAC) for Gram-positive microorganisms of

family *Bacillaceae* with the VITEK 32 system (bioMerieux Vitek, Hazelwood, MO, U.S.A.).

**PCR Amplification and Cloning of *recN* Gene Sequence**

The *recN* genes were selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal to conserved positions in the 5' and 3' regions of *Geobacillus spoIVB* and *ahrC* genes [32]. The forward primers, F1-2-(5'-CGA TTT GCG GCG ACG ATA C-3'), and reverse primers, R1-1-(5'-TAC ACC ATG CAA AAA CGG TTA C-3'), were used for amplification of the *recN* sequences. PCR reaction conditions were carried out according to Beffa *et al.* [3] and the PCR product was cloned into a plasmid using the pGEM-T Easy vector system.

**Sequencing and Phylogenetic Analysis**

Following PCR amplification and cloning of both the *recN* and 16S rRNA genes of our isolates, both the 16S rRNA and *recN* gene sequences were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea). The sequences consisting of about 1,600 nt of the *recN* gene and about 1,400 nt of the 16S rRNA gene were determined. These sequences were compared with those contained within GenBank [7] by using a BLAST search [1]. Both the 16S rRNA and *recN* gene sequences of the species most closely related to our isolates were retrieved from the database. Retrieved sequences were aligned by using the Clustal X program [28] and manually edited. Phylogenetic trees were constructed by the neigbor-joining method using the PAUP 4.0b10 [26].

**Xylanase Activity**

After cultivation of bacteria at 65°C for 24 h in LB containing 1% xylan, culture fluids were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was lyophilized and the xylanase activity was assayed by measuring the release of reducing sugar from Oat spelt xylan following the dinitrosalicylic acid (DNS) method [20]. The effect of pH on the activity of xylanases was measured by incubating 0.25 ml of enzyme and 0.75 ml of buffers, adjusted to pH from 5.0 to 11.0, containing oat spelt xylan (1%). The effect of temperature on the enzyme activity was determined by performing the standard assay as mentioned earlier for 10 min at pH 7.0 within the temperature range of 40 to 80°C.

For detection of xylanase activity, 0.2% oat spelt xylan was included in the gels before polymerization. Samples were heated for 10 min at 45°C in sample buffer before being applied to gels. After electrophoresis, the gels were soaked in 2.5% (w/v) Triton X-100 for 30 min, washed in 50 mM phosphate buffer, pH 7, for 30 min, and incubated at 65°C for 15 min in the same buffer. Gels were stained with 0.1% Congo red for 15 min and washed with 1 M NaCl until xylanase bands became visible. Gels were then immersed in 5% (w/v) acetic acid, in which the background turned dark blue, and photographed.

**Arabinofuranosidase Activity**

After cultivation of bacteria at 65°C for 24 h in LB containing 1% sugar beet arabinan, culture fluids were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was lyophilized and extracts from cells were obtained according to the methods of Belduz *et al.* [5]. The activity of Abf was assayed both with supernatant and cell extracts at 75°C in 0.5 ml (total volume) of 50 mM potassium phosphate buffer (pH 6.0). α-L-Arabinofuranosidase activity was determined by using 0.8 mM *p*-nitrophenyl α-L-arabinofuranoside as the substrate and the appropriately diluted enzyme solution. After incubation of the reaction mixture for 10 min, 0.5 ml of cold 1 M $Na_2CO_3$ was added to stop the reaction.

The endoarabinanase activity of the isolates was tested by using red debranched arabinan (RDA) as a substrate following the manufacturer's instructions. After incubation, unhydrolyzed substrate was precipitated by addition of 4 volumes of ethanol, and the absorbance of the supernatant was measured at 520 nm.

The optimum pH for α-L-arabinofuranosidase activity was determined by incubation at 75°C for 10 min in the pH range of 4 to 10. The optimum temperature for enzymatic activity was determined in 50 mM potassium phosphate buffer (pH 6.0) by using temperatures ranging from 40 to 95°C.

