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A Comparative Study: Taxonomic Grouping of Alkaline Protease Producing Bacilli

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Abstract

Alkaline proteases have biotechnological importance due to their activity and stability at alkaline pH. 56 bacteria, capable of growing under alkaline conditions were isolated and their alkaline protease activities were carried out at different parameters to determine their optimum alkaline protease production conditions. Seven isolates were showed higher alkaline protease production capacity than the reference strains. The highest alkaline protease producing isolates (103125 U/g), E114 and C265, were identified as *Bacillus licheniformis* with 99.4% and *Bacillus mojavensis* 99.8% based on 16S rRNA gene sequence similarities, respectively. Interestingly, the isolates identified as *Bacillus safensis* were also found to be high alkaline protease producing strains. Genotypic characterizations of the isolates were also determined by using a wide range of molecular techniques (ARDRA, ITS-PCR, $(GTG)_5$-PCR, BOX-PCR). These different techniques allowed us to differentiate the alkaliphilic isolates and the results were in concurrence with phylogenetic analyses of the 16S rRNA genes. While ITS-PCR provided the highest correlation with 16S rRNA groups, $(GTG)_5$-PCR showed the highest differentiation at species and intra-species level. In this study, each of the biotechnologically valuable alkaline protease producing isolates was grouped into their taxonomic positions with multi-genotypic analyses.

Key words: 16S rRNA gene sequence, alkaline protease, alkaliphilic *Bacillus*, ARDRA, ITS-PCR, *rep*-PCR

Introduction

Alkaliphilic bacteria, which are also called as extremophiles, can grow at high pH conditions. They split into two groups as alkaliphiles and alkalitolerants. While alkaliphiles grow optimally at pH 9.0 and also at higher alkaline conditions like pH 10.0, they cannot grow at or below pH 7.0. On the other hand, alkalotolerants can grow both at high (like pH 10.0) and neutral pH values. The enzymes of these alkaliphilic bacteria have a high demand for many industrial branches due to their stability at high pH values (Horikoshi, 1999; Kumar and Takagi, 1999). Of these enzymes, alkaline proteases take the lead and have been used in many areas such as detergent, medicine, food, leather, pharmaceuticals, biological waste elimination and textile industry. Alkaliphilic *Bacillus* strains, which are one of the well-known and well-studied alkaline protease producers, secrete very stable alkaline proteases against pH, temperature, and detergent additives (Ito *et al*., 1998; Horikoshi, 1999). In many countries, obtaining legal permission is necessary in order to produce industrial enzymes and the types of microorganisms that produce enzymes with industrial importance should be identified to use the enzymes commercially (Arellano-Carbajal and Olmos-Soto, 2002). Several PCR based nucleic acid fingerprinting methods have been used to characterize and differentiate *Bacillus* strains when 16S rRNA gene sequencing failed to give the information at subspecies and strain level. 16S-23S intergenic transcribed spacer region PCR (ITS-PCR), BOX and $(GTG)_5$-PCR as repetitive element sequence-based PCR (*rep*-PCR) which are powerful methods to screen the several parts of bacterial genome, have been used to identification, differentiation and comparing the bacterial genome diversity (Freitas *et al*., 2008; Cihan, 2013). In the present study, 56 rod shaped bacteria, capable of growing under highly alkaline conditions were isolated from different regions of Turkey. Carbon sources as a nutrient factor and the pH of the culture medium have a critical importance in the alkaline protease production. As a primary purpose of this study, we aimed to determine the optimal alkaline

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production and enzyme activity conditions by using different growth parameters in order to cover the needs for determining different alkaliphilic and alkalotolerant bacterial strains. Thus, two different culture media having two different pH values were used for cultivation and the enzyme activity experiments were carried with a buffer having two different pH. In addition, we aimed to compare and combine the alkaline protease production capacities of the isolates with their phylogenetic data. In this context, besides 16S rRNA gene sequence similarities, amplified rDNA (Ribosomal DNA) restriction analysis (ARDRA), internal transcribed spacer (ITS)-PCR, $(GTG)_5$ and BOX-PCR as a repetitive extragenic palindromic (*Rep*-PCR) were applied as nucleic acid fingerprinting techniques to obtain detailed information about the taxonomic position of the isolates at the subspecies and strain level.

Experimental

Materials and Methods

Strains. In this study, water and soil samples were collected from different areas of Turkey. The isolates used in this study, their origins and their isolation sources are presented in Table I. In order to isolate new alkaliphilic bacilli having alkaline protease activities, samples were mostly collected from extreme environments having alkaline and saline conditions or containing sulfur and soda. For the bacterial isolation, the soil (0.2–0.4 g) and the water samples (0.5 ml) were inoculated in to Nutrient Broth (pH 9.0) and were cultivated at 37°C by shaking at 200 rpm for 48 h. The turbid cultures were diluted with sterile saline solution and transferred onto Skim Milk Agar plates, which include 0.1% glucose, 2% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 7H_2O$, 0.5% skim milk (sterilized separately) (Denizci *et al.*, 2004). After sterilization, the pH of the medium was adjusted to 9.0 by adding 10% Na_2CO_3. The isolates, which gave a clear zone around the colonies due to the hydrolysis of skim milk were selected as an alkaline protease producing strains (Denizci *et al.*, 2004; Tekin *et al.*, 2012).

Following *Bacillus* strains were also used as reference strains; *Bacillus licheniformis* DSM 13, *Bacillus coagulans* DSM 1^T, *Bacillus subtilis* ATCC 6633^T, *Bacillus alcalophilus* DSM 485^T, *Bacillus subtilis* DSM 1971, *Bacillus clausii* DSM 8716^T, *Bacillus cohnii* DSM 6307^T, *Bacillus horikoshii* DSM 8719^T, *Bacillus gibsonii* 8722^T, *Bacillus agaradhaerens* DSM 8721^T, *Bacillus halodurans* DSM 497^T and *Bacillus pseudalcaliphilus* DSM 8725^T. The alkaline protease production capacities of all the isolates and reference strains were qualitatively screened on Skim Milk Agar medium.

Alkaline protease production and quantitative determination of enzyme activity. Two different medium was used for enzyme production. The first medium, which contains casein was prepared according to Gessesse and Gashe (1997) (0.5% Casein, 0.5% Peptone, 0.2% Yeast extract, 0.5% NaCl, 0.02% $MgSO_4 7H_2O$, 0.01% $CaCl_2 2H_2O$, 0.1% K_2HPO_4). The second medium, which contains starch, was prepared according to Denizci *et al.* (2004) (1.0% starch, 0.5% yeast extract, 0.1% K_2HPO_4 and 0.02% $MgSO_4 7H_2O$). The pH of the media was adjusted to pH 7.5 (for alkali-tolerant and facultative alkaliphiles) and 9.5 (for alkaliphiles and obligate alkaliphiles) and the incubation was carried out for 48 h and 72 h. The extracellular alkaline proteases were obtained from the culture supernatant as described by Tekin *et al.* (2012) and the same procedures were applied for the determination of alkaline protease activity. All enzyme activity assays were carried out in triplicate (technical replica) from triplicate cultivations (biological replica) and the results were calculated as mean standard values. Analysis of variance with repeated measures was performed using the Software IBM SPSS Statistics (Version 22, USA). Enzymatic activity means and standard deviation were calculated. Univariate analysis of variance was employed on the data with nutritional supplements (casein or starch)-alkaline protease activity, pH-alkaline protease activity, incubation period-alkaline protease activity, and enzyme reaction buffers having different pH values-alkaline protease activity were tested for significance. Main effects and interaction were also tested for significance.

The parameters for alkaline protease production and activity were summarized at Fig. 1. While determining the alkaline protease production capacities of the isolates, the enzyme activity values per pellet-wet weight (U/g) were determined. The micro molar extinction value of tyrosine, used at enzyme activity formula, was determined by measuring the optic densities of different concentration of tyrosine dilutions at spectrophotometry (660 nm). Tyrosine micro molar extinction value was calculated as 0.0011 µM/ml. One unit of alkaline protease activity was defined as the amount of the enzyme capable of producing 1 µg of tyrosine in 1 min under standard assay conditions.

