Turkish Journal of Biology

Volume 35 | Number 5 Article 1

1-1-2011

Isolation and characterization of xylanolytic new strains of Anoxybacillus from some hot springs in Turkey

KADRİYE İNAN

SABRİYE ÇANAKÇI

ALİ OSMAN BELDÜZ

Follow this and additional works at: https://journals.tubitak.gov.tr/biology

Part of the Biology Commons

Recommended Citation

İNAN, KADRİYE; ÇANAKÇI, SABRİYE; and BELDÜZ, ALİ OSMAN (2011) "Isolation and characterization of xylanolytic new strains of Anoxybacillus from some hot springs in Turkey," *Turkish Journal of Biology*: Vol. 35: No. 5, Article 1. https://doi.org/10.3906/biy-1003-75
Available at: https://journals.tubitak.gov.tr/biology/vol35/iss5/1

This Article is brought to you for free and open access by TÜBİTAK Academic Journals. It has been accepted for inclusion in Turkish Journal of Biology by an authorized editor of TÜBİTAK Academic Journals. For more information, please contact academic.publications@tubitak.gov.tr.

TÜBİTAK

Turk J Biol
35 (2011) 529-542
© TÜBİTAK
doi:10.3906/biy-1003-75

Isolation and characterization of xylanolytic new strains of *Anoxybacillus* from some hot springs in Turkey

Kadriye İNAN, Sabriye ÇANAKÇI, Ali Osman BELDÜZ

Department of Biology, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon - TURKEY

Received: 24.03.2010

Abstract: Some hot springs located in the west of Turkey were investigated regarding the presence of xylanolytic thermophilic microorganisms. Based on phenotyping characteristics and 16S rRNA gene sequence analysis, 9 of these xylanolytic isolates belonged to the genus *Anoxybacillus*, and grew optimally at about 50-60 °C on nutrient agar. The 16S rRNA gene sequence analysis showed that these isolates resembled the *Anoxybacillus* species ≥97 and these isolates are members of the genus *Anoxybacillus.* Based on the DNA-DNA hybridization study, SDS-PAGE profile, and biochemical and physiological features, I3, CT1Sari, and BT2.1 isolates are new strains of *Anoxybacillus gonensis*; I4.2 and B9.3 isolates are new strains of *A. voinovskiensis*; and I4.1, AC26, ACT14, and ACT2Sari isolates are new strains of *A. kestanbolensis*. The presence of xylanase activities, their optimum temperature, pH stability, and optimum pH were also investigated. The isolates ACT2Sari and ACT14 had the highest temperature optima (75 °C), and I3, CT1Sari, BT2.1, ACT2Sari, and ACT14 had the highest pH optima (pH 9.0) of xylanase. The xylanases of I3, CT1Sari, BT2.1, I4.1, ACT2Sari, AC26, and ACT14 were optimally active both at alkaline pH and elevated temperature, and xylanases of I4.1, ACT2Sari, AC26, and ACT14 also were stable at alkaline pH.

Key words: Alkaline xylanase, thermophiles, *Anoxybacillus*, 16S rRNA, DNA-DNA hybridization

Türkiye'nin bazı kaplıcalarından ksilan kullanan yeni *Anoxybacillus* suşlarının izolasyonu ve karakterizasyonu

Özet: Türkiye'nin batısında bulunan bazı kaplıcalarda, ksilan kullanan termofilik mikroorganizmaların varlığı araştırıldı. Fenotipik özelliklerine ve 16S rRNA gen dizi analizi verilerine dayanarak, kaplıcalardan izole edilen ksilan kullanan 9 izolatın *Anoxybacillus* cinsine ait oldukları ve optimum 50-60 °C üreyebildikleri belirlendi. 16S rRNA gen dizi analizine göre, bu izolatların *Anoxybacillus* türlerine ≥97'den daha fazla bir benzerlik gösterdiği ve bu izolatların *Anoxybacillus* cinsine ait oldukları belirlendi. Yapılan DNA-DNA hibridizasyonu, SDS-PAGE analizi, biyokimyasal ve fizyolojik özelliklere dayanarak, I3, CT1Sari ve BT2.1 izolatlarının *Anoxybacillus gonensis*'e; I4.2 ve B9.3 izolatlarının *A. voinovskiensis*'e; I4.1, AC26, ACT14 ve ACT2Sari izolatlarının da *A. kestanbolensis*'e ait yeni suşlar olduklarına karar verildi. İzolatların ksilan aktiveteleri, ksilanazların optimum sıcaklık, optimum pH ve pH kararlılıkları araştırıldı. ACT2Sari ve ACT14 izolatlarının ksilanazları, en yüksek optimum sıcaklığa (75 °C) sahipken; I3, CT1Sari, BT2.1, ACT2Sari ve ACT14 izolatlarının ksilanazları, en yüksek optimum pH'ya (pH 9,0) sahiptirler. I3, CT1Sari, BT2.1, I4.1, ACT2Sari, AC26 ve ACT14 izolatlarının ürettiği ksilanazlarının, hem yüksek sıcaklıkta hem de alkali pH'larda optimum aktiviteye sahip oldukları ve ayrıca I4.1, ACT2Sari, AC26 and ACT14 izolatlarının ksilanazlarının da, alkalin pH'larda yüksek kararlılık gösterdikleri belirlendi.

Anahtar sözcükler: Alkalin ksilanaz, termofilik, *Anoxybacillus,* 16S rRNA, DNA-DNA hibridizasyonu

Introduction

The taxonomy and especially the identification of thermophilic endospore-forming bacteria have generated considerable interest over recent decades. The importance of these bacteria has increased, owing to their potential as a source of thermostable enzymes, including xylanases, proteases, amylases, pullulanases, peroxidases, glucose isomerases, lipases, and DNA restriction enzymes (1-8).

Recently the interest in xylanase has markedly increased due 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 (9). The intended use of xylanases prior to the normal bleaching operation is to significantly reduce or replace the harmful alkaline extraction of hemicellulose and the need for chlorine in the bleaching process without affecting the cellulose fiber strength of paper products, thus reducing the risk of environmental pollution. Xylanase treatment breaks up the cell wall structure, thereby enabling lignin removal in subsequent stages. The fragmentation of the xylan polymer allows the free diffusion of the portions of residual lignin that are covalently attached to xylan (10). Since most of these processes are carried out at high temperature and at alkaline pH, the use of alkaline xylanases with higher temperature optima is considered advantageous, making the use of thermostable alkaline xylanases very attractive (11). The use of thermostable alkaline xylanases may allow manufacturers to cut down the amount of acid required for pH readjustment and the need for cooling and reheating of the large pulp mass, thus saving both time and money. Alkaline xylanases will also find a number of other applications (12). For example, such enzymes may also find potential application in the hydrolysis of xylan-containing waste, both as a method of waste management and as a source of fermentable sugars. Several million tons of xylan-containing waste is released annually throughout the world in the form of agricultural, industrial, and municipal waste. Because of the high solubility of xylan at alkaline pH, alkaline xylanases may have good potential for the hydrolysis of hemicellulosic wastes to fermentable sugars (13).