For detection of enzyme activity on the gel, proteins were analyzed by native gels. Gels were washed with 50 mM potassium phosphate )(pH 6.0) and then incubated in the same buffer containing 4-methylumbelliferyl-α-L-arabinofuranoside. Bands of enzymatic activity were detected by exposure to ultraviolet light (260 nm).

## RESULTS

**Isolation of Microorganisms**

Ten isolates from Dikili-Bergama Kaynarca Hot Spring (İzmir), 3 isolates from Alangullu Hot Spring (Aydin), 2 isolates from Omerbeyli Hot Spring (Aydin), and 1 isolate from Camkoy Camur Hot Spring (Aydin), in total 16 isolates, were obtained. Light microscopy revealed that cells of the isolates were rod-shaped, Gram-positive, and endospore-forming bacteria. Mature spores were abundant in aerobically grown cultures on agar plates or in agitated liquid media. Subterminal spores were observed in all twelve isolates, but central spores were also present in isolates IT3, 1.2.T, 11, and IT4.1. Sporulation was observed after 8 to 10 h of incubation at 55°C in all strains.

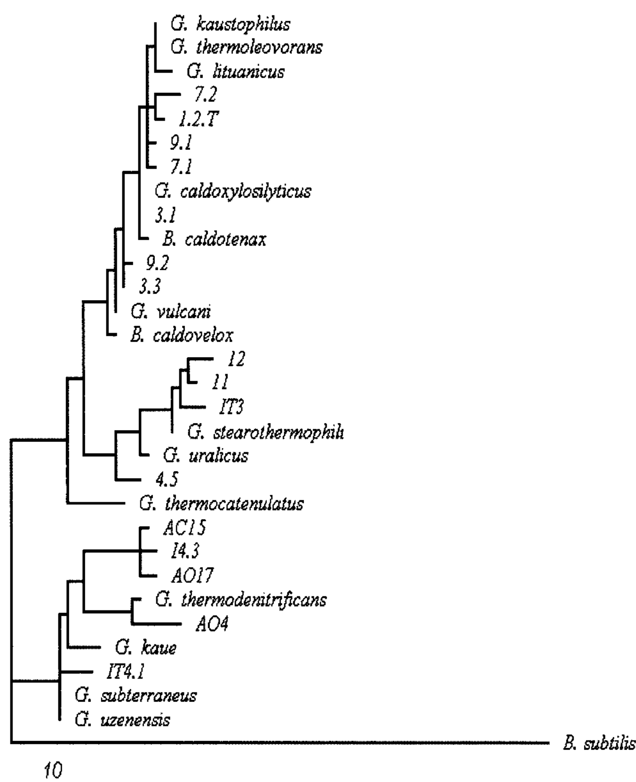


**Fig. 1.** Phylogenetic tree constructed on the basis of 16S rRNA gene sequence data of our isolates and other related organisms, using the neighbor-joining method.
Bar, 10 substitutions per 100 nucleotide positions.