Morphologic and physiologic characterization of the isolates. Actively growing cells on Nutrient Agar plates (pH 7.0 and 9.0) at 37°C were used for cell and colony morphology analyses. The formation of the spores (spore shape, position in vegetative cell and swelling property) and motility were tested by using 18–24 h Nutrient Broth cultures supplemented with 5 mg/l $MnSO_4 \cdot 4H_2O$ and observed on the phase-contrast microscope (Suzuki *et al.*, 1976). Colony morphology of the isolates determined by using 18 h-old cultures

Table I
Diversity and origin of the bacterial isolates

Bacterial isolates	Sample	Origin
APT1, APT2, APT5, APT8, APT9	Soil	Besevler, Ankara
APT10, APT11, APT12	Soil	Hazar Lake, Diyarbakır coast
APT13a, APT14, APT20b	Mud	Hazar Lake, Diyarbakır coast
APT23, APT24, APT25, APT26	Soil	Sulu Ada, Adrasan, Antalya
APT30	Soil	Acısu Deresi, Baskoy, Kastamonu
APT32	Water	Burdur Lake, Burdur
APT34	Mud	Burdur Lake, Burdur
APT35, APT36	Soil	Burdur Lake, Burdur
APT37, APT38	Water	Güvercinlik Cave, Guneysinir, Konya
APT39, APT40, APT41	Soil	Güvercinlik Cave, Guneysinir, Konya
APT42	Mud	Avlan Lake, Elmalı, Antalya
APT43	Water	Avlan Lake, Elmalı, Antalya
APT44	Mud	Kükürtlü Su, Demre İcmeleri, Antalya
APT47	Water	Kükürtlü Su, Demre İcmeleri, Antalya
APT48	Mud	Burguç Su Kaynagı, Demre, Antalya
TG11, TG20	Soil	Salt Lake, Aksaray
A107, A131, A151, A185	Sediment	Omerbeyli, Germencik, Aydin
A111, A325, A331	Soil	Omerbeyli, Germencik, Aydin
A363	Soil	Yavuzkoy, Salavatli, Aydin
B16	Water	Urganlı, Turgutlu, Manisa
B65	Soil	Urganlı, Turgutlu, Manisa
C83ca, C91, C92	Water	Buharekent, Tekkehamam/Tekkekoy, Denizli
C234, C235, C236, C241, C244, C251, C265	Soil	Buharekent, Tekkehamam/Tekkekoy, Denizli
D311	Water	Doganbey, Seferhisar, İzmir
E114, E287	Sediment	Altinsu, Kozakli, Nevsehir
E215	Soil	Baglica, Kozakli, Nevsehir

on Nutrient Agar plates at 37°C. Gram staining, catalase and amylase activities were carried out according to the methods of Claus and Berkeley (1986). The optimal pH for growth was defined in Nutrient Broth, which was adjusted to different pH values from 7.0 to 13.0 after incubating at 37°C for 24–48 h. The temperature

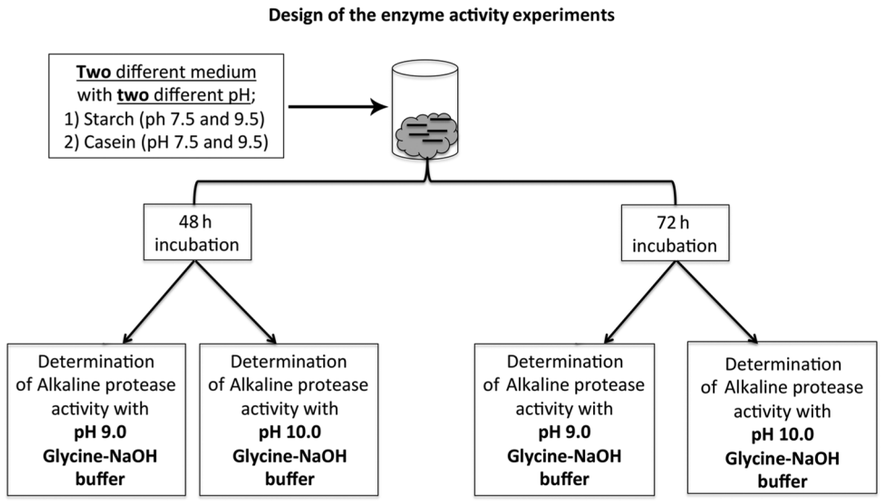

Fig. 1. The design of the enzyme activity experiments.

requirements were tested in Nutrient Broth at pH 10.0 after 24–48 h incubation at different temperatures (5–70°C). The salinity (0–10% NaCl) tolerance for growth was tested in Nutrient Broth and after 24–48 h incubation at 37°C (Nielsen *et al.*, 1995). The growth properties of the isolates were determined according to their optic density at 660 nm. All characterization assays were performed with monocultures in triplicates.

Amplification and sequencing of 16S rRNA gene. Genomic DNA extraction, 16S rRNA gene amplification, purification of the PCR products and sequencing reactions were carried out as previously described (Tekin *et al.*, 2012). In phylogenetic analyses, the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and the evolutionary history was derived using the Neighbor-Joining method (Saitou and Nei, 1987) with the bootstrap values based on 1000 replicates (Felsenstein, 1985). The phylogenetic tree was constructed with the MEGA package version 4 (Tamura *et al.*, 2007).

Amplified ribosomal DNA restriction analysis (ARDRA) of 16S rRNA gene. The same PCR amplicons that used for sequencing reactions were taken into ARDRA analysis of the 16S rRNA gene primed by 27F/1492R (Tekin *et al.*, 2012). The PCR products were digested with the restriction enzymes with Fast digest *Alu*I, *Hae*III and *Taq*I restriction enzymes (MBI Fermentas). ARDRA profiles were detected and statistically analyzed as previously described (Cihan *et al.*, 2011). Finally, the individually examined all the ARDRA analyses were then taken into a cumulative cluster analysis which combined all these tests in a dendrogram by using the GelCompar II software packages (Applied Maths, Belgium). In clustering analyses, according to the presence or absence of DNA bands and also to their densities, the similarity shade limits of 16.66, 33.33, 49.99, 66.66 and 83.3% values were used with GelCompar II software (Applied Maths, Belgium). In this context, the bacteria displaying lower similarities than 97% were denoted as having unique distinctive profiles; the ones having similarities between 97.0% and 100% were determined as sharing similar profiles and then were implied as displaying the same profiles.

PCR based fingerprinting analyses of *Rep* elements and intergenic 16S-23S rRNA gene. Repetitive elements (*Rep*-PCR) genomic fingerprintings (BOXA1- and $(GTG)_5$-PCR) and intergenic transcribed spacers (ITS) between 16S and 23S rRNA genes were performed according to Cihan (2013). Distinctive ITS-PCR, BOXA1 and $(GTG)_5$-PCR fingerprintings were analyzed by the GelCompar II software packages (Applied Maths, Belgium). Similarities of the digitized profiles were calculated using Dice correlation and an average linkage (UPGMA) dendrogram was obtained. The individually examined all the ITS-, $(GTG)_5$- and BOX-PCR fingerprintings were then taken into a cumulative cluster analysis which combined all these tests in a dendrogram by using the GelCompar II software packages (Applied Maths, Belgium). This combined results containing dendrogram was presented in this paper, instead of showing all the three individual cluster analyses of these fingerprinting tests. In clustering analyses according to the presence or absence of DNA bands and also to their densities, the similarity shade limits of 16.66, 33.33, 49.99, 66.66 and 83.3% values were used in GelCompar II software. In this context, the bacteria displaying lower similarities than 83 % were denoted as having unique distinctive profiles, the ones having similarities between 83.3.0% and 99.9% were determined as showing similar profiles and the ones with 100% similarity were implied as displaying the same profiles.

For the all PCR based reactions, the DNA templates were analyzed spectrophotometrically on Nanodrop (NanoDrop 1000 Spectrophotometer V3.7, Thermo Fisher Scientific Inc, Wilmington, DE, USA). Then the concentrations of genomic DNA samples were diluted to 200 ng/µl as stock DNA. For the each PCR based experiment same amount of DNA was used from 200 ng/µl stocks to standardize the band density.

Results

Alkaline protease producing isolates. All of the 56 isolates determined as alkaline protease producers due to growing and producing proteolytic zone on skim milk agar plates at different pH values (7.0, 9.0 and 10.0) as reference *Bacillus* strains. Qualitative proteolytic activity was expressed as a diameter of clear zones in mm (data not shown).

Quantitative alkaline protease production. Enzyme activity values of the isolates and reference strains per pellet-wet weight (U/g) generally were differed between 353-103125 U/g. Isolates E114 and C265 were produced the highest amounts of alkaline protease (103125 U/g) when compared with the other isolates and reference strains. Besides these two isolates APT11, B65, APT43, C251 and C234 were also showed higher alkaline protease production than the reference strains at the different parameters which were summarized in Fig. 1. Alkaline protease production capacities of the seven highest enzyme producing strains and the reference strains were showed in graphs on Fig. 2. Prominently, it's assessed that 70% of the bacteria showed high alkaline protease activity after 48 h incubation. In addition, 76% of the isolates displayed high enzyme activity after incubating at the casein containing medium. Also, 73% of the isolates were carried out the highest enzyme activity measurements at the Glycine-NaOH reaction buffer at pH 9.0. However, it's revealed that

