Journal of Applied Microbiology 2004, **97,** 766–773 doi:10.1111/j.1365-2672.2004.02357.x

Identification of extracellular enzyme producing alkalophilic bacilli from Izmir province by 16S-ITS rDNA RFLP

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2004/0231: received 1 March 2004, revised 12 May 2004 and accepted 12 May 2004

ABSTRACT

G. AKBALIK, H. GUNES, E. YAVUZ, I. YASA, S. HARSA, Z.S. ELMACI AND A.F. YENIDUNYA. 2004.

Aims: To screen industrially important extracellular enzymes from the newly isolated alkalophilic bacilli and to characterize them by phenotypic and 16S-internal transcribed spacer (ITS) rDNA restriction pattern analysis.

Methods and Results: Three different environmental samples, soil, leather and horse faeces, were collected within the province of Izmir. Isolates grown on Horikoshi-I medium for 24 h at 37°C were screened for extracellular enzyme activity by using eight different substrates: birchwood xylan, carboxymethylcellulose, casein, citrus pectin, polygalacturonic acid, soluble starch, and Tween 20 and 80. In total, 115 extracellular enzyme-producing bacilli were obtained. Casein was hydrolysed by 78%, soluble starch by 67%, citrus pectin by 63%, polygalacturonic acid by 62%, Tween 20 by 34%, birchwood xylan by 16%, Tween 80 by 12%, and carboxymethylcellulose by 3% of the isolates. The isolates were differentiated into 19 distinct homology groups by the 16S-ITS rDNA restriction pattern analysis.

Conclusions: Eight different extracellular enzyme activities were determined in 115 endospore forming bacilli. The largest 16S-ITS rDNA homology group (HT1) included 36% of the isolates, 98% of which degraded casein, polygalacturonic acid, pectin and starch.

Significance and Impact of the Study: This study is the first report on the characterization of the industrial enzyme-producing alkalophilic bacilli by 16S-ITS rDNA restriction fragment length polymorphism (RFLP). Restriction profiles of 64% of the isolates were found to be different from those of five reference strains used.

Keywords: 16S-ITS rDNA-RFLP, alkalophilic *Bacillus*, extracellular enzyme screening, similarity analysis.

INTRODUCTION

Alkalophiles have adopted a wide pH spectrum ranging from neutral to 12 (Krulwich and Guffanti 1989). Nevertheless, they are described as micro-organisms which grow optimally at pH values ranging between 10 and 12 (Horikoshi 1999a). Some other definitions have also been made (Krulwich and Guffanti 1989; Kroll 1990). Alkalophilic bacteria display a wide biodiversity (Horikoshi 1991) and *Bacillus* spp. can be considered the main representatives (Ntougias and Russell 2000). Many strains of alkalophilic *Bacillus* have been isolated from diverse environments including neutral soil, soda lakes, soda soils, soda deserts and animal manure (Horikoshi 1999a; Kumar and Takagi 1999). Isolation of novel *Bacillus* members from different environments may thus result in the finding of extracellular enzymes with novel properties that could be useful for diverse industrial applications.

Alkalophilic bacteria, especially *Bacillus* strains, have the ability to produce industrially important extracellular enzymes which are active and stable at high pH values (Martins *et al.* 2001). For example, alkaline proteases are used in detergent formulations (Anwar and Saleemuddin 1998; Kumar and Takagi 1999), for dehairing and bating processes in leather manufacturing (Loperena *et al.* 1994; Hameed *et al.* 1996), silver recovery from used X-ray films

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(Singh *et al.* 1999), processing of proteinaceous waste (Dalev 1994), for the preparation of protein hydrolysates (Kumar and Takagi 1999), and for the esterification reactions in organic solvents (Pedersen *et al.* 2003). Alkaline amylases, alkaline lipases and alkaline cellulases have also been included in detergent formulations (Hoshino and Ito 1997; Ito *et al.* 1998; Sharma *et al.* 2001). Alkaline xylanases have been mainly used in the prebleaching of paper pulp (Beg *et al.* 2001). Other enzymes which are also used in the papermaking industry are the alkaline pectinases (Reid and Ricard 2000). Textile industry is another important application area of alkaline pectinases and amylases (Kim *et al.* 1995; Kashayp *et al.* 2001).