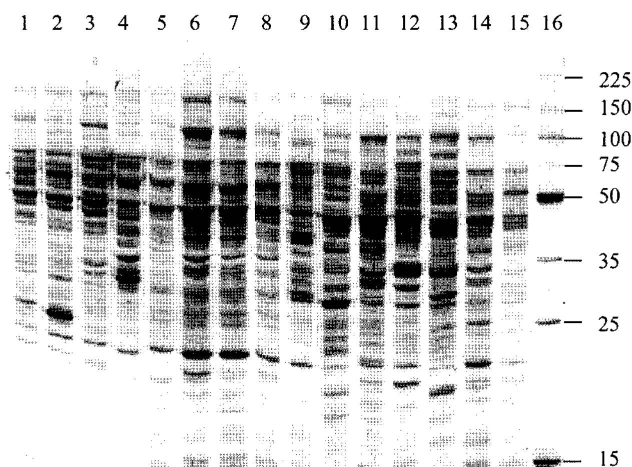


**Fig. 2.** SDS-PAGE whole-cell protein profiles of I4/3 (lane 1), *G. thermodenitrificans* (DSM 465$^T$) (lane 2), IT4/1 (lane 3), *G. subterraneus* (DSM 135522$^T$) (lane 4), *G. caldoxylosilyticus* (DSM 12041$^T$) (lane 5), 9.1 (lane 6), *G. kaustophilus* (CECT 4264) (lane 7), *Bacillus caldotenax* (DSM 406) (lane 8), *G. thermoleovorans* (DSM 5366$^T$) (lane 9), *G. thermocatenulatus* (DSM 730$^T$) (lane 10), 4.5 (lane 11), *Geobacillus stearothermophilus* (ATCC 7953) (lane 12), IT3 (lane 13), 12 (lane 14), 11 (lane 15), and Marker (lane 16).

## 16S rRNA Gene Sequence Analysis

A total of 1,400 nucleotides of the 16S rRNA from 16 isolates were aligned and compared with sequences of related bacteria. The phylogenetic tree was constructed using the neigbor-joining method (Fig. 1). On the basis of 16S rRNA gene sequence analysis, our 16 isolates showed ≥97% similarity to other *Geobacillus* species, and this sequences similarity showed that the 16 isolates are members of the genus *Geobacillus*.

## SDS-PAGE Analysis

The electrophoretic patterns of soluble cellular proteins of 16 isolates, as determined by SDS-PAGE (data not shown), showed that isolates 1-2T, 3.3, 3.1, 7.2, 7.1, 9.2, and 9.1 are similar to each other, and 9.1 was selected as a type isolate. Moreover, isolates AO17, AO4, AC15, and I4.3 are similar to each other, and I4.3 was selected as a type isolate. The other five isolates (4.5, 11, 12, IT3, IT4.1) are not similar to either each other or the other two groups of the isolates.

The selected two type isolates (9.1 and I4.3) and the other five isolates (4.5, 11, 12, IT3, IT4.1) were compared to the *Geobacillus* species that are most closely related to our isolates (Fig. 2). The profiles of the type isolate 9.1 resembled those of *G. kaustophilus*, and the profiles of the other type isolate I4.3 are similar to *G. thermodenitrificans*. The other five isolates are not similar to *Geobacillus* spp.

## Physiological and Biochemical Characterization of Isolates

Based on SDS-PAGE analysis, seven different isolates were selected and phenotypic characterization of these isolates was determined. Biochemical and physiological features of seven isolates are reported in Table 1. All isolates were Gram-positive, moderately thermophilic, and motile. All isolates grew optimally in NB medium where pH values were 6.5 to 8. The seven isolates tolerated up to 1% NaCl, and the isolates IT3, 12, 9.1, and 4.5 tolerated to 2%. Most isolates grew well at 45 to 65°C, and isolates 4.5 and IT4.1 grew well at 35°C. Isolates 9.1, 12, and IT3 grew well at 70°C, and IT3 also grew well at 75°C.

Except for IT3, the other isolates were catalase positive and isolates 4.5, I4.3, and 11 were oxidase negative; the other isolates were oxidase positive. All isolates were capable of fermenting xylose, sodium acetate, and ribose. Physiological properties of these isolates are more or less different from each other. These results placed these strains into three groups: 12 and IT4.1; 11, IT3, and 9-1; 4-5 and I4.3.

## *recN* Gene Sequence Analysis

Following PCR amplification, *recN* gene sequences of the seven isolates were determined. The *recN* sequences of the seven isolates were aligned and compared with sequences