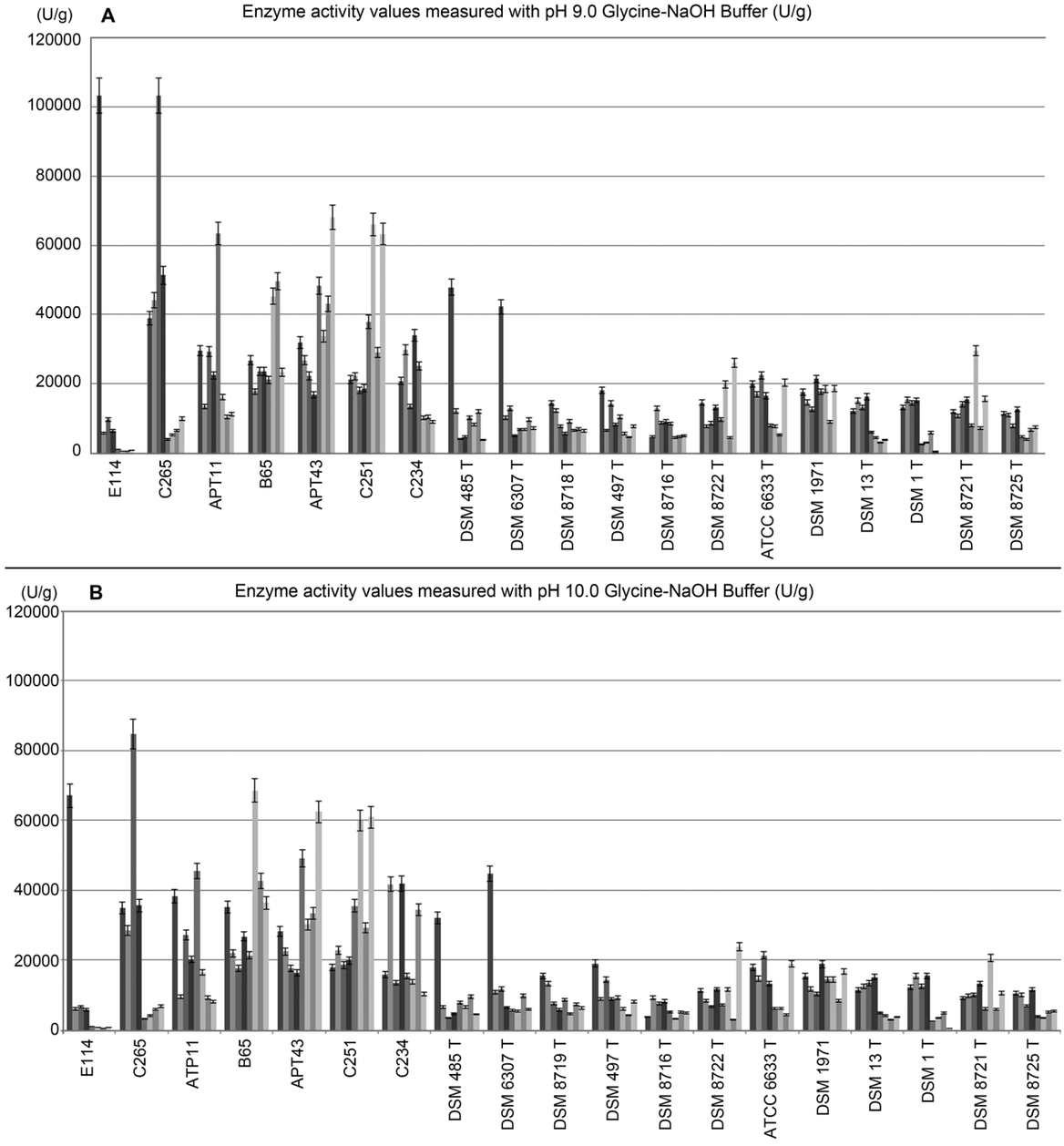

Fig. 2. Alkaline protease activities of the highest enzyme producing bacterial isolates and reference strains with (A) pH 9.0 and (B) 10.0 Glycine-NaOH enzyme reaction buffer.

effect of pH on growth medium wasn't significant. As a conclusion, 56 isolates and 12 reference strains generally showed their highest alkaline protease activity at casein containing medium after 48 h incubation with activity at pH 9.0 Glycine-NaOH buffer. Besides these findings, it can reveal that the pH of the media showed variability according to the physiological requirements of the isolates and reference strains. Additionally, we can conclude that the detection of proteolytic zone at skim milk agar does not reflect the alkaline protease production capacity of the isolates; however, proteolytic zone detection can be assessed to determine the ability of alkaline protease production.

Statistical analysis of quantitative alkaline protease activity. Effect of different carbon (starch) and nitrogen (casein) sources, pH (7.5 and 9.5), incubation period (48–72 h) and Glycine-NaoH buffers adjusted with different pH values (pH: 9.0 and 10.0) on the production of alkaline protease activity is summarized and illustrated in Figure 3A-C. Three sets of experiments were carried out for all the strains and it was observed that there was a drastic statistical difference between

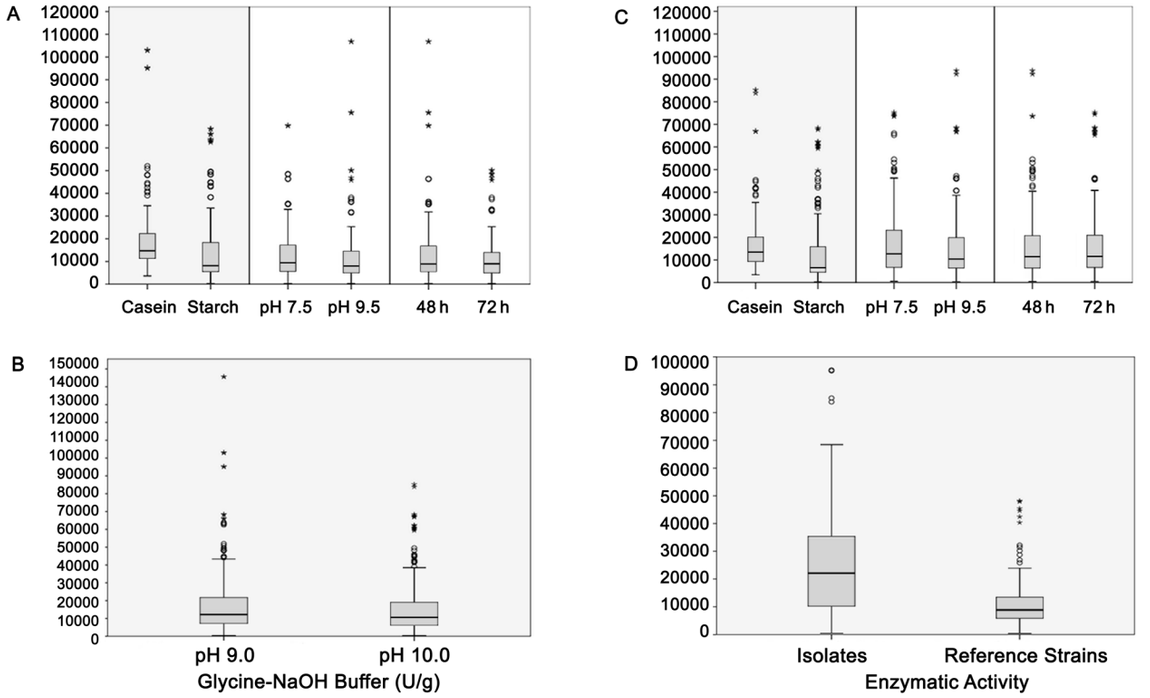

Fig. 3. Statistical analysis of alkaline protease activities.

(A) pH 9.0 Glycine-NaOH buffer; Casein-starch supplement, pH 7.5–9.5 media pH, 48–72 h incubation; (B) pH 10.0 Glycine-NaOH buffer; Casein-starch supplement, pH 7.5–9.5 media pH, 48–72 h incubation; (C) Overall enzyme activity comparisons between Glycine-NaOH buffer having pH 9.0 and 10.0; (D) Overall enzyme activity comparisons between isolates and reference strains.

the casein and starch for alkaline protease production ($p < 0.01$) (Fig. 3A, B). Depending on enzymatic assays performed with both pH 9.0 Glycine-NaOH and pH 10.0 Glycine-NaOH Buffers, it was concluded that supplementation with casein (casein) enhanced production of the enzyme only, and there was no significant differences between pH 9.0 and pH 10.0 Glycine-NaOH buffers (Fig. 3C). Also, the alkaline protease activity between the groups of pH (7.5–9.5) – alkaline protease activity and incubation period (48–72 h) – alkaline protease activity was not found to be statistically significant with both Glycine-NaOH buffers [(pH 9.0 buffer = pH-alkaline protease; $p = 0.776$, incubation period-alkaline protease; $p = 0.076$), (pH 10.0 buffer = pH-alkaline protease; $p = 0.174$, incubation period-alkaline protease; $p = 0.087$)] (Fig. 3A, B). Finally, pH, buffer conditions, and incubation period had no significant effect on alkaline protease activity, but overall alkaline protease activity of the isolates was ~2 fold in comparison with the overall alkaline protease activity of the reference strains ($p < 0.01$) (Fig. 3D).

Morphological and physiological characterization of the isolates. All of the 56 isolates were found to be Gram-positive, endospore-forming and motile bacilli. They were also found positive for catalase activity except strain APT26. Colony morphologies and spore formation differed depending on the species. Forty-seven of the isolates showed amylase activity. Growth was observed at pH 9.0–12.0 (optimum pH 7.0–10.0), at 25–70°C (optimum 30–50°C) and at 0 to 10% concentrations of NaCl (optimum 0–7%) (Data not shown).