So far no reports describing the characterization of extracellular enzyme producing alkalophilic bacteria from Turkey have been available in the literature. The present study was therefore the first attempt to characterize and build a culture collection of alkalophilic bacteria in Turkey.

MATERIALS AND METHODS

Reference strains

Five reference strains of *Bacillus* were obtained from Agricultural Research Service Culture Collection (ARS/NRRL; Peoria, IL, USA): *Bacillus alcalophilus* (NRRL B-14309) has been described by Vedder (1934) and isolated from human faeces. *Bacillus clausii* (NRRL B-23342), *B. gibsonii* (NRRL B-23346), *B. halmapalus* (NRRL B-23347) and *B. pseudofirmus* (NRRL B-23349) have been described by Nielsen *et al.* (1995). *Bacillus clausii* (NRRL B-23342) has been isolated from garden soil (Ghana). The latter three strains have been isolated from soil (Ghana).

Sampling and isolation

Soil samples were collected from Izmir-Camalti salt production site, and leather samples were from Izmir-Menemen and Ege University Leather Processing Unit. Horse faeces samples were collected from Izmir-Bostanli. The samples were first incubated for 10 min at 80°C in a water bath to kill most of the vegetative cells (Mora *et al.* 1998). Different dilutions of the samples were then plated on Horikoshi-I agar (pH 10·2) (Horikoshi 1999a) media and incubated at 37°C for 24–72 h. Single colonies showing different morphologies were picked and re-streaked for a number of times on the agar medium until single and uniform colonies were obtained. Isolates were then stored in 20% glycerol at −80°C.

Phenotypic studies

Gram staining was performed according to the method of Murray *et al.* (1994). Catalase and oxidase reactions were performed as described by Smibert and Krieg (1994) and Tarrand and Gröschel (1982), respectively. Endospores were examined under the phase-contrast microscope (Olympus, Tokyo, Japan). Some physiological tests were then carried out for 4–5 days on alkaline nutrient agar: growth in the presence of 5% (pH 9·9), 7% (pH 9·7) and 10% NaCl (pH 9·7) at 37°C, and growth at 45, 50 and at 55°C (pH 10·3). Growth at pH 7 was also carried out using nutrient agar for 2 days at 37°C.

All the media used for enzyme screening were supplemented with 10 g l^{-1} Na_2CO_3 (20% Na_2CO_3 solution was prepared in distilled water, autoclaved separately, and then added to the medium). Protease screening was performed according to the method described by Horikoshi (1999b). For amylase, xylanase and cellulase screening, the mineral medium described by Fritze *et al.* (1990) was modified (vitamin solution was replaced with 0·5% yeast extract) and used: 5 g l^{-1} of yeast extract, 7 g l^{-1} of K_2HPO_4; 2 g l^{-1} of KH_2PO_4, 0·1 g l^{-1} of $MgSO_4{\cdot}7H_2O$; 1 g l^{-1} of $(NH_4)_2SO_4$ and 5 g l^{-1} of NaCl. The medium was supplemented either with 5 g l^{-1} soluble starch or birchwood xylan or with carboxymethylcellulose for amylase, xylanase and cellulase screening, respectively. Plates were incubated for 2 days for amylase, 2 days for xylanase and 3 days for cellulase screening. Amylase zones were detected as described by Horikoshi (1999b). Xylanase and cellulase zones were detected according to Gessesse and Gashe (1997). For pectinolytic enzyme-screening pectate agar medium described by Kobayashi *et al.* (1999) was used (yeast nitrogen base was not included in the medium), and both citrus pectin and polygalacturonic acid were used as the substrates. The method of Haba *et al.* (2000) was adopted for lipase screening by using Tween 20 and 80 as the substrates, and the plates were incubated for 2 days at 37°C.