**Table 1.** Physiological and biochemical characteristic of the seven isolates.

| | 12 | IT4.1 | 11 | IT3 | 9.1 | 4.5 | I4.3 |
|---|---|---|---|---|---|---|---|
| Shape of cells | Rod | Rod | Rod | Rod | Rod | Rod | Rod |
| Spore formation | + | + | + | + | + | + | + |
| Temperature range (°C) | 45–70 | 35–65 | 40–65 | 40–70 | 45–70 | 35–65 | 40–75 |
| pH range | 5.5–8 | 5.5–9 | 6–8 | 5.5–8.5 | 6.5–9 | 5.5–8.5 | 5.5–8.5 |
| Tolerance to NaCI (%) | 2.0 | 1.0 | 1.0 | 2.0 | 2.0 | 2.0 | 1.0 |
| Catalase activity | + | + | + | – | + | + | + |
| Oxidase activity | + | + | – | + | + | – | – |
| Starch | – | – | + | + | + | – | – |
| Galactose | – | – | – | + | – | – | – |
| Amygdalin | – | – | – | – | – | – | – |
| Sorbitol | – | – | – | – | – | – | – |
| Oleandomycin | – | – | – | – | – | – | – |
| Sucrose | + | + | + | + | – | – | – |
| Arabinose | + | + | + | – | + | – | – |
| Inulin | – | – | – | – | – | – | – |
| *N*-Acetyl-D-glucosamine | + | – | – | – | + | – | – |
| Sodium acetate | + | + | + | + | + | + | + |
| Tetrazolium red | – | – | – | – | – | – | – |
| Xylose | + | + | + | + | + | + | + |
| Ribose | + | + | + | + | + | + | + |
| Amylopectin | – | – | – | – | – | – | – |
| Arabitol | – | – | – | – | – | – | – |
| Tagatose | – | – | – | – | – | – | – |
| Mannitol | + | + | + | + | + | – | – |
| Maltose | + | + | + | + | + | – | + |
| Potassium thiocyanate | + | + | + | + | + | – | – |
| Polyamidohygrostreptin | – | – | – | – | – | – | – |
| Glucose | + | + | + | + | + | – | + |
| Raffinose | – | – | – | – | – | – | – |
| Trehalose | + | + | + | + | + | – | – |
| Nalidixic acid | – | – | – | – | – | – | – |
| Inositol | – | – | – | – | – | – | – |
| Salicin | + | + | – | – | – | – | – |
| Palatinose | + | + | – | + | – | – | – |
| Mandelic acid | + | + | + | + | + | + | – |
| Esculin | – | – | – | – | – | – | – |

of related bacteria. The phylogenetic tree was constructed using the neigbor-joining method (Fig. 3). On the basis of the results of this analysis, IT4.1 showed 99%, 99%, and 98% similarity to the sequence of *G. subterranus*, *G. uzenensis*, and *G. kaue*, respectively; I4.3 exhibits 99% similarity to that of *G. thermodenitrificans*; 9.1 has 99% similarity to *G. thermoleovorans*, *G. kaustophilus*, *G. caldotenax*, *G. vulcani*, *G. lituanicus*, *G. caldovelox*, and *G. thermocatenulatus*; isolates 11, IT3, and 12 have 99% similarity to *G. stearothermophilus*; isolate 4.5 shows 96% similarity to that of *G. stearothermophilus*, *G. lituanicus*, and *G. thermoleovorans* respectively.

On the basis of *recN* sequence similarity analysis, isolates 11, IT3, and 12 are strains of *G. stearothermophilus*. Isolate I4.3 is a strain of *G. thermodenitrificans*. Isolate 9.1 is the strain of one of the following species: *G. thermoleovorans*, *G. kaustophilus*, *G. caldotenax*, *G. vulcani*, *G. lituanicus*, *G. caldovelox*, or *G. thermocatenulatus*. IT4.1 is the strain of either *G. subterraneus* or *G. uzenensis* or *G. kaue*. The species identity for the isolate 4.5 is uncertain.