Phylogenetic analysis. Sequences of the isolates of 16S rRNA gene were analyzed in order to determine their phylogenetic position. Approximately 1500 bp length 16S rRNA gene sequence data of all the isolates have been deposited in the GenBank databases and all the accession numbers were given in the phylogenetic tree (Fig. 4). The isolates and reference strains were phylogenetically clustered into 28 groups on the basis of their individual 16S rRNA gene sequence homologies to their closest relatives (Fig. 4). Groups of the isolates of 16S rRNA gene and their similarity percents to their closest relatives were also detailed in Table II. The isolates were found to be belonged to the families *Bacillaceae*, *Planococcaceae* and *Paenibacillaceae* from order *Bacillales*. The isolates which clustered into *Bacillaceae* family diverged among 4 different genera (*Bacillus*, *Virgibacillus*, *Lysinibacillus* and *Exiguobacterium*). The isolates belonging to *Planococcaceae* and *Paenibacillaceae* families were also clustered into 2 different genera from *Sporosarcina* and *Paenibacillus*, respectively. In comparison analyses, isolate APT23 displayed more heterogenic 16S rRNA gene sequence similarity to *Bacillus cereus* DSM 31^T (98.3%) and *Bacillus anthracis*

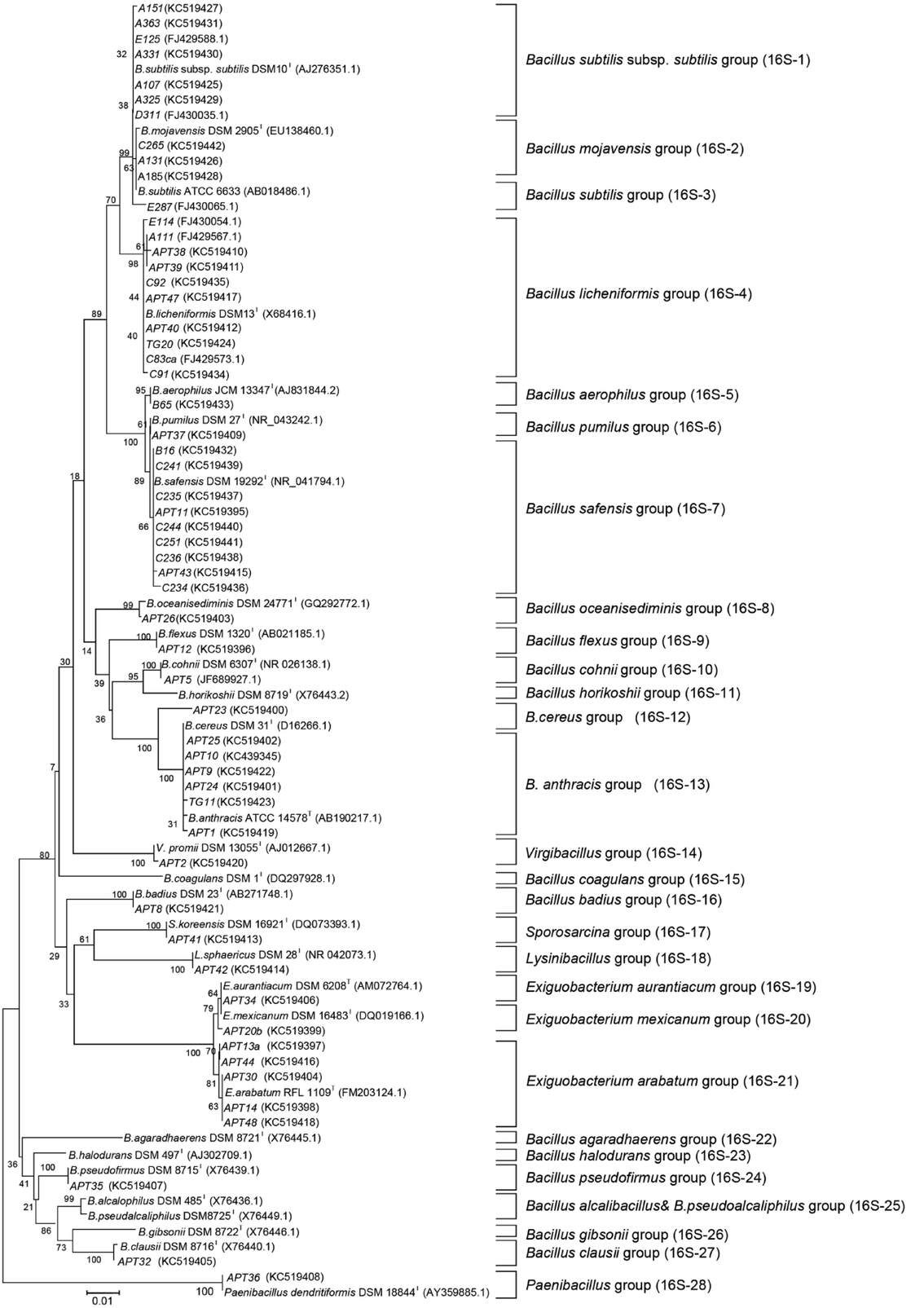

Fig. 4. A phylogenetic tree based on the 16S rRNA gene sequences.

The tree was generated by neighbor-joining method. Bootstrap values (%) are based on 1.000 replicates and shown for branches with more than 30% bootstrap support. Bar indicates 0.01 substitutions per 100 nucleotide positions.

Table II
The species groups of the isolates and the number of the bacteria belonging to these groups derived from 16S rRNA gene nucleotide sequences

16S rRNA Gene Groups	Number of the isolates	Isolates and similarity percentages (%)
Bacillus subtilis	1	E287 (97)
Bacillus subtilis subsp. *subtilis*	7	A363 (99.9), E215 (99.8), A151 (99.9), D311 (99.9), A325 (99.9), A107 (99.8), A331(99.6)
Bacillus mojavensis	3	A185 (99.8), C265 (99.8), A131 (99.8)
Bacillus licheniformis	10	C83ca (99.9), C92 (99.9), C91 (99.8), A111 (99.8), APT38 (99.7), APT39 (99.7), APT47 (99.6), TG20 (99.5), E114 (99.4), APT40 (99.3)
Bacillus aerophilus	1	B65 (100)
Bacillus pumilus	1	APT37 (99.8)
Bacillus safensis	9	B16 (100), APT43 (99.9), C235 (100), C244 (100), C241 (100), C234 (99.9), C236 (99.9), C251 (99.9), APT11 (99.7)
Bacillus oceanisediminis	1	APT26 (99.6)
Bacillus pseudofirmus	1	APT35 (99.9)
Bacillus clausii	1	APT32 (99.7)
Bacillus flexus	1	APT12 (99.9)
Bacillus cohnii	1	APT5 (99.9)
Bacillus cereus	1	APT23 (98.3)
Bacillus anthracis	6	APT10 (100), APT24 (100), APT9 (100), APT25 (100), APT1 (99.9), TG11 (99.9)
Virgibacillus proomii	1	APT2 (99.9)
Sporosarcina koreensis	1	APT41 (99.7)
Lysinibacillus sphaericus	1	APT42 (99.8)
Exiguobacterium arabatum	5	APT14 (99.9), APT13a (99.9), APT44 (99.9), APT48 (99.9), APT30 (99.8)
Exiguobacterium aurantiacum	1	APT34 (99.9)
Exiguobacterium mexicanum	1	APT20b (99.4)
Paenibacillus dendritiformis	1	APT36 (99.1)

(99.2%) reference strains. The rest of the isolates clustered between % 99.1 and 100 similarity values to their type strains as indicated at Table II.

Benchmark of alkaline protease production with phylogenetic clusters. It is obvious that genus *Bacillus* heads a list in the alkaline protease in producing bacteria. Similarly in our study, the highest alkaline protease producing isolates, E114 (103125 U/g) and C265 (103125 U/g) were clustered in *B. licheniformis* and *Bacillus mojavensis*, respectively (Fig. 2 and 4). Other highest alkaline protease producing strain B65 (68506 U/g) was clustered with *Bacillus aerophilus*, and isolates APT11 (63429 U/g), APT43 (68082 U/g), C251 (65939 U/g) and C234 (41834 U/g) were clustered with *Bacillus safensis*. In addition, the isolate APT36 was grouped within *Paenibacillus dendritiformis* (3824 U/g), identified as the lowest alkaline protease producer within all isolates and the reference strains. Notwithstanding, while APT23 was clustered with *B. cereus*, APT1, APT9, APT10, APT24, APT25 and TG11 were clustered with *B. anthracis*, which are class III pathogen strains. Despite of the alkaline protease production capacities of these isolates, as they produced higher amount of protease than most of the strains, they are not sufficient for the industrial enzyme production due to their pathogenic identity (Table IIIA, B). Consequently, 49 isolates except these 7 isolates were clustered within non-pathogenic strains. Within the context of optimal alkaline protease production conditions, it was assessed that while *B. safensis* group isolates produced highest alkaline protease in the starch medium, the isolates belonging to *B. licheniformis*, *B. anthracis* and *Exiguobacterium* groups produced their highest alkaline protease in the casein medium. According to alkaline protease production capacity of the isolates against to reference strains, it is observed that all of the isolates belonging to *B. subtilis* and *B. subtilis* subsp. *subtilis* group displayed higher enzyme production levels than the reference strains of *B. subtilis* DSM 1971 and *B. subtilis* ATCC 6633^T. Similarly, the isolate APT36 grouped as *B. clausii*, and the isolate APT5 belonging to *B. cohnii*, showed higher alkaline protease production capacities than their reference strains *B. clausii* DSM 8716^T and *B. cohnii* DSM 6307^T, respectively (Table IIIA, B).