Genomic DNA extraction and amplification

The method of Ausubel *et al.* (1994) was used for genomic DNA isolation. 16S rDNA-ITS region of rRNA genes of the isolates and reference strains were amplified using the following DNA oligoprimers: forward, 5′-AGAGTTTGATCCTGGCTCAG-3′ (Mora *et al.* 1998), and reverse 5′-CAAGGCATCCACCGT-3′ (Jensen *et al.* 1993). PCR reaction mixture contained *ca* 500 μg of genomic DNA, 10 pmol each of the forward and reverse primers, 0·2 mM dNTPs, 1·5 mM $MgCl_2$, 1x PCR buffer (Fermentas, Lithuania), and 1·25 U *Taq* DNA polymerase (Fermentas) in a reaction volume of 50 μl. PCR conditions were as follows: an initial denaturation step for 5 min at 94°C followed by 40 amplification cycles including 1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min elongation at 72°C, steps. Final extension was performed for 10 min at 72°C. PCR amplifications were carried out in a Mini Cycler System (MJ Research Inc., MA, USA).

Amplified 16S-ITS rDNA RFLP analysis

Amplified 16S-ITS rDNA fragments, *ca* 2000 bp in length, were digested with 10 U *Taq*I (Fermentas) or *Hae*III (Fermentas). Restriction products were separated in 2% agarose gels (low EEO; Applichem, Darmstadt, Germany) and were then analysed by Bio-1D++ software (Vilber-Lourmat, Torcy, France) at 13% homology coefficient after normalizing the restriction patterns against a DNA size marker (1 kb GeneRuler; Fermentas). The formula of Nei and Li was specified to automatically determine the similarity between the strains. The unweighted pair group method with arithmetic averages (UPGMA) was used for clustering analysis (Vilber-Lourmat).

RESULTS

In total 115 isolates were obtained. Seventy-three per cent of the isolates were from leather, 23% from soil and 4% were from horse faeces samples (Table 1). Isolates were named according to the source (L, isolates from leather; S, from soil and F, from horse faeces; Table 1). The isolates were classified into 19 distinct homology groups on the basis of 16S-ITS rDNA restriction pattern analysis (Table 2).

Phenotypic characterization

Morphology. All the isolated strains were found to be spore-forming, Gram-positive, catalase-positive and rod-shaped bacteria. Oxidase presence was determined in 92% of the isolates because the isolates L15, L21, L69, L70, L74, L79, L80, S4 and S19, did not show oxidase activity.

Physiological properties. Twenty-three per cent of the isolates (L14–L17, L20–L22, L32, L33, L54, L57, L60, L61, L63, L64, L67–L70, L74, L77–L80, L82, L83; Table 1) did not grow at pH 7 after 2 days of incubation. This result could indicate that isolates were obligate alkalophiles. Remaining of the isolates (77%) grew at pH 7 after 24 h incubation suggesting that they could be facultative alkalophiles as these isolates also grew at alkaline pH above 9·5 (Krulwich and Guffanti 1989).

Most of the isolates appeared to be halotolerant because 97% of the isolates grew at 5% NaCl (pH 9·9) (Table 1).

Seventy-three per cent of the isolates grew at 45°C (pH 10·3) whereas 50% of the isolates grew at 50°C (pH 10·3) (Table 1). However no growth was obtained at 55°C (pH 10·3) suggesting that these isolates could be thermotolerant.