As a result, the use of thermostable alkaline xylanases is very attractive from economical and technical points of view. However, relatively few microorganisms produce xylanases, which are optimally active either at elevated temperature or alkaline conditions. Xylanases, which are optimally active and stable both at elevated temperature and pH, are rare (14). Therefore, it is important to find novel microorganisms that can produce alkali-active thermostable xylanases without any cellulose production, which can be used in the paper and pulp industry (15). 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 (16). Therefore, thermophilic organisms are of special interest as a source of novel thermostable enzymes (17-20).

The genus *Anoxybacillus* is one of the genera in so called *Bacillus* sensu lato group and the species type of this genus is *Anoxybacillus pushchinensis* DSM 12423^T (21). The genus *Anoxybacillus* has 11 species: *Anoxybacillus pushchinoensis* (21), *Anoxybacillus flavithermus* (21), *Anoxybacillus gonensis* (22), *Anoxybacillus contaminans* (23), *Anoxybacillus kamchatkensis* (24), *Anoxybacillus voinovskiensis* (25), *Anoxybacillus ayderensis* (26), *Anoxybacillus kestanbolensis* (26), *Anoxybacillus rupiensis* (27), *Anoxybacillus amylolyticus* (28), and *Anoxybacillus bogrovensis* (29).

The genus *Anoxybacillus* represents aerobic or facultatively anaerobic, neutrophilic, obligately thermophilic, endospore-forming bacteria. The identification of these species by traditional biochemical techniques is imprecise and time consuming. The sequence comparison of 16S rRNA genes, which is highly conserved throughout prokaryotic organisms, has been most widely used to determine phylogenetic relationships (30,31). However, the 16S rRNA gene has often proved to be insufficient for resolving phylogenetic relationships between some closely related species (32). The determination of total genomic DNA-DNA homology values has persisted as a dominant component of taxonomic analyses. In fact, although many

such techniques exist, the analysis of DNA-DNA hybridization values remains the "gold standard" for defining bacterial species (33-35).

Anoxybacillus species are widely distributed and readily isolated from geothermally heated environments, with a continually increasing industrial interest for their thermostable gene products. Turkey is a country rich in geothermal water sources, largely varying in their temperatures (ranging from 45 to 100 °C) and pH. Recently, thermophilic producers of biotechnologically valuable enzymes have been isolated from Turkish hot springs (36-39). Therefore, isolating the new strains of this novel bacterial genus is not only a taxonomical concern, but also a necessity in order to exploit its biotechnological potential completely.

Materials and methods

Culture medium

Agar plate A consisted of 2% Bacto-trytone, 1% Bacto-yeast extract, 1% NaCl, and 2% agar at pH 7.0. This medium was used for growth of bacteria. Agar plate B contained 1% xylan, 0.2% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$, and 2% agar at pH 7.0. This medium was used for screening xylanase-producing bacteria (9).

Isolation of xylanase-producing micro-organisms and culture maintenance

The thermophilic bacteria used in this study were isolated from mud and water samples of Dikili-Bergama Kaynarca Hot Spring (İzmir), Çamköy Çamur Hot Spring (Aydın), Ömerbeyli Hot Spring (Aydın), and Alangüllü Hot Spring (Aydın) in Turkey. The water temperature of these hot springs is between 70 and 130 °C. The mud and water samples of the hot springs were brought to the laboratory at 4 °C and these samples were poured and spread onto agar plates A. These plates were incubated at 55-60 °C for 2 days. Observed colonies were transferred to agar plates B. After incubating the plates at 55 °C for 36 h, these plates were flooded with 0.1% aqueous Congo red for 15 min followed by repeated washing with 1 mol L^{-1} NaCl (40). Xylanase screening was performed based on the formation of clear zones around the colonies.

Nutrient agar was used to maintain these isolates having xylanase activities. The purity of the obtained strains was confirmed routinely by microscopic examination and uniform colony formation on the agar plates.

Morphological characterization of the isolated strains

Colonial morphology of the bacterial isolates was observed on LB agar medium by direct and stereomicroscopic observations of single colonies. The cell morphology and motility were studied by the light microscopy of native preparations.

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 the agar plates at different incubation periods.

Physiological and biochemical characterization 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 within the pH range 5.0-12.0 in the nutrient broth medium. Media were adjusted to the initial pH indicated with either 1M NaOH or 1M HCl. Growth was determined by measuring the OD_{600} of the cultures at 1, 3, and 7 days.

Four sets of nutrient broth were prepared containing 1%, 2%, 3%, 4%, 5%, 7%, 8%, and 9% of NaCl, respectively. The growth of isolate at different salt concentrations was tested using NB as an organic substrate and a control broth without any NaCl supplementation.

Catalase and oxidase were detected by the method devised by Cowan and Steel (41).

Conventional biochemical tests were determined using the API 20E and API 50CHB system (bioMérieux), according to the manufacturer's instructions. The API test strips were handled according to the manufacturer's instructions (bioMérieux, France) with the exception of incubation temperature (55 °C) for growing thermophilic bacterial isolates. The bacterial colonies of each isolate were diluted in 0.85% NaCl solution. The amount of bacteria was adjusted to 1 McFarland

standard. Two hundred microliters of this solution was transferred into each well of the API 50CHB and 20E panels. In order to prevent any contact with air, the wells were filled up with mineral oil. Then the panels were incubated for 18-24 h at 55 °C. The results of the tests were performed by software based on 'IdBact v. 1.1, G. Kronvall, with Matrix for API20E from bioMérieux, France'.

16S rRNA gene sequence analysis

The 16S rRNA genes were selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal to conserve positions in the 3' and 5' regions of the 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'-ATGGTACCGTGTGACGGGCGGTGTTGTA), corresponded to the complement of positions 1411 to 1393 of the *Escherichia coli* 16S rRNA (10). PCR reaction conditions were carried out according to work by Beffa et al. (42) and the PCR product was cloned to the pGEM-T vector system.

Following the PCR amplification and cloning of the 16S rRNA genes of our isolates, the 16S rRNA sequences were determined with an Applied Biosystems model 373A DNA sequencer using an ABI PRISM cycle sequencing kit (Macrogen, Korea). Sequences consisting of about 1400 nt of 16S rRNA gene were determined. These sequences were compared with those contained within GenBank (43) using BLAST search (44). The 16S rRNA gene sequences of the species most closely related to our isolates were retrieved from the database. The retrieved sequences were aligned using the Clustal X program (45) and manually edited. Phylogenetic trees were constructed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis version 4.0 (MEGA 4.0) (46) (Figure 1).