**Xylanase Activities**

Xylanase activities were detected in the strains investigated at the end of 24 h. At the end of the study, we determined that only 11 (3.3, 7.1, 9.1, AO4, AC15, 12, I4.3, AO17, 9.2, 7.2, 1-2T) of our strains have xylanase activity. We also showed these activities in the SDS-PAGE (Fig. 4). The xylanases we identified forms three groups in respect to their molecular masses. The xylanases of the strains 7.1,

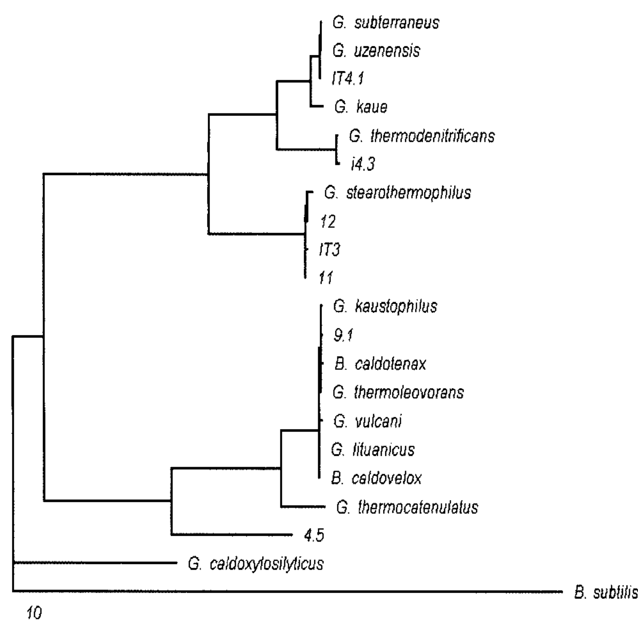


**Fig. 3.** Phylogenetic tree constructed on the basis of *recN* gene sequence data of our 7 isolates and other related organisms, using the neighbor-joining method.
Bar, 10 substitutions per 100 nucleotide positions.

7.2, 9.1, 9.2, 1-2T, 12 form the 1st group; the strains AO4, AC15, I4.3, and AO17 form the 2nd group. The strain AO17 has an extra heavier xylanase band than the ones of

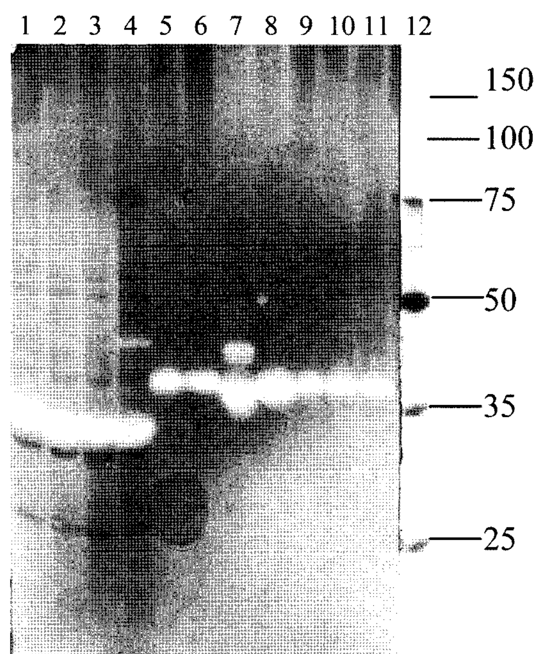


**Fig. 4.** Xylanase activities of 11 isolates in SDS-PAGE gel.
Lane (1) AO4, lane (2) AC15, lane (3) I43, lane (4) AO17, lane (5) 1-2T, lane (6) 9.1, lane (7) 3.3, lane (8) 7.1, lane (9) 9.2, lane (10) 7.2, lane (11) 12, and lane (12) Marker.

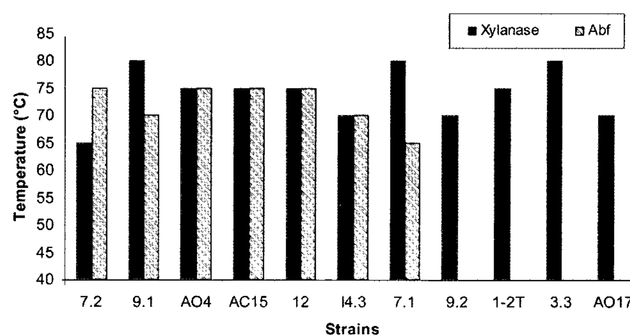


**Fig. 5.** Optimal temperatures of both the xylanases and arabinofuranosidases of our isolates.

the 2nd group but with weaker xylanase activity. Molecular masses of the 1st group are higher than the 2nd group. Strain 3.3 is the only member of the 3rd group that has one faint and two intense xylanases as molecular mass.