Extracellular enzyme screening. Proteolytic activity was the most abundant extracellular enzyme activity which was observed from 78% of the isolates at pH 10·2 (Table 2). Soluble starch hydrolysis was detected from the 67% of the isolates at pH 10. Pectin was degraded by 63% of the isolates at pH 9·5, polygalacturonic acid by 62% at pH 9·1, Tween 20 by 34% at pH 10·3 and Tween 80

Table 1 Physiological characteristics of the isolates

Name of the isolates	Growth at						
	pH 7	5% NaCl	7% NaCl	10% NaCl	45°C	50°C	55°C
L79	−	−	−	−	−	−	−
L67	−	+	+	−	+	−	−
L20, L33, L57, L77, L78, L83	−	+	+	+	−	−	−
L14, L32, L54, L60–L64, L68–L70, L74, L80, L82	−	+	+	−	−	−	−
L15–L17, L21, L22	−	+	+	+	+	−	−
S6, S12–S15, S19–S21, S23, S26	+	+	+	−	+	−	−
F4, S7, S22	+	+	−	−	+	+	−
S8, S24	+	+	+	−	−	−	−
F2, L1, L3–L9, L18, L19, L23–L25, L28–L31, L35, L39, L40, L42, L46, L47, L50, L53, L55, L58, L65, L66, L71–L73, L75, L76, S3	+	+	+	+	+	+	−
F1, F3, L2, L10, L12, L26, L27, L34, L37, L38, L41, L44, L45, L48, L51, L52, L56, L81, S25	+	+	+	−	+	+	−
L11, L36	+	−	−	−	+	−	−
L13	+	+	−	−	−	−	−
L43, L49, L59, L84, S2, S4, S17	+	+	+	+	−	−	−
F5, S1, S5, S9, S10, S11, S16, S18	+	+	−	−	+	−	−

Isolates were named according to the source (L, isolates from leather being processed; S, from soil; and F, from horse faeces). Growth properties at different physiological conditions (right) were indicated (+ or −).

Table 2 Grouping of the isolates with respect to RFLP profiling and extracellular enzyme activity results

RFLP homology groups	Name of the strain	No. isolates	Substrate
HT1	*B. clausii* (NRRL B23342), F1, F3, L1–L3, L6–L10, L12, L28–L30, L31, L34, L35, L37, L38, L41, L44–L48, L50–L53, L56, L58, L66, L71–L73, L75, L76, L81, S3, S25	40	Casein, pectin, PGA, starch
	L65	1	Casein, pectin, PGA
HT2	L42	1	Pectin, PGA, starch, Tween 20
	F2	1	Casein, pectin, PGA
HT3	S18, S6	2	Casein
	L13	1	Casein, Tween 20
	F4, S1, S5, S12, S13, S14, S15, S16, S20, S21, S22, S23, S24, S26	14	Casein, Tween 20 and 80
HT4	S4	1	Casein, cellulose, pectin, starch, Tween 20, xylan
HT5	S8	1	Casein, starch
	S2	1	Casein, starch, pectin, PGA
HT6	L11, L36	2	Casein, cellulose, starch, Tween 20, xylan
T1	L62	1	Pectin, PGA, starch,
T2	L60, L61, L63, L64, L67, L68, L69, L74, L77, L78, L80, L82, L83	13	Pectin, PGA, Tween 20, xylan
	L79	1	Starch, Tween 20, xylan
	L14	1	PGA, Tween 20, xylan
T3	F5, S7, S9, S10, S11, S19	6	Starch
T4	L70	1	Pectin, PGA, starch
H1	L4, L5, L24, L25, L39, L40, L55	7	Casein, pectin, PGA, starch
H2	L18, L19, L23	3	Casein, pectin, PGA, starch
	L15, L16, L17, L21, L22	5	Casein, starch
H3	L26	1	Casein, PGA, Tween 20
	L27	1	Casein, Tween 20
H4	L43	1	Casein, pectin
	L84	1	Casein
H5	L54	1	Casein, pectin, starch
XXA	L49, L59	2	Casein, starch, Tween 20
	L57	1	Casein, starch
XXB	L33	1	Casein, starch
XXC	L20	1	Starch
	S17	1	Casein, starch
XXD	L32	1	Casein, Tween 20

Isolates were named according to the source (L, isolates from leather being processed; S, from soil and F, from horse faeces). Nineteen homology groups (left) on the basis of 16S-ITS rDNA RFLP pattern analysis and extracellular enzyme activities specific to each of these homology groups were indicated (right). PGA, polygalacturonic acid.

only by 12% of the isolates at pH 10·3. Xylanolytic activity was observed from 16% of the isolates at pH 10. Carboxymethylcellulose was hydrolysed only by 3% of the isolates at pH 10, thus it was the least frequent enzyme activity (Table 2).