The *Rep*-PCR and ITS-PCR fingerprintings of the isolates. The isolates and the reference strains were

Table IIIA
16S rRNA groups of the isolates and their individual optimum alkaline protease activity conditions

16S rRNA species groups	Bacteria	Alkaline protease activity (U/g)	The highest alkaline protease activity conditions			
			Medium	pH of the medium	Incubation time (h)	pH of glycine-NaOH buffer
Bacillus subtilis subsp. *subtilis*	A363	44864	Casein	7.5	48	9.0
	E215	43725	Starch	9.5	48	9.0
	A151	29906	Starch	7.5	48	9.0
	D311	53350	Starch	7.5	48	10.0
	A325	27726	Casein	7.5	72	9.0
	A107	22471	Casein	9.5	48	9.0
	A331	22367	Casein	9.5	72	9.0
Bacillus subtilis	E287	41708	Casein	9.5	48	9.0
	DSM1971	21450	Casein	9.5	72	9.0
	ATCC6633	22458	Casein	9.5	48	9.0
Bacillus mojavensis	A185	33950	Starch	7.5	48	9.0
	C265	103125	Casein	9.5	48	9.0
	A131	30425	Casein	7.5	48	9.0
Bacillus licheniformis	C83ca	12844	Casein	7.5	72	10.0
	C92	18116	Casein	9.5	48	9.0
	C91	18700	Casein	7.5	48	10.0
	A111	17233	Casein	9.5	72	9.0
	APT38	7398	Casein	7.5	48	10.0
	APT39	9888	Casein	7.5	48	9.0
	APT47	26308	Casein	7.5	48	9.0
	TG20	10529	Casein	7.5	48	10.0
	E114	103125	Casein	7.5	48	9.0
	APT40	11648	Casein	7.5	48	10.0
	DSM13	16323	Casein	9.5	72	9.0
Bacillus aerophilus	B65	68506	Starch	7.5	72	10.0
Bacillus pumilus	APT37	31656	Casein	9.5	72	9.0
Bacillus safensis	B16	42961	Starch	7.5	48	9.0
	APT43	68082	Starch	9.5	72	9.0
	C235	37492	Starch	9.5	48	9.0
	C244	65939	Starch	7.5	72	9.0
	C241	34490	Starch	7.5	48	10.0
	C234	34375	Starch	9.5	48	10.0
	C236	53763	Starch	9.5	72	9.0
	C251	65939	Starch	7.5	72	9.0
	APT11	63429	Starch	7.5	48	9.0

implemented to the *Rep*-PCR and ITS fingerprinting analyses. These fingerprinting results, the fingerprinting groups and the individual 16S rRNA gene groups were presented in Fig. 5, Table IV, Table V A-B respectively. The isolates, having unique distinctive profiles, were indicated in Table V A-B. In the individual cluster analyses of the *Rep*-PCR containing BOX- and $(GTG)_5$-PCR fingerprintings, totally 33 to 51 clusters were obtained, whereas 28 clusters were obtained from ITS-PCR fingerprintings. According to these results *Rep*-PCR products, especially $(GTG)_5$-PCR fingerprintings were generated a high number of bands giving discriminative information below species and subspecies level between the endospore-forming bacilli isolates when compared with ITS-PCR fingerprintings and 16S rRNA phylogenetic analyses. Additionally to these findings, the clusters obtained with ITS-PCR fingerprints and 16S rRNA phylogenetic analyses showed codependency and sustained the relation between the isolates. In conclusion, the cluster analyses of the *Rep*- and ITS-PCR

Table IIIB
16S rRNA groups of the isolates and their individual optimum alkaline protease activity conditions

16S rRNA species groups	Bacteria	Alkaline protease activity (U/g)	The highest alkaline protease activity conditions			
			Medium	pH of the medium	Incubation time (h)	pH of glycine-NaOH buffer
Bacillus pseudofirmus	APT35	38019	Casein	9.5	48	9.0
Bacillus clausii	APT32	34035	Starch	9.5	48	9.0
	DSM 8716^T	12950	Casein	7.5	72	9.0
Bacillus flexus	APT12	31549	Casein	9.5	48	9.0
Bacillus cohnii	APT5	15400	Casein	7.5	48	10.0
	DSM 6307^T	44688	Casein	7.5	48	10.0
Bacillus cereus	APT23	14491	Casein	7.5	48	9.0
Bacillus anthracis	APT10	20689	Casein	9.5	48	9.0
	APT24	22756	Casein	9.5	72	9.0
	APT9	23623	Casein	7.5	48	9.0
	APT25	16800	Casein	9.5	48	9.0
	APT1	20442	Casein	9.5	48	9.0
	TG11	29081	Casein	7.5	48	9.0
Bacillus badius	APT8	24420	Casein	7.5	48	9.0
Virgibacillus proomii	APT2	22358	Casein	9.5	48	9.0
Sporosarcina koreensis	APT41	13180	Casein	7.5	48	10.0
Lysinibacillus sphaericus	APT42	27762	Starch	9.5	48	10.0
Exiguobacterium aurantiacum	APT34	20006	Casein	9.5	72	9.0
Exiguobacterium mexicanum	APT20b	8054	Casein	9.5	48	10.0
Exiguobacterium arabatum	APT14	9598	Casein	7.5	48	9.0
	APT13a	24844	Casein	7.5	48	9.0
	APT44	36300	Casein	9.5	48	9.0
	APT48	13292	Casein	7.5	48	10.0
	APT30	16454	Casein	7.5	48	10.0
Paenibacillus dendritiformis	APT36	3824	Casein	9.5	48	9.0
B. alcaliphilus	DSM 486^T	47850	Casein	7.5	48	9.0
B. pseudalcaliphilus	DSM 8715^T	12668	Casein	9.5	72	9.0
B. agaradhaerens	DSM 8721^T	29578	Starch	7.5	72	9.0
B. halodurans	DSM 497^T	18993	Casein	7.5	48	10.0
B. horikoshii	DSM 8719^T	15461	Casein	7.5	48	10.0
B. gibsonii	DSM 8722^T	26171	Starch	9.5	72	9.0
B. coagulans	DSM 1^T	15538	Starch	7.5	72	9.0

Table IV
Number of clusters for both isolates and reference strains obtained from individual 16S rRNA genes, ARDRA profiles and ITS-, BOX- and GTG-PCR DNA fingerprintings

Number of	16S rRNA	ITS-PCR	BOX-PCR	GTG-PCR	*Alu*I	*Taq*I	*Hae*III
Standard Clusters	6	7	5	10	6	3	0
Isolate Clusters	22	21	28	41	20	21	27
Total Clusters	28	28	33	51	26	24	27

fingerprintings allowed us to differentiate these isolates and reference strains genetically from each other.

***Alu*I, *Hae*III and *Taq*I-ARDRA analyses of the *Anoxybacillus* isolates.** The amplified PCR products of the isolates were subjected to digestion with *Alu*I, *Hae*III and *Taq*I restriction enzymes in comparison with reference strains. The individual *Alu*I-, *Hae*III- and *Taq*I-ARDRA cluster analyses of the digitized band-