We investigated the optimal temperatures and pH of the xylanases of our isolates. The strains 3.3, 7.1, and 9.1 had the highest temperature optima (80°C) among the strains whereas the strains 9.1, AO4, and AO17 had the optimum pH of 8.0. The optimum temperature and pH of the enzymes of the other strains are given in Figs. 5 and 6.

## Arabinofuranosidase Activities

Arabinofuranosidase activities were detected after 24 h incubation. At the end of the study, we determined that only 7 (7.1, 7.2, 9.1, AO4, AC15, 12, I43) of our isolates had arabinofuranosidase activity. We also showed the activities in the native gel (Fig. 7). The arabinofuranosidases (Abfs) we identified formed two groups in respect to their molecular masses. Abfs of the strains 7.1, 7.2, 9.1 forms the 1st group; the strains AO4, AC15, I4.3, 12 form the 2nd group. Molecular masses of the 2nd group are higher. The strain 12 of the 2nd group has the other Abf with a so much lower molecular mass than the ones of the 1st group.

We investigated the optimal temperatures and pH of the arabinofuranosidase of the isolates, shown in Figs. 5 and 6. Arabinofuranosidases of the strains 7.2, AO4, AC15, and 12 were the most thermophilic Abfs (75°C).

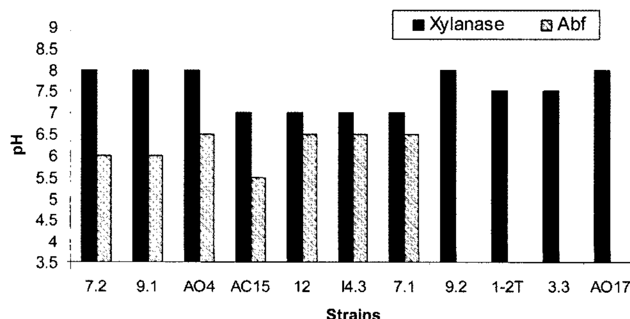


**Fig. 6.** Optimal pH of the arabinofuranosidases and xylanases.

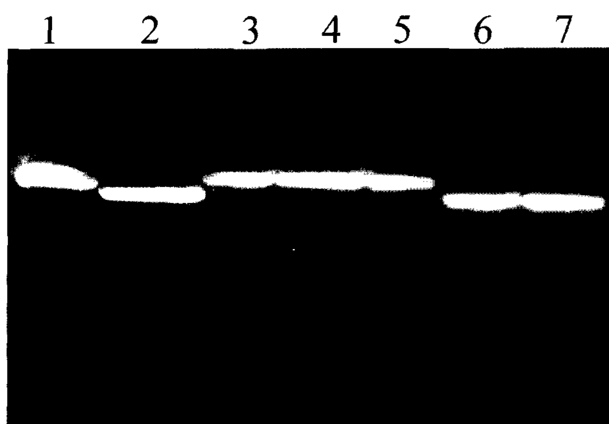


**Fig. 7.** Arabinofuranosidase activities of seven isolates in the native PAGE.
Lane (1) 12 , lane (2) 9.1, lane (3) AC15, lane (4) I4.3, lane (5) AO4, lane (6) 7.2, and lane (7) 7.1.

## DISCUSSION

Environmental strains of thermophilic bacilli from some hot springs of Turkey were isolated and characterized on the basis of some morphologic and physiological properties, 16S rRNA and *recN* gene sequence analysis, and thermostable xylanase and arabinofuranosidase production.