SDS-PAGE analysis

Extracts from cells growing actively on the nutrient broth medium were obtained according to the method by Belduz et al. (47). Protein concentration in the extracts was measured according to the Bradford method (1976) (48) and 40 µg crude extract was loaded per lane. Electrophoresis on 12% SDS-PAGE was carried out, as described by Laemmli (1970) (49). Proteins were stained in a solution that contained coomassie blue R-250 (0.125%), methanol (50%), and acetic acid (10%) for 2-4 h, and visualized by destaining the gels in a solution of methanol (5%) and acetic acid (7%).

G+C content and DNA-DNA hybridization analyses

Genomic DNAs were extracted and purified using the method by Marmur (50). The G + C contents of these DNA were determined by the thermal denaturation method (51) using an Agilent 8453E UV-visible spectrophotometer.

The DNA was isolated by chromatography on the hydroxylapatite. DNA-DNA hybridization was determined at the DSMZ, as described by De Ley et al. (52), with modifications described by Huß et al. and Escara and Hutton (53,54). A Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter was used. Renaturation rates were computed with the TRANSFER.BAS program (55).

Xylanase activity

Xylanase activity was determined in the extracts of the 9 isolates. For this experiment, cells were cultivated in LB containing 1% xylan, at 60 °C for 24 h and culture fluids were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was lyophilized and xylanase activity was assayed by measuring the release of reducing sugar from oat spelt xylan following the dinitrosalicylic acid (DNS) method (56).

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 12.0, containing oat spelt xylan (1%). Stability of the enzyme at different pH values was also studied by incubating the enzyme at various pH values ranging from 5.0 to 12.0 for 36 h at 4 °C and then residual activity was estimated.

The effect of temperature on the enzyme activity was determined by performing the standard assay as mentioned earlier for 10 min at pH 8.0 within the temperature range of 40 to 90 °C.

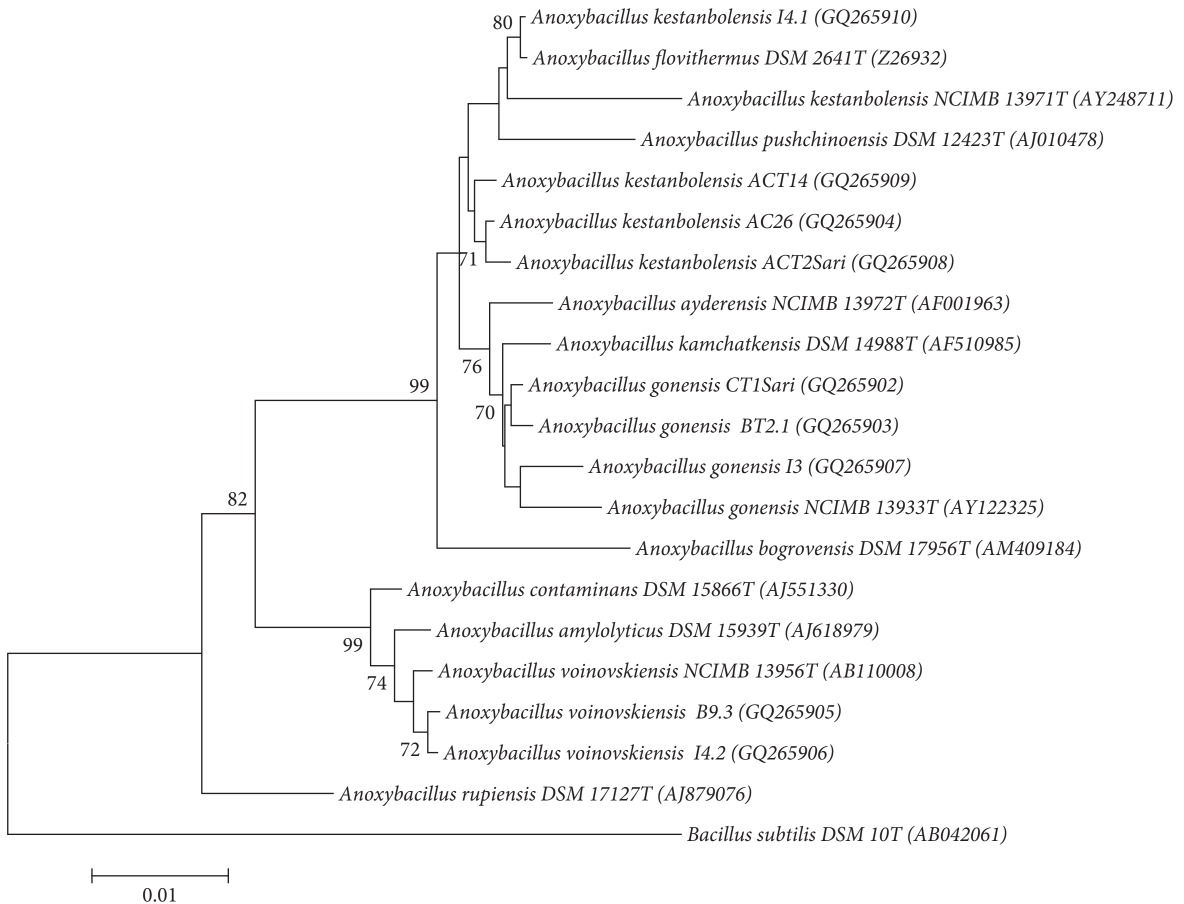

Figure 1. Dendrogram estimated phylogenetic relationships on the basis of 16S rRNA gene sequence data of the thermophilic bacteria isolated from various hot springs in Turkey and some reference strains, using the neighbor-joining method. The accession numbers are given in parentheses. The scale bar represents 1% divergence.

Results and discussion

Isolation of microorganisms

The isolation and purification procedures carried out with both water and mud samples from hot springs allowed the isolation of 9 bacteria with xylanase activities. A total of 9 strains were finally selected and characterized by morphological, physiological, and biochemical characteristics and genetic features. All the strains developed colonies within 48 h of incubation at 55 °C. The diameter of the colonies for the strains varied between 1.0 and 7.0 mm. The cells of the strains appeared gram positive and rod shaped. The cell sizes of isolates I3, CT1Sari, BT2.1, I4.2, B9.3, I4.1, ACT2Sari, AC26, and ACT14 were 0.75 × 3.0 µm, 0.5 × 4.0-6.0 µm, 0.4-0.6 × 7.0 µm, 1.0 × 6.5-8.0 µm, 0.8 × 6.0 µm, 0.8-1.0 × 7.0 µm, 0.3-0.7 × 5.5-8.0 µm, 0.4-0.9 × 8.5 µm, and 0.7 ×5.0-8.9 µm, respectively. On the agar plates, 3 isolates (CT1Sari, I3, B9.3) were yellow, and the others had white colony, flat or convex colonies after 1 day of incubation at 55 °C.

Sporulation was observed after 25 h of incubation at 55 °C in all strains. Subterminal spores were observed in all 9 strains, but central spores were also present in strains I4.2, B9.3, and I4.1.