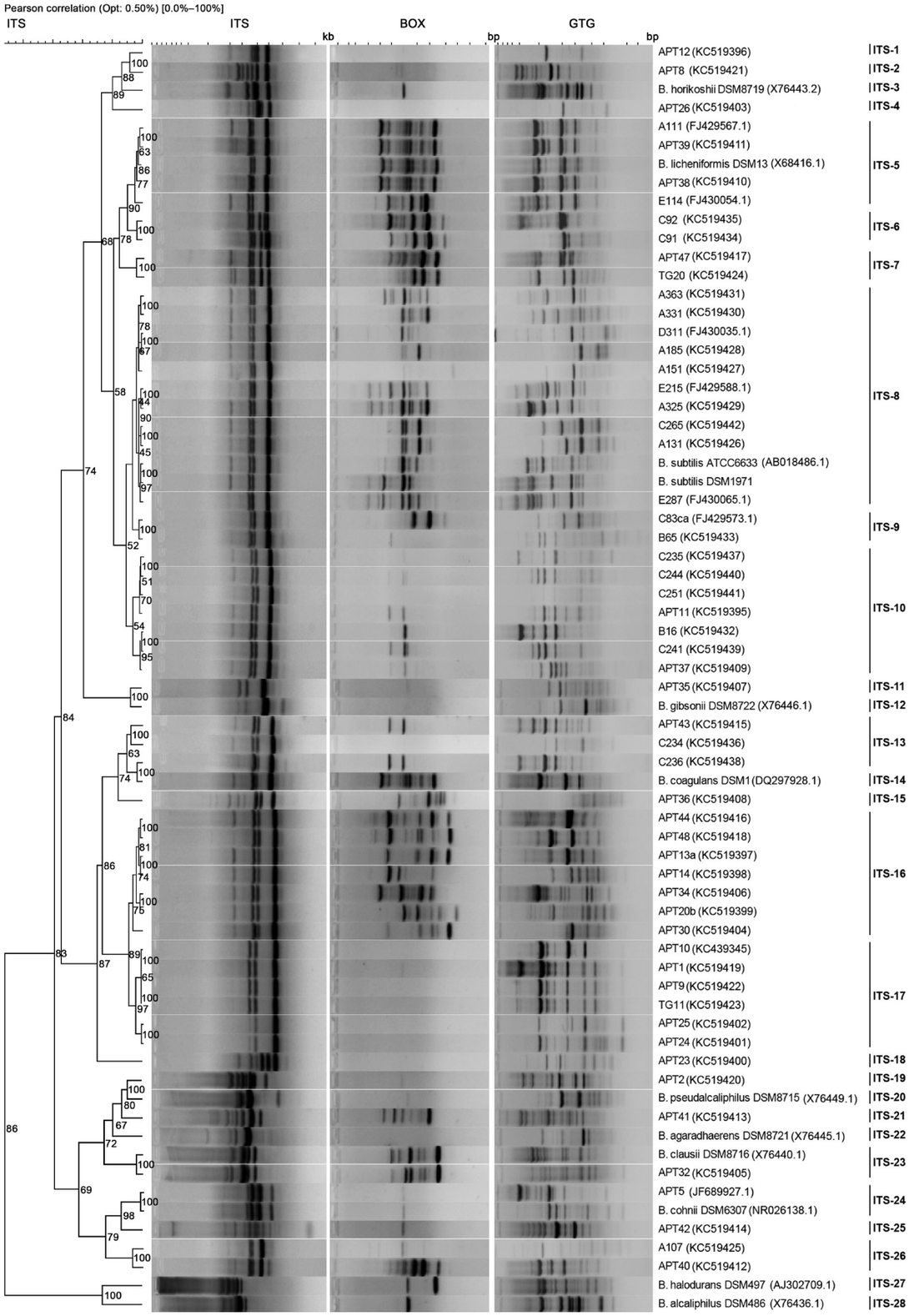

Fig. 5. The cumulative cluster analysis of representative digitized banding patterns, generated by ITS-, BOX- and GTG-PCR profiles from isolates and reference strains.

The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. Due to the correlation between 16S rRNA groups, the numbers of clusters obtained from the ITS-PCR cumulative analysis were indicated in the right side of the figure.

Table VA
Individual *Rep*- and ITS-PCR fingerprinting groups against to 16S rRNA groups

16S rRNA species groups	Isolate/reference strain	16S rRNA group	ITS group	BOX group	GTG group
Bacillus subtilis subsp. *subtilis*	A363	16S-1	ITS-8	BOX-27	GTG-44
	E215	16S-1	ITS-8	BOX-28*	GTG-44
	A151	16S-1	ITS-8	BOX-25*	GTG-16*
	D311	16S-1	ITS-8	BOX-30	GTG-45
	A325	16S-1	ITS-8	BOX-11	GTG-41*
	A107	16S-1	ITS-26	BOX-31*	GTG-46*
	A331	16S-1	ITS-8	BOX-11	GTG-1*
Bacillus subtilis	E287	16S-2	ITS-8	BOX-29*	GTG-43*
	DSM1971	–	ITS-8	BOX-27	GTG-42*
	ATCC6633	16S-2	ITS-8	BOX-30	GTG-45
Bacillus mojavensis	A185	16S-3	ITS-8	BOX-26	GTG-10
	C265	16S-3	ITS-8	BOX-26	GTG-10
	A131	16S-3	ITS-8	BOX-26	GTG-10
Bacillus licheniformis	C83ca	16S-4	ITS-9	BOX-10	GTG-14*
	C92	16S-4	ITS-6	BOX-9	GTG-21
	C91	16S-4	ITS-6	BOX-10	GTG-12*
	A111	16S-4	ITS-5	BOX-4	GTG-22
	APT38	16S-4	ITS-5	BOX-4	GTG-22
	APT39	16S-4	ITS-5	BOX-4	GTG-22
	APT47	16S-4	ITS-7	BOX-8*	GTG-24*
	TG20	16S-4	ITS-7	BOX-7	GTG-20*
	E114	16S-4	ITS-5	BOX-9	GTG-21
	APT40	16S-4	ITS-26	BOX-7	GTG-25*
	DSM13	16S-4	ITS-5	BOX-5*	GTG-22
Bacillus aerophilus	B65	16S-5	ITS-9	BOX-24*	GTG-9*
Bacillus pumilus	APT37	16S-6	ITS-10	BOX-32	GTG-6*
Bacillus safensis	B16	16S-7	ITS-10	BOX-32	GTG-4
	APT43	16S-7	ITS-13	BOX-33	GTG-7
	C235	16S-7	ITS-10	BOX-23	GTG-3
	C244	16S-7	ITS-10	BOX-23	GTG-4
	C241	16S-7	ITS-10	BOX-32	GTG-4
	C234	16S-7	ITS-13	BOX-14	GTG-3
	C236	16S-7	ITS-13	BOX-33	GTG-7
	C251	16S-7	ITS-10	BOX-23	GTG-5
	APT11	16S-7	ITS-10	BOX-33	GTG-5

The isolates, having unique distinctive profiles, were indicated with "*"

ing patterns derived from the isolates and the reference strains can be seen in Fig. 6. The numbers of clusters obtained from these three ARDRA profiles and the isolates, having unique distinctive profiles, were shown in Table VIA and VIB. In individual cluster analysis based on the *Alu*I-, *Hae*III- and *Taq*I-ARDRA profiles of the amplified 16S rRNA genes, totally 26, 27 and 24 clusters were observed when the presence or absence of the restriction fragments and also the density of these DNA bands were considered. The highest group numbers were determined by *Hae*III-ARDRA analyses. However, *Hae*III-ARDRA band patterns did not show any correlation with 16S rRNA clusters. Both *Hae*III- and *Taq*I-ARDRA groups showed a similar restriction band patterns between phylogenetically distinct strains and reference strains, and could not give any discriminative result even in genus level. This can be the result of the evolutionary conserved property of 16S rRNA gene. On the contrary to *Hae*III- and *Taq*I-ARDRA analyses, *Alu*I-ARDRA analysis was found to be superior on the

Table VB
Individual *Rep*- and ITS-PCR fingerprinting groups against to 16S rRNA groups

16S rRNA species groups	Isolate/reference strain	16S rRNA group	ITS group	BOX group	GTG group
Bacillus oceanisediminis	APT26	16S-8	ITS-4*	BOX-14	GTG-15*
Bacillus pseudofirmus	APT35	16S-24	ITS-11*	BOX-14	GTG-11*
Bacillus clausii	APT32	16S-27	ITS-23	BOX-2*	GTG-19*
	DSM8716	16S-27	ITS-23	BOX-1*	GTG-26*
Bacillus flexus	APT12	16S-9	ITS-1*	BOX-14	GTG-13*
Bacillus cohnii	APT5	16S-10	ITS-24	BOX-14	GTG-51*
	DSM6307	16S-10	ITS-24	BOX-14	GTG-38*
Bacillus cereus	APT23	16S-12	ITS-18*	BOX-14	GTG-37*
Bacillus anthracis	APT10	16S-13	ITS-17	BOX-14	GTG-36*
	APT24	16S-13	ITS-17	BOX-14	GTG-35
	APT9	16S-13	ITS-17	BOX-14	GTG-33
	APT25	16S-13	ITS-17	BOX-14	GTG-35
	APT1	16S-13	ITS-17	BOX-14	GTG-34*
	TG11	16S-13	ITS-17	BOX-14	GTG-33
Bacillus badius	APT8	16S-16	ITS-2*	BOX-14	GTG-28*
Virgibacillus proomii	APT2	16S-14	ITS-19*	BOX-14	GTG-50*
Sporosarcina koreensis	APT41	16S-17	ITS-21*	BOX-12*	GTG-47*
Lysinibacillus sphaericus	APT42	16S-18	ITS-25*	BOX-14	GTG-2*
Exiguobacterium aurantiacum	APT34	16S-19	ITS-16	BOX-17*	GTG-39*
Exiguobacterium mexicanum	APT20b	16S-20	ITS-16	BOX-18*	GTG-48*
Exiguobacterium arabatum	APT14	16S-21	ITS-16	BOX-19*	GTG-29
	APT13a	16S-21	ITS-16	BOX-22	GTG-27
	APT44	16S-21	ITS-16	BOX-20*	GTG-27
	APT48	16S-21	ITS-16	BOX-21*	GTG-30*
	APT30	16S-21	ITS-16	BOX-22	GTG-29
Paenibacillus dendritiformis	APT36	16S-28	ITS-15*	BOX-13*	GTG-17*
B. alcaliphilus	DSM486	16S-25	ITS-28*	BOX-16*	GTG-49*
B. pseudalcaliphilus	DSM8715	16S-25	ITS-20*	BOX-14	GTG-18*
B. agaradhaerens	DSM8721	16S-22	ITS-22*	BOX-14	GTG-31*
B. halodurans	DSM497	16S-23	ITS-27*	BOX-3*	GTG-40*
B. horikoshii	DSM8719	16S-11	ITS-3*	BOX-15*	GTG-8*
B. gibsonii	DSM8722	16S-26	ITS-12*	BOX-14	GTG-32*
B. coagulans	DSM1	16S-15	ITS-14*	BOX-6*	GTG-23*

The isolates, having unique distinctive profiles, were indicated with "*"

other restriction enzyme digestions for differentiating the reference strains within 6 unique clusters. Also, the differences at species and genus level were correlated with 16S rRNA phylogenetic groups.