Stackebrandt and Goebel [25] reached the conclusion that strains belonging to the same genus that exhibit less than 97% 16S rRNA gene sequence similarity should be considered members of different species. However, it is also known that analysis of 16S rRNA sequences may be insufficient to distinguish between some species [30]. On the basis of 16S rRNA gene sequence analysis, our 16 isolates showed ≥97% similarity to other *Geobacillus* species. However, we also determined that some physiological, morphological, and biochemical characteristics and electrophoretic patterns of soluble cellular proteins of our isolates differed from those of the species of the genus *Geobacillus*. Cato *et al.* [11] suggested that strains with 80% DNA-DNA relatedness have the same protein profiles, and strains with 70% DNA-DNA relatedness have a few differences. Based on protein profile similarity, seven of our isolates formed one group, and another 4 isolates formed another group. The other 5 isolates were not similar to either each other or the other two groups of isolates. Isolate 9.1, which has a similar protein profile to *G. kaustophilus*, was selected as a type strain of the former group. The I4.3 protein profile resembled that of *G. thermodenitrificans* and it was designated as the type strain of the latter group. The other strains were not similar to any *Geobacillus* spp.

Zeigler [32] suggested that *recN* sequence comparisons could accurately measure genome similarities for the *Geobacillus* genus, and if the *recN* DNA sequences for two bacterial strains or isolates are less than 84% identical, we can be 95% confident that their genome sequences are less than 70% identical and that the bacteria belong to different species. If the *recN* DNA sequences are more than 96% identical, then by the same reasoning we can be 95% confident that the bacteria belong to the same species. If the *recN* sequences are between 84% and 96% identical, it is questionable whether the genome sequence identity is greater or less than 70%, making the species identity for these strains uncertain. Zeigler [33] clustered this set of thermophilic *Geobacillus* strains into nine homology groups. Groups 1A, 2, 4A, 5, and 6A appear to correspond unambiguously to the species *G. thermodenitrificans*, *G. stearothermophilus*, *G. thermoglucosidasius*, *G. toebii*, and *G. caldoxylosilyticus*, respectively. Identification of the other four homology groups (1B, 3, 4B, and 6B) with currently recognized species is somewhat more difficult, however. On the basis of a *recN* sequence similarity analysis, we suggest that isolates 11, IT3, and 12 belong to Group 2 and are strains of *G. stearothermophilus*; isolate I4.3 belongs to Group 1A and is a strain of *G. thermodenitrificans*; isolates 9.1 and IT4.1 belong to Group 3 and Group 1B, respectively. Group 3 contains the type strains of *G. kaustophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. vulcani*, and *G. lituanicus*. The group also includes *B. caldotenax* (96A4) and *B. caldovelax* (96A5). Group 1B contains the type strain of *G. subterraneus* (BGSC 9.1A1T), and also contains *G. uzenensis* (BGSC 92A2) and *G. kaue* W9A25. Since *recN* sequences of 9.1 and IT4.1 place these bacteria into Group 3, to determine the species identities, further analysis is needed. Further analysis is also needed for the strain 4.5, because its *recN* sequence has 96% similarity to the *Geobacillus* species discussed above.

We investigated the production of thermostable xylanases of our 16 isolates and showed that only 11 isolates have xylanase activity. Based on their SDS-PAGE profile, 16S rRNA gene sequence analysis, and *recN* gene analysis, we decided that I4.3 and the other similar isolates belong to *Geobacillus thermodenitrificans*, isolate 12 belongs to *G. stearothermophilus*, 9.1 and the other relatives isolates belong to *Geobacillus* Group 3, and IT4.1 is a member of *Geobacillus* Group 1B. It is known that some *Geobacillus* species (for example, *G. thermodenitrificans*, *G. stearothermophilus*) have thermostable xylanase activity. However, we compared the optimum temperatures of *Geobacillus* xylanase enzymes with the ones of our isolates and we observed differences. As shown in Fig. 8, our *G. thermodenitrificans* isolates have only 30 kDa, and *G. stearothermophilus* isolate 12 and *Geobacillus* Group 3 isolates have 35 kDa xylanase on the zymogram of SDS-PAGE. Although we could not find any data about the molecular mass of *G. thermodenitrificans* xylanase, Khasin *et al.* [16] suggested that *G. stearothermophilus* T-6 has