Physiological and biochemical characterization of isolates

The biochemical and physiological features of the 9 isolates are reported in Table 1. All isolates were gram positive, moderately thermophilic, and motile.

All isolates grew optimally in the NB medium, where the pH values were 5.5-9. The 9 isolates tolerated up to 5% NaCl, and the isolates I3, B9.3, and CT1Sari tolerated up to 8%. All isolates grew well at 30-65 °C, and isolates I4.2 and B9.3 grew well at 70 °C.

Except for I4.2 and B9.3, the isolates hydrolyzed starch, isolates AC26, ACT2Sari, and ACT14 were oxidase negative, and the other isolates were oxidase positive. All isolates were capable of fermenting glucose, fructose, maltose, and sucrose. The physiological properties of these isolates were more or less different from each other. We also determined that some physiological, morphological, and biochemical characteristics of our isolates differentiated from those of the species of the genus *Anoxybacillus* (Table 2).

16S rRNA gene sequence analysis

A total of 1400 nucleotides of the 16S rRNA from 9 isolates were aligned and compared to sequences of related bacteria. The phylogenetic tree was constructed using the neighbor-joining method (Figure 1). On the basis of the 16S rRNA gene sequence analysis, the 9 isolates showed ≥97% similarity to other *Anoxybacillus* species, and this sequence similarity showed that the 9 isolates are members of the genus *Anoxybacillus*. The accession numbers of the 16S rRNA gene sequences of the 9 isolates were assigned by GenBank (Table 3).

SDS-PAGE analysis

The electrophoretic patterns of the soluble cellular proteins of the 9 isolates, as determined by SDS-PAGE, showed that isolates AC26, ACT2Sari, ACT14, and I4.1 were similar to each other. Isolates B9.3 and I4.2 were similar to each other, and BT2.1 and CT1Sari were similar to each other. The other isolate (I3) was not similar to any other or the other 3 groups of isolates (Figure 2).

The SDS-PAGE analysis, and biochemical and physiological features of the 9 isolates showed that isolates AC26, ACT2Sari, ACT14, and I4.1 were similar to each other, and I4.1 was selected as a type isolate. Moreover, isolates B9.3 and I4.2 were similar to each other and I4.2 was selected as a type isolate. Finally, BT2.1 and CT1Sari were similar to each other and CT1Sari was selected as a type isolate. The other isolate (I3) was not similar to the other 3 groups of isolates.

G+C content and DNA-DNA hybridization analyses

Based on the SDS-PAGE analysis, biochemical and physiological features, 4 different isolates (I3, I4.2, I4.1, and CT1Sari) were selected and the G + C content and DNA-DNA hybridization analyses of these isolates were determined (Table 4). The G + C contents of CT6Sari, I3, I4.1. and I4.2 were 47.3%, 46.9%, 42.3%, and 44.9%, respectively.

Based on the 16S rRNA sequence analysis, these thermophilic 4 isolates resembled ≥97% *Anoxybacillus flavithermus, A. gonensis, A. kestanbolensis, A. ayderensis, A. voinovskiensis, A. kamchatkensis, A. pushinoensis,* and *A. contaminans*. Therefore, the DNA-DNA hybridization study was performed among I3, I4.2, I4.1, and CT1Sari, and *Anoxybacillus flavithermus, A. gonensis, A. kestanbolensis, A. ayderensis, A. voinovskiensis, A. kamchatkensis, A. pushinoensis,* and *A. contaminans.*

In this study, we found an 87.9% similarity between CT1Sari and *Anoxybacillus gonensis*, a 94.7% similarity between I3 and *Anoxybacillus gonensis*, a 94.3% similarity between I4.1 and *Anoxybacillus kestanbolensis*, and a 71.4% similarity between I4.2 and *Anoxybacillus voinovskiensis* on the basis of the DNA-DNA hybridization study. Wayne et al. (57) suggested that strains of a species are similar to each other with a 70% or greater similarity. If a strain has less than 70% similarity, it belongs to a different species.

Based on these data we suggest our thermophilic isolates CT1Sari and I3 are a strain of *Anoxybacillus gonensis*, I4.1 is a strain of *Anoxybacillus kestanbolensis*, and I4.2 is a strain of *Anoxybacillus voinovskiensis.*

Xylanase activities

Xylanase activities were detected in strains investigated at the end of 24 h. We investigated the optimal pH and temperatures of the xylanases of our isolates. The xylanases of strains I3, CT1Sari, BT2.1, ACT2Sari, and AC26 showed optimal pH at 9.0, whereas the xylanases of strains I4.1, ACT14, I4.2, and B9.3 represented optimal pH at 8.0, 8.0, 7.0, and 7.0, respectively.

Table 1. Biochemical and physiological characters of thermophilic bacterial strains.

Biochemical and physiological characters	Strain No.								
	I3	B9.3	I4.1	I4.2	C26	ACT2 Sari	ACT14	BT2.1	CT1Sari
Catalase	+	+	+	+	+	+	+	+	+
Amylase	+	-	+	-	+	+	+	+	+
Hydrolysis of gelatine	+	+	+	+	+	+	+	+	+
Voges-Proskauer	+	-	+	+	+	+	-	-	+
MK Test	-	+	+	+	-	-	-	+	+
Indole	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	+	-	+	-	-	-	+	+
Growth with lysozyme present	-	+	-	+	-	+	+	-	-
Growth in 2%-8% NaCl	+	+	+	+	+	+	+	+	+
Growth in 9% NaCl	-	-	-	-	-	-	-	-	-
Growth at pH 4.5	-	+	-	-	-	-	-	-	-
Growth at pH 5	-	+	-	-	-	+	-	-	-
Growth at between pH 5.5 and pH 9	+	+	+	+	+	+	+	+	+
Growth at pH 9.5	-	+	+	-	+	-	-	-	-
Growth at pH 10	-	-	-	-	-	-	-	-	-
Optimum pH	6.5-7	6.5	8-8.5	6.5	7.5-8	8-Jul	7.5-8.5	6.5-7.5	7.5
Growth at 25 °C	-	-	-	-	-	-	-	-	-
Growth at 30-70 °C	+	+	+	+	+	+	+	+	+
Growth at 75 °C	-	-	-	-	-	-	-	-	-
Optimum growth (°C)	60 °C	60 °C	60˚C	60 °C	50 °C	60 °C	55 °C	60 °C	60 °C
Glycerol	+	+	+	+	+	+	+	+	-
Erythritol	-	-	-	+	-	-	-	-	-
D-Arabinose	-	+	-	+	-	-	-	-	-
L-Arabinose	-	-	+	-	+	+	+	-	-
D-Ribose	+	+	+	+	+	+	+	-	+
D-Xylose	+	-	+	-	+	+	+	+	+
L-Xylose	+	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	+	-	-	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	-	-	-	-	-	-
D-Galactose	+	-	+	-	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+
D-Mannose	-	+	-	+	-	-	-	-	+
L-Sorbose	+	-	-	-	-	+	-	-	-