Discussion

Because of the high value of the alkaline proteases, many new data regarding the alkaline protease producing *Bacillus* strains have been publishing during the last years (Niyonzima and More, 2014; Sari *et al.*, 2015). Especially *B. licheniformis*, *B. subtilis*, *B. amyloliquifaciens* and *B. mojavensis* are the most preferred alkaline protease producing *Bacillus* species due to their high enzyme production capacities and their non-toxic properties. Some of the alkaline proteases, produced by *B. licheniformis* were patented and they have been widely using as laundry detergent additives (Kumar and Takagi, 1999). Also, Haddar *et al.* (2009) announced that serine alkaline proteases produced from *B. mojavensis*, are effaceable for the industrial usage. Likewise, in our study the highest alkaline protease producer strains E114 and C265 were identified as *B. licheniformis* and

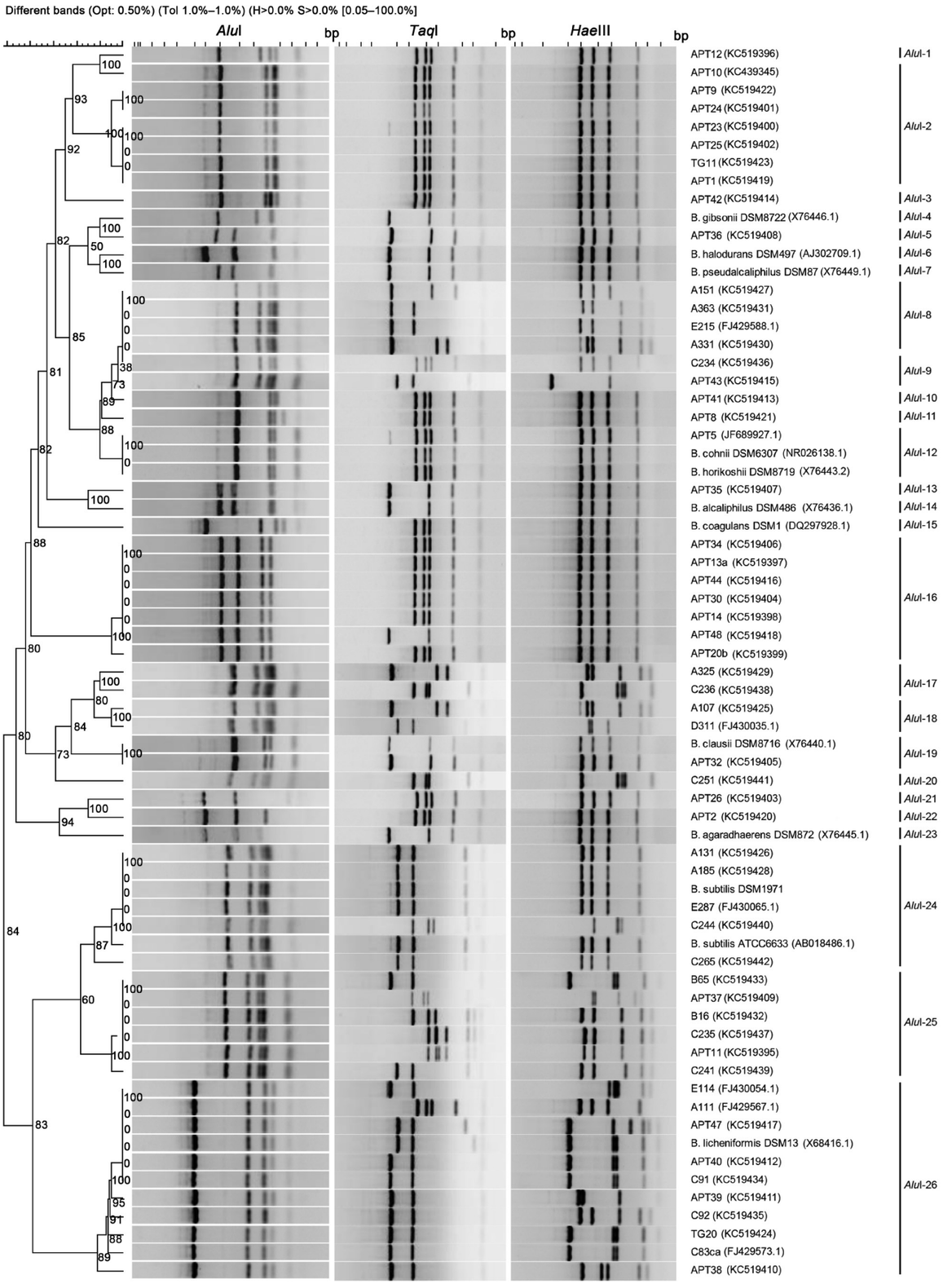

Fig. 6. The cumulative cluster analysis of representative digitized banding patterns, generated by ARDRA profiles from isolates and reference strains.

The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. Due to the correlation between 16S rRNA groups, the numbers of clusters obtained from the *Alu*I profile cumulative analysis were indicated in the right side of the figure.

Table VIA
Individual ARDRA profile groups against to 16S rRNA groups

16S rRNA species groups	Bacteria	16S rRNA group	*Alu*I group	*Taq*I group	*Hae*III Group
Bacillus subtilis subsp. *subtilis*	A363	16S-1	*Alu*I-8	*Taq*I-5	*Hae*III-1*
	E215	16S-1	*Alu*I-8	*Taq*I-5	*Hae*III-14*
	A151	16S-1	*Alu*I-8	*Taq*I-17*	*Hae*III-10
	D311	16S-1	*Alu*I-18	*Taq*I-2	*Hae*III-5*
	A325	16S-1	*Alu*I-17	*Taq*I-8	*Hae*III-9*
	A107	16S-1	*Alu*I-18	*Taq*I-8	*Hae*III-17*
	A331	16S-1	*Alu*I-8	*Taq*I-8	*Hae*III-15*
Bacillus subtilis	E287	16S-2	*Alu*I-24	*Taq*I-1	*Hae*III-26*
	DSM1971	–	*Alu*I-24	*Taq*I-1	*Hae*III-6
	ATCC6633	16S-2	*Alu*I-24	*Taq*I-1	*Hae*III-6
Bacillus mojavensis	A185	16S-3	*Alu*I-24	*Taq*I-2	*Hae*III-6
	C265	16S-3	*Alu*I-24	*Taq*I-2	*Hae*III-6
	A131	16S-3	*Alu*I-24	*Taq*I-2	*Hae*III-6
Bacillus licheniformis	C83ca	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-22
	C92	16S-4	*Alu*I-26	*Taq*I-5	*Hae*III-25*
	C91	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-22
	A111	16S-4	*Alu*I-26	*Taq*I-15*	*Hae*III-10
	APT38	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-3*
	APT39	16S-4	*Alu*I-26	*Taq*I-5	*Hae*III-12*
	APT47	16S-4	*Alu*I-26	*Taq*I-2	*Hae*III-23*
	TG20	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-22
	E114	16S-4	*Alu*I-26	*Taq*I-7*	*Hae*III-7*
	APT40	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-24
	DSM13	16S-4	*Alu*I-26	*Taq*I-4*	*Hae*III-24
Bacillus aerophilus	B65	16S-5	*Alu*I-25	*Taq*I-6	*Hae*III-19*
Bacillus pumilus	APT37	16S-6	*Alu*I-25	*Taq*I-22*	*Hae*III-13*
Bacillus safensis	B16	16S-7	*Alu*I-25	*Taq*I-24	*Hae*III-16*
	APT43	16S-7	*Alu*I-9	*Taq*I-3	*Hae*III-27*
	C235	16S-7	*Alu*I-25	*Taq*I-23	*Hae*III-11
	C244	16S-7	*Alu*I-24	*Taq*I-24	*Hae*III-20*
	C241	16S-7	*Alu*I-25	*Taq*I-3	*Hae*III-11
	C234	16S-7	*Alu*I-9	*Taq*I-13*	*Hae*III-8
	C236	16S-7	*Alu*I-17	*Taq*I-21	*Hae*III-2*
	C251	16S-7	*Alu*I-20*	*Taq*I-21	*Hae*III-21*
	APT11	16S-7	*Alu*I-25	*Taq*I-23	*Hae*III-18*

The isolates, having unique distinctive profiles, were indicated with "*"

B. mojavensis, respectively, according to their 16S rRNA gene sequence similarities. Additionally to these species strain B65 identified as *B. aerophilus*, strains APT11, APT43, C251 and C234 were identified as *B. safensis*. Both *B. aerophilus* and *B. safensis* species previously have not been defined as alkaline protease producing *Bacillus* species. Therefore, in the scope of this study, we conclude that these species may be new alkaline protease producing *Bacillus* species and also can be a potential for new alkaline protease sources. Their non-pathogenic property also supports their industrial usage.