Most of the isolates showed more than one extracellular enzyme activity (Table 2). Sixty per cent of the isolates could degrade both citrus pectin and polygalacturonic acid. Forty-three per cent of the isolates could hydrolyse casein, citrus pectin, polygalacturonic acid, and soluble starch (Table 2). Only one of the isolates, S4, could hydrolyse six different substrates (Table 2).

Amplified 16S-ITS rDNA analysis

One hundred and fifteen isolated, and five reference strains were characterized according to their 16S-ITS rDNA restriction profiles. Restriction reactions were carried out by using two frequent cutter endonucleases, *Taq*I and *Hae*III. Digestion products were first resolved in a

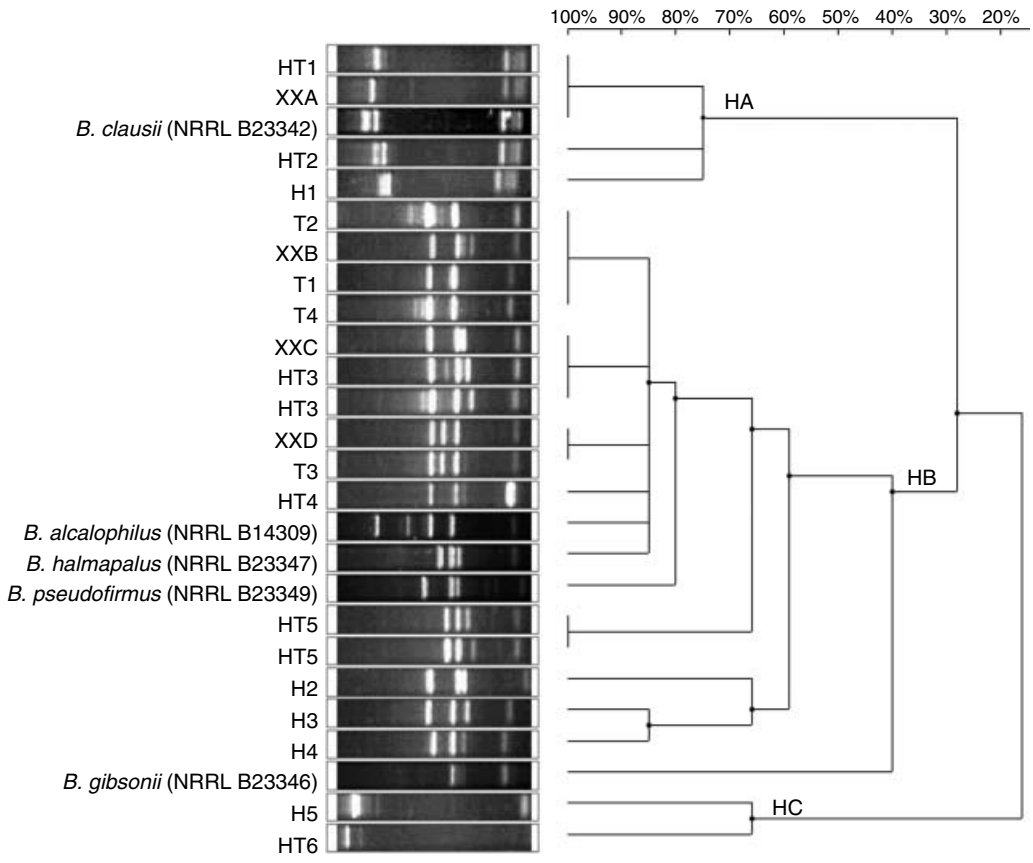

Fig. 1 Dendrogram of *Hae*III 16S-ITS homology groups. A DNA size marker (1 kb DNA ladder; Fermentas) was used to normalize restriction profiles of each of the homology groups. Restriction profiles of reference strains and homology groups were analysed at 13% homology coefficient by using a computer program (Bio-1D++; Vilber-Lourmat). The names of homology groups and reference strains were indicated (left). HA, HB and HC indicated the three main clusters revealed by *Hae*III restriction enzyme digestion

preparative agarose gel and homology groups (Table 2) were identified on the basis of identical restriction profiles. Representatives of these digestion product groups were then electrophoresed together with those of five reference strains and group profiles were analysed at 13% homology coefficient.