Table 1. (Continued)

Biochemical and physiological characters	Strain No.								
	I3	B9.3	I4.1	I4.2	C26	ACT2 Sari	ACT14	BT2.1	CT1Sari
L-Rhamnose	-	+	-	+	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-
Inositol	-	+	-	+	-	-	-	-	-
Methyl-αD-Glucopyranoside	+	+	+	+	+	+	+	+	-
N-Acetyl glucosamine	+	-	-	-	-	-	-	-	+
Amygdalin	+	+	+	+	+	+	+	-	-
Arbutin	+	+	+	+	+	+	+	-	+
Esculin ferric citrate	+	+	+	+	+	+	+	-	+
Salicin	+	+	+	+	+	+	+	-	+
D-Cellobiose	+	+	+	+	+	+	+	-	+
D-Maltose	+	+	+	+	+	+	+	+	+
D-Lactose	-	-	+	+	+	-	+	-	+
D-Melibiose	+	-	+	-	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-
D-Melezitose	+	-	+	-	+	+	+	+	+
D-Raffinose	+	-	+	-	+	+	+	+	+
Amidon (Starch)	+	-	+	-	+	+	+	+	+
D-Mannitol	+	+	-	+	-	-	-	+	+
D-Sorbitol	-	+	-	+	-	-	-	-	-
Glycogen	+	-	+	-	+	+	+	+	+
Xylitol	-	+	-	+	-	-	-	-	-
Gentiobiose	-	+	+	+	+	+	+	-	-
D-Turanose	+	+	+	+	+	+	+	+	+
D-Lyxose	-	-	-	-	-	-	-	-	-
D-Tagatose	+	-	+	+	+	+	+	-	-
D-Fucose	-	-	-	-	-	-	-	-	-
L-Fucose	-	+	-	+	-	-	-	-	-
D-Arabitol	-	+	-	+	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-
Potassium gluconate	+	-	+	-	+	-	-	+	-
Potassium 2-Ketogluconate	-	-	-	-	-	-	-	-	-
Potassium 5-Ketogluconate	+	+	+	+	+	+	+	+	+
Methyl-αD-mannopyranoside	-	-	-	-	-	-	-	-	-

Table 2. Comparison of the phenotypic characteristics to our nine isolates and the other related *Anoxybacillus* species.

Biochemical and physiological characters	Strain No																
	13	B9.3	14.1	14.2	AC26	ACT2 Sari	ACT14	BT2.1	CT1 Sari	1	2	3	4	5	6	7	8
Growth at pH 5.5	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Growth at pH 10.5	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-
Growth at 30 °C	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	ND	-
Growth at 70 °C	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-
Amylase	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-
Hydrolysis of gelatine	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	-
Nitrate reduction	-	+	-	+	-	-	-	+	+	+	+	+	-	+	-	+	+
Growth in 8% NaCl	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
D-Mannose	-	+	-	+	-	-	-	-	+	+	ND	+	-	+	+	+	-
L-Rhamnose	-	+	-	+	-	-	-	-	-	-	ND	-	-	-	-	-	ND

1 *A. flavithermus* DSM 2641^T (Pikuta et al. 2000); 2 *A. pushchionensis* DSM 12423^T (Pikuta et al. 2000); 3 *A. voinovskiensis* NCIMB 13956^T (Yumoto et al. 2004); 4 *A. gonensis* NCIMB 13933^T (Belduz et al. 2003); 5 *A. kestanbolensis* NCIMB 13971^T (Dulger et al. 2004); 6 *A. ayderensis* NCIMB 13972^T (Dulger et al. 2004); 7 *A. contaminans* DSM 15866^T (De Clerck et al. 2004), 7 *A.kamchatkensis* DSM 14988^T (Kevbrin et al. 2005).
+ positive, - negative, ND not determined

Table 3. Accession numbers of 16S rRNA gene sequences of 9 isolates.

Strain No.	Accession No.
Anoxybacillus gonensis CT1Sari	GQ265902
Anoxybacillus gonensis BT2.1	GQ265903
Anoxybacillus kestanbolensis AC26	GQ265904
Anoxybacillus voinovskiensis B9.3	GQ265905
Anoxybacillus voinovskiensis I4.2	GQ265906
Anoxybacillus gonensis I3	GQ265907
Anoxybacillus kestanbolensis ACT2Sari	GQ265908
Anoxybacillus kestanbolensis ACT14	GQ265909
Anoxybacillus kestanbolensis I4.1	GQ265910

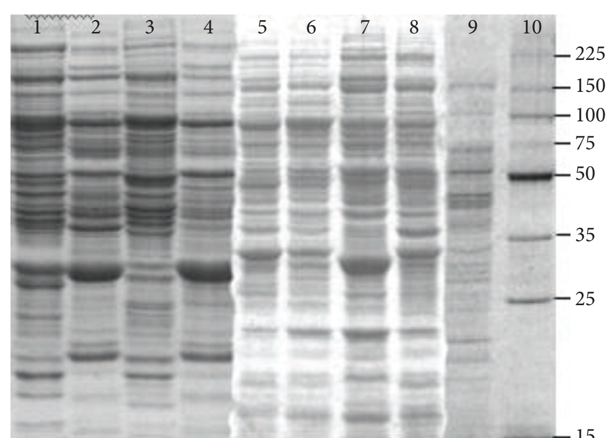

Figure 2. SDS–PAGE whole-cell protein profiles of CT1Sari (lane 1), I4.2 (lane 2), BT2.1 (lane 3), B9.3 (lane 4), AC26 (lane 5), ACT14 (lane 6), I4.1 (lane 7), ACT2Sari (lane 8), I3 (lane 9), Marker (lane 10).

To find the optimum temperature of the xylanolytic activity, xylanases were assayed at pH 8.0 in a range between 40 and 90 °C and the strains ACT2Sari and ACT14 had the highest temperature optima (75 °C) among the strains, whereas the others showed optimal temperatures at 65-70 °C. The optimum temperature and pH of the enzymes of the strains are given in Table 5.

Xylanase stabilities of these strains were studied between pH 5.0 and 12.0 at 4 °C and the enzymes of I4.2 and B9.3 showed about 20%-30% of their original activities after 12 h. The enzymes of I3, CT1Sari, and BT2.1 had 75% residual activity at pH 9.0 and the enzymes activities of these strains were about 10% of their original activity at the others pHs after 36 h. However, the enzymes of I4.1, ACT2Sari, AC26, and ACT14 retained full activity in the pH range of 5.0-12.0 after 48 h.

Environmental strains of thermophilic bacilli from some hot springs in Turkey were isolated and characterized on the basis of some morphologic and physiological properties, 16S rRNA gene sequence analysis, G + C content, and DNA-DNA relatedness.