Due to its evolutionary protected property, 16S rRNA gene sequences provide distinguishing the microorganisms at the genus level but also its conserved property fails while differentiating the closely related species at subspecies level (Clarridge, 2004). Therefore, when 16S rRNA gene sequence similarities show 97.0% or more similarity within the closest relative species, the Ad Hoc Committee recommends the DNA-DNA hybridizations to determine the novel species (Stackebrandt *et al.*, 2002; Logan *et al.*, 2009). In our study, all of the isolates show higher 16S rRNA gene sequence

Table VIB
Individual ARDRA profile groups against to 16S rRNA groups.

16S rRNA species groups	Bacteria	16S rRNA group	*Alu*I group	*Taq*I group	*Hae*III Group
Bacillus oceanisediminis	APT26	16S-8	*Alu*I-21*	*Taq*I-10	*Hae*III-4
Bacillus pseudofirmus	APT35	16S-24	*Alu*I-13*	*Taq*I-20	*Hae*III-8
Bacillus clausii	APT32	16S-27	*Alu*I-19	*Taq*I-19	*Hae*III-8
	DSM8716	16S-27	*Alu*I-19	*Taq*I-20	*Hae*III-8
Bacillus flexus	APT12	16S-9	*Alu*I-1	*Taq*I-10	*Hae*III-4
Bacillus cohnii	APT5	16S-10	*Alu*I-12	*Taq*I-11	*Hae*III-4
	DSM6307	16S-10	*Alu*I-12	*Taq*I-10	*Hae*III-4
Bacillus cereus	APT23	16S-12	*Alu*I-2	*Taq*I-11	*Hae*III-8
Bacillus anthracis	APT10	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	APT24	16S-13	*Alu*I-2	*Taq*I-14*	*Hae*III-4
	APT9	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	APT25	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	APT1	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	TG11	16S-13	*Alu*I-2	*Taq*I-10	*Hae*III-8
Bacillus badius	APT8	16S-16	*Alu*I-11*	*Taq*I-12	*Hae*III-4
Virgibacillus proomii	APT2	16S-14	*Alu*I-22*	*Taq*I-12	*Hae*III-4
Sporosarcina koreensis	APT41	16S-17	*Alu*I-10*	*Taq*I-10	*Hae*III-4
Lysinibacillus sphaericus	APT42	16S-18	*Alu*I-3*	*Taq*I-10	*Hae*III-4
Exiguobacterium aurantiacum	APT34	16S-19	*Alu*I-16	*Taq*I-12	*Hae*III-4
Exiguobacterium mexicanum	APT20b	16S-20	*Alu*I-16	*Taq*I-9	*Hae*III-4
Exiguobacterium arabatum	APT14	16S-21	*Alu*I-16	*Taq*I-9	*Hae*III-4
	APT13a	16S-21	*Alu*I-16	*Taq*I-12	*Hae*III-8
	APT44	16S-21	*Alu*I-16	*Taq*I-9	*Hae*III-8
	APT48	16S-21	*Alu*I-16	*Taq*I-20	*Hae*III-8
	APT30	16S-21	*Alu*I-16	*Taq*I-9	*Hae*III-8
Paenibacillus dendritiformis	APT36	16S-28	*Alu*I-5*	*Taq*I-19	*Hae*III-8
B. alcaliphilus	DSM486	16S-25	*Alu*I-14*	*Taq*I-16	*Hae*III-8
B. pseudalcaliphilus	DSM8715	16S-25	*Alu*I-7*	*Taq*I-18	*Hae*III-8
B. agaradhaerens	DSM8721	16S-22	*Alu*I-23*	*Taq*I-16	*Hae*III-8
B. halodurans	DSM497	16S-23	*Alu*I-6*	*Taq*I-16	*Hae*III-8
B. horikoshii	DSM8719	16S-11	*Alu*I-12	*Taq*I-10	*Hae*III-4
B. gibsonii	DSM8722	16S-26	*Alu*I-4*	*Taq*I-18	*Hae*III-8
B. coagulans	DSM1	16S-15	*Alu*I-15*	*Taq*I-9	*Hae*III-4

The isolates, having unique distinctive profiles, were indicated with "*"

similarities than 97% with their closest relative species. Especially in order to use the high alkaline protease producing strains at industrial purposes, their species have to be determined with hybridizing their DNA to their closest relative species. However, before using this expensive method, their suitable closest reference strains have to determine correctly. In this study, the nucleic acid fingerprinting techniques used allow us to determine the most suitable reference strain and avoid the DNA:DNA hybridization between the less similar strains (Cihan *et al.*, 2011).

Intergenic Transcribed Spacers PCR (ITS-PCR) is one of the most suitable nucleic acid fingerprinting technique by distinguishing the species and intraspecies levels. It provides high range variety according to evolutionary highly protected 16S rRNA gene sequences. In parallel with our study, ITS-PCR groups were better correlated with 16S rRNA groups than $(GTG)_5$ and BOX-PCR groups. Logan *et al.* (2009) and Daffonchio *et al.* (1998a; 1998b) reported that 6 species of the *B. cereus* group (*B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis*) were showed similar ITS band profile. Therefore, they concluded that this technique is not effective to differentiate *B. cereus* species. Similarly, Huang *et al.* (2012) reported that ITS-PCR is not effective to differentiate

B. cereus and *B. subtilis* specie. However, contrary to these studies *B. cereus*, *B. anthracis* and *B. subtilis* species showed different ITS band profiles and distinguished from each other successfully in this study (Fig. 5). Our results also showed that ITS-PCR was not discriminative for *B. subtilis* and *B. mojavensis* species, which were identified by 16S rRNA gene sequences. However, it obviously illuminated the diversity for *B. licheniformis* and *B. safensis* groups at intraspecies level.

The main difference of *Rep*-PCR from the other fingerprinting techniques is that it provides scanning of the repetitive sequences on complete bacterial genomes (Versalovic *et al.*, 1994). The discriminative efficacy of BOX and $(GTG)_5$ elements on taxonomic classification of endospore forming bacilli were showed at various studies (Freitas *et al.*, 2008; Logan *et al.*, 2009; Cihan *et al.*, 2011). According to our results, BOX-PCR profiles correlated with 16S rRNA groups. Especially the species of *B. subtilis*, *B. subtilis* subsp. *subtilis*, *B. licheniformis*, *B. safensis* and the species of genus *Exiguobacterium* discriminated at intraspecies level. However, BOX elements of *B. anthracis* and *B. cereus* could not amplified with the same PCR conditions of other samples. Similarly Freitas *et al.* (2008) reported that BOX elements of some samples were not amplified while the reactions were successful for the other isolates. We concluded that new PCR conditions have to be determined for *B. anthracis* and *B. cereus* species when BOX-PCR was used as a discriminative fingerprinting method. Contrary to the BOX-PCR, $(GTG)_5$-PCR efficiently distinguished the isolates at species and intraspecies level. Especially *B. cohnii*, *B. anthracis* and *Exiguobacterium* groups, which were not discriminated by ITS and BOX-PCR, were distinguished at intraspecies level with a higher resolution. Similarly, Freitas *et al.* (2008) reported that they were carried genomic fingerprint analysis with $(GTG)_5$, BOXA1R and ERIC (Enterobacterial Repetitive Intergenic Consensus) PCR primers on many different *Bacillus* isolates and of these techniques $(GTG)_5$-PCR provided a wide variety of band profiles. Again similarly, at their study $(GTG)_5$-PCR was not efficient grouping the microorganism according to BOX and ERIC-PCRs. Also, De Clerck and De Vos (2004) highlighted the efficiency of $(GTG)_5$-PCR when distinguishing the intraspecies level. ARDRA analyses are carried on evolutionarily highly conserved 16S rRNA gene. Despite the conserved property of 16S rRNA gene, *Alu*I restriction fragments correlated with 16S rRNA gene sequence analyses and the isolates partly distinguished at species and intraspecies level. rRNA genes are organized as multiple gene families and it is known that they express from 1 to 15, different copy numbers (Klappenbach and Dunbar, 2000). The mixed groups obtained by *Hae*III and *Taq*I ARDRA analysis may occur because of the different copy numbers of 16S rRNA gene. Many studies were carried out on ARDRA analysis of 16S rRNA gene with different restriction enzymes. But, according to our knowledge this is the first study analyzing the ARDRA profiles of alkaline protease producing strains belonging to the *Bacillaceae* family. In conclusion, by this study, we determined the alkaline protease production capacities of the each isolates and reference strains according to; their carbon source requirements (casein or starch containing growth media), the effect of growth time on enzyme activities (48 and 72 h incubation) and also the effect of pH on enzyme activities (Glycine-NaOH buffer at pH 9.0 and 10.0) by applying various parameters. Moreover, we identified the taxonomic positions of these numerous endospore-forming bacilli, alkaline protease producing isolates in a polyphasic approach which leads to determine their appropriate taxonomic levels by investigating their phenotypic and genotypic diversity (White *et al.*, 1993; Mora *et al.*, 1998). According to the literature, this is the first report that compares many DNA fingerprint techniques with on alkaline protease production capacities of the *Bacillus* strains. Moreover, many novel *Bacillus* species were introduced as alkaline protease producers and enumerated according to their enzyme production capacities.

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