HaeIII digestion. Twenty-six homology group patterns, 21 isolates and five reference strains, were used for the evaluation of *Hae*III digestion profiles. Analysis of the *Hae*III restriction patterns revealed 17 distinct homology groups (Fig. 1) and four of these groups were formed by the reference strains. These homology groups were included in three major clusters, designated as HA, HB and HC (Fig. 1). Reference strains were included into HA (*B. clausii*) and HB (*B. alcalophilus*, *B. halmapalus*, *B. pseudofirmus*, and *B. gibsonii*). HC contained only three of the isolates which were also included in homology groups H5 and HT6 (Fig. 1, Table 2). HB was the largest cluster which included 51% of the isolates (Fig. 1). Forty-four isolates in HT1 and XXA (HA; Fig. 1, Table 1) were clustered with the reference strain *B. clausii*.

TaqI digestion. Twenty-six homology group patterns, 21 isolates and five reference strains, were used for the evaluation of *Taq*I digestion profiles. *Taq*I digestion resulted in 15 distinct homology groups and three of these were formed by the reference strains (Fig. 2). These homology groups were included in three major clusters, designated as TA, TB and TC (Fig. 2). Reference strains were included into TA (*B. clausii*, *B. gibsonii*, and *B. pseudofirmus*) and TC (*B. alcalophilus* and *B. halmapalus*). Sixty-four of the isolates were clustered with two of the reference strains: 51 isolates, in H1, HT1, XXB and XXC (cluster TA; Fig. 2), with *B. clausii*, and 13 isolates, in H2, H5, XXA and XXD (cluster TA; Fig. 2), with *B. pseudofirmus*.

Combined analysis of the homology groups. The results of the dendrograms generated from both of the restriction enzyme profiles were combined and 19 distinct