Stackebrandt and Goebel (35) 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 a different species. On the basis of 16S rRNA gene sequence analysis, our 9 isolates showed ≥97% similarity to other *Anoxybacillus* species, and this sequence similarity showed that the 9 isolates are members of the genus *Anoxybacillus*. However, it is also known that analysis of 16S rRNA sequences

Table 4. DNA-DNA relatedness (%).

	CT1Sari	I3	I4.1	I4.2
A. pushinoensis DSM 12423^T	57.8	43.6	63.8	13.3
A. contaminans DSM 15866^T	40.1	-	-	-
A. flavithermus DSM 2641^T	19.9	-	-	-
A. gonensis NCIMB 13933^T	87.9	94.7	47.2	37.7
A. kestanbolensis NCIMB 13971^T	54.7	20.5	94.3	23.3
A. ayderensis NCIMB 13972^T	61.0	49.3	9.3	15.5
A. voinovskiensis NCIMB	27.9	12.4	-	71.4
A. kamchatkensis DSM 14988^T	57.8	58.6	-	2.3

Table 5. Optimal temperatures and optimal pH of the xylanases of our isolates.

Strain	Optimum pH	Optimum temperature (°C)
I3	9	70
CT1Sari	9	70
BT2.1	9	65
I4.2	7	70
B9.3	7	65
I4.1	8	65
ACT2Sari	9	75
AC26	9	65
ACT14	8	75

may be insufficient to distinguish between some species (58). We determined that some physiological, morphological, and biochemical characteristics of our isolates differed from those of the species of the genus *Anoxybacillus*. The 9 isolates differed from their closest relatives in terms of the following phenotypic characteristics: gelatin hydrolysis, nitrate reduction, pH and temperature range of growth, and spectrum of fermentable carbohydrates. Most type strains of *Anoxybacillus* do not hydrolyze gelatin, but the 9 isolates did. Also no type strain of *Anoxybacillus* grows at ≥5% NaCl but all of our isolates showed positive results for growth at 8% NaCl. Most type strains of *Anoxybacillus* are not able to grow at 30 °C and pH 5.5 but the 9 isolates could grow at 30 °C and pH 5.5. None of the 9 isolates grow at pH 10.5 but most type strains of *Anoxybacillus* are able to grow at this pH. Except for *A. voinovskiensis*, all type strains of *Anoxybacillus* hydrolyze starch, but B9.3 and I4.2 did not. Most type strains of *Anoxybacillus* show positive results for nitrate reduction and also are able to utilize D-mannose. However, I3, I4.1, AC26, ACT2Sari, and ACT14 did not utilize D-mannose and were negative for nitrate reduction. Although none of the type strains of *Anoxybacillus* uses L-rhamnose, B9.3 and I4.2 were able to utilize this sugar.

Cato et al. (59) 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, and biochemical and physiological features, 4 of our isolates formed one group, another 2 isolates formed another group, and another 2 isolates formed another group, but the final isolate (I3) was not similar to the other 3 groups of isolates. I4.1 was selected as type strain of the 1st group (I4.1, AC26, ACT14, ACT2Sari). CT1Sari was designated as type strain of the 2nd group (CT1Sari, BT2.1) and I4.2 was selected as a type strain of the 3rd group (I4.2, B9.3). The strain I3 formed the last group. Also biochemical and physiological features of these isolates supported these grouping. According to biochemical and physiological features of these isolates, it was determined that there were significant similarities within groups, whereas there were differences between groups. While the 3rd group used D-arabinose, D-mannose, L-rhamnose, inositol, D-sorbitol, xylitol, L-fucose, and D-arabitol as sole carbon and energy sources, the other groups did not use these sugars for growth. The 3rd group showed negative reaction for D-xylose, D-galactose, D-melezitose, D-raffinose, starch, and glycogen but the other groups presented positive reaction for these sugars. The last group (I3) was able to utilize L-xylose, L-sorbose, N-asetylglucosamine, and potassium gluconate but the other groups were not able to. The 1st group utilized L-arabinose, whereas the others did not utilize this sugar. The 2nd group was not able to grow on amygdalin and D-tagatose but the others were able to do so. The 2nd group and the last group did not ferment gentiobiose, whereas the others did. Also the 2nd and the 3rd group showed positive results for nitrate reduction, but the other groups showed negative results. The 3rd group was not sensitive to lysozyme, whereas the others were sensitive to lysozyme.

The species concept for prokaryotes has been developed in parallel to the design of laboratory techniques that permitted the retrieval of useful information. For the time being, the analysis of DNA-DNA hybridization values remains the "gold standard" for defining bacterial species (34). Therefore, DNA-DNA hybridization studies were performed among our 4 selected isolates and 7 type strains of *Anoxybacillus* of which the 16S rRNA sequence of the 4 selected isolates resembled ≥97%.

In conclusion, in spite of some differences in phenotypic, morphological, biochemical, and physiological characteristics, based on the DNA-DNA hybridization study, we decided that I3, CT1Sari, and the other similar isolates belong to *Anoxybacillus gonensis*; isolate I4.2 and the other relative isolates belong to *A. voinovskiensis*; and I4.1 and the other relative isolates belong to *A. kestanbolensis*.

So far, many attempts have been made to screen thermophilic and alkaliphilic bacteria from natural sources (37,38,60-66). The use of enzymes stable at a high pH and temperature especially contributes to technical and economical feasibilities of the hydrolysis process (62,67).

Many xylanase-producing thermophilic bacteria have been reported from different laboratories. However, the xylanases from most of these thermophilic bacteria have their optimum pH around neutrality. Although some strains are known to produce xylanases having good activity at pHs

greater than 8.0, the optimum temperature for activity and stability is at or below 50 to 55 °C. So far, only a few xylanases with optimum temperature for activity exceeding 70 °C at or above pH 9.0 have been reported (13). In this study, xylanase production was performed with thermophilic strains isolated from hot-springs. The xylanases of only I4.2 and B9.3 had optimum activity at neutral pH but the other isolates produced xylanases having good activity at pHs greater than 7.0. The optimum pH of xylanases of I3, CT1Sari, BT2.1, ACT2Sari, and AC26 was 9.0; the optimum pH of the others was 8.0. Also xylanases of all strains showed good activity at or over 65 °C. The optimum temperature of ACT2Sari and ACT14 was 75 °C; the optimum temperature of I3, CT1Sari, and I4.2 was 70 °C; and that of the others was 65 °C. Xylanase stabilities of strains were studied between pH 5.0 and 12.0 at 4 °C and the enzymes of I4.1, ACT2Sari, AC26, and ACT14 fully stable at this pH range after 48 h, after 120 h incubation at this pH range, xylanase from AC26 and ACT14 had 95% and I4.1 and ACT2Sari had 88% residual activity (data not shown). The xylanases of I3, CT1Sari, BT2.1, I4.1, ACT2Sari, AC26, and ACT14 were optimally active both at alkaline pH and elevated temperature, and xylanases of I4.1, ACT2Sari, AC26, and ACT14 also were stable at alkaline pH.