43 kDa xylanase but Nanmori *et al.* [21] characterized a 21 kDa xylanase in a different strain of *G. stearothermophilus* 21. Although the two enzymes have different molecular masses, Khasin *et al.* [16] suggested that xylanase 21 and xylanase T6 have similar modes of action. The optimum pH of the xylanase of *G. stearothermophilus* T-6 and our strain 12 differ from each other. The optimum pH of the xylanase of *G. stearothermophilus* T6 is 6.5 and that of our strain 12 is 7.0. Dupont *et al.* [13] reported the optimum temperature of *G. thermodenitrificans* SB1 as 70°C, but the strains A04 and AC15 have an optimum temperature of 75°C, and A017 and I4.3 have an optimum temperature of 70°C. Based on these data, we can conclude that the xylanases of our *G. thermodenitrificans* isolates have higher optimum temperatures than *G. thermodenitrificans* SB1. Xylanases of 7.2, 3.3, $9_1$, 12, and 1-2T maintained at least 50% of their activities at pH 11, but 7.1 and 9.2 maintained more than 75% of their activities at pH 11. Xylanase stabilities of strains 7.1 and 9.2 were studied between pH 7 to 11 at 25°C and the enzymes were fully stable at this pH range after 48 h, and after 120 h incubation at pH 11, xylanase from 7.1 had 90% and 9.1 had 77% residual activity (data is not shown). The optimum temperature of 7.1 was 60°C, and the optimum temperature of 9.2 was 80°C.

The use of alkaline active xylanases allows direct enzymatic treatment of the alkaline pulp and avoids the cost of incurring and the time-consuming steps of pH re-adjustment. In particular, alkaline xylanases that are operationally stable at higher temperature are more beneficial because of savings in cooling cost and time. In this regard, the present xylanases, 7.1 and 9.2, are expected to operate under conditions close to those of most mills; i.e., high pH and temperature. So far, only a few xylanases with optimum temperature for activity exceeding 70°C at or above pH 9 have been reported [14]. Owing to better solubility of xylan under alkaline conditions, alkaline-active xylanases may also find other potential applications in addition to pulp bleaching. When compared, the features of our xylanases make them good candidates to meet the requirements of the mills and Kraft pulp treatment, better than the present enzymes in the market.

Several arabinofuranosidases from different microorganisms, including yeast, fungi, and bacteria, have been documented and characterized to date. In the genus of the *Geobacillus*, two different arabinofuranosidases from *G. stearothermophilus* have been characterized [9, 15]. Whereas *G. stearothermophilus* L1 and T-6 strains are each producing only one arabinofuranosidase, our *G. stearothermophilus* strain 12 is producing two arabinofuranosidases (Fig. 7). Whereas the optimum temperatures of arabinofuranosidases of *G. steorothermophilus* L1 and T-6 are 70°C, arabinofuranasidase of *G. stearothermophilus* strain 12 has 75°C optimum temperature, which makes it better than the reported arabinofuranosidases of *G. stearothermophilus* in terms of optimum working temperature.

Takao *et al.* [27] have isolated a *G. thermodenitrificans* TS-3 strain and this strain produced an extracellular endoarabinanase and two arabinofuranosidases when grown at 60°C on a medium containing sugar beet arabinan. In this study, we isolated four *G. thermodenitrificans* strains and three of them have extracellular arabinofuranosidase activity. Moreover, we investigated for endoarabinanase activity, but none of these strains has endoarabinanase activity.

This study reports new isolates belonging to thermophilic *Geobacillus* species. These isolates were investigated in terms of their xylanase and arabinofuranosidase activities. As a result, we determined the isolates have xylanases and arabinofuranosidases with better features compared with the enzymes of their reported relatives. We think that some of these enzymes may meet the needs of industry.

To facilitate further studies with the enzymes, cloning and expression of the genes for both enzymes are in progress.

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