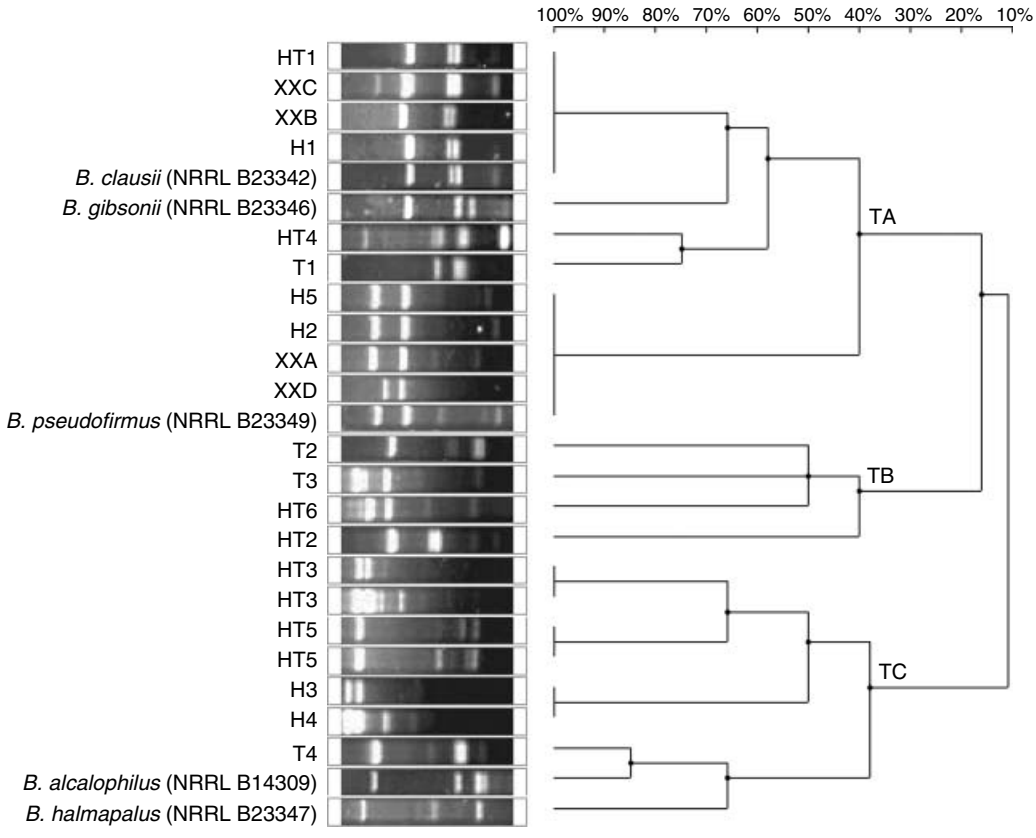

Fig. 2 Dendrogram of *Taq*I 16S-ITS homology groups. A DNA size marker (1 kb DNA ladder; Fermentas) was used to normalize restriction profiles of each of the homology groups. Restriction profiles of reference strains and homology groups were analysed at 13% homology coefficient by using a computer program (Bio-1D++; Vilber-Lourmat). The names of homology groups and reference strains were indicated (left). TA, TB and TC indicated the three main clusters revealed by *Taq*I restriction enzyme digestion

homology groups of the isolated strains were obtained. Sixty-five of the isolates were found to be differentiated into six identical homology groups by both enzymes and these homology groups were designated as HT (Figs 1and 2, Table 2). Similarly the groups that were generated by only one of the restriction enzymes were named as either T (*Taq*I-specific groups) or H (*Hae*III-specific groups) (Figs 1 and 2, Table 2). Four more homology groups (XXA, XXB, XXC and XXD) were generated by using the isolates which could not be included into any of the HT, T or H groups (Figs 1 and 2, Table 2).

Combined analysis of the homology groups indicated that some species of *Bacillus* could be better differentiated by only one of the restriction enzymes. For example, while *B. clausii* (NRRL B-23342) and *B. gibsonii* (NRRL B-23346) were clustered more closely by *Taq*I (cluster TA; Fig. 2), *Hae*III could locate them into much more distant clusters (clusters HA and HC, respectively, Fig. 1). Similarly, while *Hae*III digestion clustered *B. pseudofirmus* and homology groups, HT3, HT4, T1, T2, T3, T4, XXB and XXC, within the same major cluster (cluster HB, Fig. 1), these groups appeared to be dispersed along the *Taq*I dendrogram (Fig. 2).

The largest group, HT1 (Table 2), contained 41 isolates which displayed the same restriction patterns with those of the reference strain *B. clausii* (NRRL B-23342) (Figs 1 and 2). Some homology groups were represented by only one isolate (HT4, T1, T4, H5, XXB and XXD; Table 2).

Some of the homology groups included isolates with identical extracellular enzyme activities. For example, 99% of the HT1 isolates degraded casein, pectin, polygalacturonic acid and starch (Table 2), while 82% of the HT3 isolates hydrolysed casein, Tween 20 and 80. HT3 was the only homology group which contained isolates with Tween 80 hydrolysing activity. Two of the homology groups which were represented by only one isolate, differed in extracellular enzyme activity. For example, the isolate L54 (H5) could degrade casein, pectin and starch, while S4 (HT4) isolate could degrade casein, cellulose, pectin, starch, Tween 20 and xylan (Table 2).

16S-ITS rDNA RFLP pattern similarity among the five reference strains varied from 10% (between *B. clausii*/

B. gibsonii and *B. alcalophilus/B. halmapalus*, Fig. 2) to 85% (between *B. alcalophilus* and *B. halmapalus*, Fig. 1). These results could imply that RFLP groups showing <86% similarity in either of the dendrograms, could indicate a new species or existing species.