The use of alkaline active xylanases allows the direct enzymatic treatment of the alkaline pulp and avoids the cost incurring and time consuming steps of pH re-adjustment. In particular, alkaline xylanases, which are operationally stable at higher temperatures, are more beneficial because of savings in cooling costs and time (13). In this regard, the xylanases of our isolates are expected to operate under conditions close to those of most mills; i.e. high pH and temperature. When compared, the features of our xylanases make them good candidates for biotechnological applications.

This study reports new strains belonging to thermophilic *Anoxybacillus* species. The strains were investigated in term of their xylanase activities. As a result, we determined that these strains have xylanases with better features compared with the enzymes of their reported relatives. Since the thermal and alkaline pH activity of xylanase is a very important property due to its potential applications in several industrial processes, these strains could be a good candidate for biotechnology and these strains may be good sources of novel industrial enzymes.

Acknowledgments

This work was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK), Grant No: 104T286. The doctoral scholarship to Kadriye İnan from TÜBİTAK is duly acknowledged.

Corresponding author:

Ali Osman BELDÜZ

Department of Biology,

Faculty of Arts and Sciences,

Karadeniz Technical University,

61080 Trabzon - TURKEY

E-mail: belduz@ktu.edu.tr.

References

1. Kuisiene N, Raugalas J, Stuknyte M et al. Identification of the genus *Geobacillus* using genus-specific primers, based on the 16S-23S rRNA gene internal transcribed spacer. FEMS 277: 165-172, 2007.
2. Mamo G, Gashe BA, Gessesse A. A highly thermostable amylase from a newly isolated thermophilic Bacillus sp. WN11. J Appl Microbiol 86: 557-560, 1999.
3. Hawumba JF, Theron J, Brözel VS. Thermophilic protease-producing *Geobacillus* from Buranga hot springs in Western Uganda. Curr Microbiol 45: 144-150, 2002.
4. Messaoud BE, Ammar BY, Mellouli L et al. Thermostable pullulanase type I from new isolated *Bacillus thermoleovorans* US105: cloning, sequencing and expression of the gene in E. coli. Enzyme Microb Technol 31: 827-832, 2002.
5. Derekova A, Sjøholm C, Mandeva R et al. Biosynthesis of a thermostable gellan lyase by newly isolated and characterized strain of *Geobacillus stearothermophilus* 98. Extremophiles 10: 321-326, 2006.
6. Soliman NA, Knoll M, Abdel-Fattah YR et al. Molecular cloning and characterization of thermostable esterase and lipase from *Geobacillus thermoleovorans* YN isolated from desert soil in Egypt. Process Biochem 42: 1090-1100, 2007.

7. Maugeri TL, Gugliandolo C, Caccamo D et al. A polyphasic taxonomic study of thermophilic bacilli from shallow, marine vents. Systematic and Applied Microbiology 24: 572-587, 2001.

8. Liu P, Ewis HE, Tai PC et al. Crystal structure of the *Geobacillus stearothermophilus* carboxylesterase Est55 and its activation of prodrug CPT-11. J Mol Biol 367: 212-223, 2007.

9. Cordeiro CAM, Martins MLL, Luciano ABL et al. Production and properties of xylanase from thermophilic *Bacillus* sp. Braz. Arch Biol Techn 45: 413-418, 2002.

10. Somogyi M. Notes on sugar determination. J Biol Chem 195: 19-23, 1952.

11. Sonnleitner B, Fiechter A. Advantages of using thermophiles in biotechnological processes: expectations and reality. Trends in Biotechnol 1: 74-80, 1983.

12. Grant WD, Horikoshi K. Alkaliphiles: ecology and biotechnological applications. In: Herbert RA and Sharp RJ. ed. Molecular Biology and Biotechnology of Extremophiles. New Yoırk: Chapman and Hall; 1992: pp. 143-162.

13. Gessesse A. Purification and properties of two thermostable alkaline xylanases from an alkaliphilic *Bacillus* sp. Appl Environ Microbiol 64: 3533-3535, 1998.

14. Mamo G, Hatti-Kaul R, Mattiasson B. A thermostable alkaline active endo-β-1-4-xylanase from *Bacillus halodurans* S7: Purification and characterization. Enzyme Microb Techn 39: 1492-1498, 2006.

15. Avcioglu B, Eyupaoglu B, Bakir U. Production and characterization of xylanases of a *Bacillus* strain isolated from soil. World J Microb Biot 21: 65-68, 2005.

16. Bergquist PL, Morgan HW. The molecular genetics and biotechnological application of enzyme from extremely thermophilic eubacteria. In: Herbert RA and Sharp RJ ed. Molecular Biology and Biotechnology of Extremophiles. Chapman & Hall; 1992: pp. 44-75.

17. Becker P, Abu-Reesh I, Markossian S et al. Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus* sp. IHI-91 on olive oil. Appl Microbiol Biotechnol 48: 184-190, 1997.

18. Beg OK, Bhushan B, Kapoor M et al. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. J Ind Microbiol Biotechnol 24: 396-402, 2000.

19. Lee D, Koh YS, Kim KJ et al. Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol Lett 179: 393-400, 1999.

20. Touzel JP, O'Donohue M, Debeire P et al. *Thermobacillus xylanilyticus* gen. nov., sp. nov., a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil. Int J Syst Evol Microbiol 50: 315-320, 2000.

21. Pikuta E, Lysenko A, Chuvilskaya N et al. *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. Int J Syst Evol Microbiol 50: 2109-2117, 2000.

22. Belduz AO, Dulger S, Demirbag Z. *Anoxybacillus gonensis* sp. nov., a moderately thermophilic, xylose-utilizing, endospore-forming bacterium. Int J Syst Evol Microbiol 53: 1315-1320, 2003.

23. De Clerck E, Rodriguez-Diaz M, Vanhoutte T et al. *Anoxybacillus contaminans* sp. nov. and *Bacillus gelatini* sp. nov. isolated from contaminated gelatin batches. Int J Syst Evol Microbiol 54: 941-946, 2004.

24. Kevbrin VV, Zengle K, Lysenko AM et al. *Anoxybacillus kamchatkensis* sp. nov., a novel thermophilic facultative aerobic bacterium with a broad pH optimum from the Geyser vallay, Kamchatka. Extremophiles 9: 391-398, 2005.

25. Yumoto I, Hirota K, Kawahara T et al. *Anoxybacillus voinovskiensis* sp. nov., a moderately thermophilic bacterium from a hot spring in Kamchatka. Int J Syst Evol Microbiol 54: 1239-1242, 2004.

26. Dulger S, Demirbag Z, Belduz AO. *Anoxybacillus ayderensis* sp. nov. and *Anoxybacillus kestanbolensis* sp. nov., Int J Syst Evol Microbiol 54: 1499-1503, 2004.

27. Derekova A, Sjoholm C, Mandeva R et al. *Anoxybacillus rupiensis* sp. nov., a novel thermophilic bacterium isolated from Rupi basin (Bulgaria). Extremophiles 11: 577-583, 2007.

28. Poli A, Esposito E, Lama L et al. *Anoxybacillus amylolyticus* sp. nov., a thermophilic amylase producing bacterium isolated from Mount Rittmann (Antarctica). Syst Appl Microbiol 29: 300-307, 2006.

29. Atanassova M, Derekova A, Mandeva R et al. *Anoxybacillus bogrovensis* sp. nov., a novel thermophilic bacterium isolated from a hot spring in Dolni Bogrov, Bulgaria. Int J Syst Evol Microbiol 58: 2359-2362, 2008.

30. Meintanis C, Chalkou KI, Kormas KA et al. Application of *rpoB* sequence similarity analysis, REP-PCR and BOX-PCR for the differentiation of species within the genus *Geobacillus*. Lett Appl Microbiol 46: 395-401, 2008.

31. Woese CR, Winker S, Gutell RR. Architecture of ribosomal RNA: constraints on the sequence of 'tetraloops'. Proc Natl Acad Sci USA 87: 8467-8471, 1990.

32. La Duc MT, Satomi M, Agata N et al. *gyrB* as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. J Microbiol Meth 56: 383-394, 2004.

33. Zeigler DR. Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int J Syst Evol Microbiol 53: 1893-1900, 2003.

34. Stackebrandt E, Frederiksen W, Garrity GM et al. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52: 1043-1047, 2002.

35. Stackebrandt E, Goebel BM. Taxonomic Note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44: 846-849, 1994.

36. Canakci S, Belduz AO, Saha BC et al. Purification and characterization of a highly thermostable α-L-Arabinofuranosidase from *Geobacillus caldoxylolyticus* TK4. Appl Microbiol Biot 75: 813-820, 2007.

37. Canakci S, Inan K, Kacagan M et al. Evaluation of arabinofuranosidase and xylanase activities of *Geobacillus* spp. isolated from some hot springs in Turkey. J Microbiol Biotech 17: 1262-1270, 2007.

38. Kacagan M, Canakci S, Sandalli C et al. Characterization of a xylanase from a thermophilic strain of *Anoxybacillus pushchinoensis* A8. Biologia 63: 599-606, 2008.

39. Yavuz E, Gunes H, Harsa S et al. Identification of extracellular enzyme producing thermophilic bacilli from Balcova (Agamemnon) geothermal site by ITS rRDNA RFLP. J Appl Microbiol 97: 810-817, 2004.

40. Gessesse A, Gashe BA. Production of alkali xylanase by an alkaliphilic *Bacillus* sp. isolated from an alkaline soda lake. J Appl Microbiol 83: 402-406, 1997.

41. Cowan ST, Steel KJ. Manual for the Identification of Medical Bacteria, 2nd ed.: Cambridge University Press. Cambridge; 1974.

42. Beffa T, Blanc M, Lyon PF et al. Isolation of *Thermus* strains from hot composts (60 to 80 °C). Appl Environ Microbiol 62: 1723-1727, 1996.

43. Benson DA, Boguski MS, Lipman DJ et al. GenBank. Nucleic Acids Res 27: 12-17, 1999.

44. Altschul SF, Gish W, Miller W et al. Basic local alignment search tool. J Mol Biol 215: 403-410, 1990.

45. Thompson JD, Gibson TJ, Plewniak F et al. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876-4882, 1997.

46. Tamura K, Dudley J, Nei M et al. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599, 2007.

47. Belduz AO, Lee EJ, Harman JG. Mutagenesis of the cyclic AMP receptor protein of *Escherichia coli:* targeting positions 72 and 82 of the cyclic nucleotide binding pocket. Nucleic Acids Res 21: 1827-1835, 1993.

48. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.

49. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature 227: 680-685, 1970.

50. Marmur J. A Procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3: 208-218, 1961.

51. Mandel M, Marmur J. Use of ultraviolet light temperature profile for determination of the guanine plus cytosine content of DNA. Enzymol 12: 195-206, 1968.

52. De Ley J, Cattoir H, Reynaerts A. The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem 12: 133-142, 1970.

53. Huß VAR, Festl H, Schleifer KH. Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst Appl Microbiol 4: 184-192, 1983.

54. Escara JF, Hutton JR. Thermal stability and renaturation of DNA in dimethyl sulphoxide solutions: acceleration of the renaturation rate. Biopolymers 19: 1315-1327, 1980.

55. Ahmad S, Scopes K, Rees GN et al. *Saccharococcus caldoxylolyticus* sp. nov., an obligately thermophilic, xylose-utilizing, endospore forming bacterium. Int J Syst Evol Microbiol 50: 517-523, 2000.

56. Miller GL. Use of dinitrosalicyclic acid reagent for determination of reducing sugars. Anal Chem 31: 426-428. 1959.

57. Wayne LG, Brenner DJ, Colwell RR et al. Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37: 463-464, 1987.

58. Vandamme P, Plot B, Gillis M et al. Polyphasic taxonomy, a consensus approach to bacterial systematic. Microbiol Rev 60: 407-438, 1996.

59. Cato EP, Hash DE, Holdeman LV et al. Electroforetic study of *Clostridium* species. J Clin Microbiol 15: 688-702, 1982.

60. Bataillon M, Cardinali APN, Duchiron F. Production of xylanases from a newly isolated alkalophilic thermophilic *Bacillus* sp. Biotechnol Lett 20: 1067-1071, 1998.

61. Horikoshi K. Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol R 63: 735-750, 1999.

62. Mamo G, Gessesse A. Purification and characterization of two raw-starch-digesting thermostable amylases from a thermophilic *Bacillus*. Enzyme Microb Tech 25: 433-438, 1999.

63. Niehaus F, Bertoldo C, Kahler M et al. Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biot 51: 711-729, 1999.

64. Haki GD, Rakshit SK. Developments in industrially important thermostable enzymes: a review. Bioresource Technol 89: 17-34, 2003.

65. Yavuz E, Gunes H, Harsa S et al. Identification of extracellular enzyme producing thermophilic bacilli from Balcova (Agamemnon) geothermal site by ITS rDNA RFLP. J Appl Microbiol 97: 810-817, 2004.

66. Sharma A, Pandey A, Shouche YS et al. Characterization and identification of *Geobacillus* spp. isolated from Soldhar hot spring site of Garhwal Himalaya, India. J Basic Microb 48: 1-8, 2008.

67. Yu EKC, Tan LUL, Chan MKH et al. Production of thermostable xylanase by a thermophilic fungus, *Thermoascus aurantiacus*. Enzyme Microb Tech 9: 16-24, 1987.