DISCUSSION

Alkaline enzymes from alkalophilic bacteria, especially from *Bacillus* genus, have been widely studied in recent years and they have found many diverse areas of application in industry (Horikoshi 1999a). Nevertheless, no reports have been available for Turkey's alkalophilic bacteria or their alkaline enzymes. So far, genetic methods used in the characterization of alkalophilic *Bacillus* have included 16S rDNA sequence data analysis, comparison of DNA–DNA hybridization patterns and G–C content analysis (Nielsen *et al.* 1995; Takami *et al.* 1999; Martins *et al.* 2001). On the basis of 16S rDNA sequence analysis the major alkalophilic *Bacillus* species have been proposed to be: *B. pseudofirmus*, *B. agaradhaerens*, *B. clarkii*, *B. halodurans*, *B. clausii*, *B. halmapalus*, *B. horikoshii*, *B. pseudoalcalophilus* and *B. gibsonii* in addition to previously known species, *B. cohnii* and *B. alcalophilus* (Fritze *et al.* 1990; Nielsen *et al.* 1995). However, there has been no report in the literature on the characterization of alkalophilic *Bacillus* by 16S-ITS rDNA pattern analysis. This method was developed and first used for the identification of nine lactobacilli reference strains (Yavuz *et al.* 2004). It was found to be easy to perform and it also clearly differentiated five alkalophilic *Bacillus* reference strains used in the present study. As this study constituted the first attempt to characterize extracellular enzyme-producing alkalophilic bacilli in Turkey, a large number of strains had to be used. The results indicated that 16S-ITS rDNA RFLP could be a rapid method in revealing genetic diversity of the large collections of alkalophilic bacteria at species level.

The isolated strains were clustered into 19 distinct homology groups by the combined analysis of 16S-ITS rDNA restriction profiles. Both *Taq*I and *Hae*III were found to be necessary for the discrimination of the strains by this method.

All the isolates in homology group HT1 displayed the same restriction patterns with those of the reference strain *B. clausii* (NRRL B-23342), and also showed the same enzyme activity profiles except isolate L65 (Table 2). However, physiological properties of 59% of the HT1 isolates were the same with those of *B. clausii*: they could all grow at 10% NaCl (pH 9·7), and at 50°C (pH 10·3). However, the remaining of the HT1 isolates differed in salt tolerance as they could not grow at 10% NaCl (pH 9·7). Nevertheless it could be suggested that HT1 isolates were strains of *B. clausii*.

Twenty-six of the isolates could not grow at pH 7 whereas they grew well in alkaline pH (10·2), indicating that these isolates could be obligate alkalophiles (Table 1). These isolates however distributed into eight different homology groups (H2, H5, T2, T4, XXA, XXB, XXC and XXD; Table 2), and only five of these homology groups (H5, T2, T4, XXB and XXD) contained obligately alkalophilic isolates. The 26 isolates were all from leather samples suggesting that obligate alkalophiles could be found at the liming step of leather processing where the pH was measured at 12.

Extracellular enzymes from facultative or obligate alkalophilic micro-organisms can be stable and active at alkaline pH (Gessesse and Gashe 1997; Kobayashi *et al.* 1999; Ghanem *et al.* 2000). For example, most of the bacterial alkaline proteases show optimal activity between pH 10 and 12 (Takami and Horikoshi 2000). Extracellular enzyme activities using eight different substrates were detected in alkaline media. Most of the isolates (91%) displayed more than one extracellular enzyme activity. This finding might suggest that all these extracellular enzymes could function at high pH. However, to determine optimum pH points for each of these enzyme activities, further studies are required. Furthermore, different carbon and nitrogen sources, metal ions and various chemical reagents in the growth media can also affect the enzyme activity (Kumar and Takagi 1999). Therefore, enzyme activities in different media also remain to be determined.

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