

The genotypic diversity and lipase production of some thermophilic bacilli from different genera

Melih Koc¹, Cumhuri Cokmus², Arzu Coleri Cihan¹

¹Biology Department, Faculty of Science, Ankara University, Ankara, Turkey.

²Konya Food & Agriculture University, Dede Korkut Mah., Konya, Turkey.

Submitted: November 13, 2014; Approved: April 12, 2015.

Abstract

Thermophilic 32 isolates and 20 reference bacilli were subjected to *Rep*-PCR and ITS-PCR fingerprinting for determination of their genotypic diversity, before screening lipase activities. By these methods, all the isolates and references could easily be differentiated up to subspecies level from each other. In screening assay, 11 isolates and 7 references were found to be lipase producing. Their extracellular lipase activities were measured quantitatively by incubating in both tributyrin and olive oil broths at 60 °C and pH 7.0. During the 24, 48 and 72-h period of incubation, the changes in the lipase activities, culture absorbance, wet weight of biomass and pH were all measured. The activity was determined by using *p*NPB in 50 mM phosphate buffer at pH 7.0 at 60 °C. The lipase production of the isolates in olive oil broths varied between 0.008 and 0.052, whereas these values were found to be 0.002-0.019 (U/mL) in the case of tyributyrim. For comparison, an index was established by dividing the lipase activities to cell biomass (U/mg). The maximum thermostable lipase production was achieved by the isolates F84a, F84b, and *G. thermodenitrificans* DSM 465^T (0.009, 0.008 and 0.008 U/mg) within olive oil broth, whereas *G. stearothermophilus* A113 displayed the highest lipase activity than its type strain in tyributyrim. Therefore, as some of these isolates displayed higher activities in comparison to references, new lipase producing bacilli were determined by presenting their genotypic diversity with DNA fingerprinting techniques.

Key words: endospore-forming bacilli, screening, thermostable lipase, ITS-PCR, *Rep*-PCR.

Introduction

Isolation of novel thermophilic bacilli by polyphasic approach has received considerable attention since they include the species of industrial, biotechnological and environmental interest (Derekova *et al.*, 2008). These thermophiles play an important role in applications such as enzymatic synthesis of novel oligosaccharides, in hydrolysis of starch to glucose, in industrial fermentation processes, in biopolymer and biodiesel production as well as in textile and detergent industries due to their unique thermostable enzymes (Hough and Danson, 1999; Haki and Rakshit, 2003). In order to screen isolates producing novel thermostable enzymes that might be useful in industrial applications, the taxonomic studies are of importance in determining the phenotypic and genotypic diversity of these

microorganisms, and placing these bacilli in appropriate taxonomic levels by characterizing novel species from these natural thermal habitats, harbouring undiscovered microorganisms (Mora *et al.*, 1998). This approach clusters a great number of similar bacteria belonging to the same genus, and includes obtaining information about these clusters with both definitive phenotypic and DNA-directed genotypic fingerprinting methods which amplify the intergenic transcribed spacers (ITS) and the repetitive extragenic palindromic elements (*Rep*-elements), (Vanechoutte *et al.*, 1992; White *et al.*, 1993).

Moreover, lipases (triacylglycerol hydrolases, E.C.3.1.1.3) catalyze the hydrolysis of triacylglycerol to free fatty acids and glycerol. In addition, lipases can achieve esterification, interesterification, acidolysis, alcoholysis and aminolysis reactions (Joseph *et al.*, 2008).

Thermophilic bacilli are the natural source of many thermostable enzymes, and of those from thermozymes, thermostable lipases have received attention in both structural studies and industrial applications as they show high stability at elevated temperatures and in organic solvents (Bornscheuer *et al.*, 1994; Jaeger *et al.*, 1994). Thermostable lipases have been described and characterized in a few thermophilic species, including *Geobacillus thermo-leovarans* ID-1 (Lee *et al.*, 1999), *Geobacillus stearothermophilus* L1 (Kambourova *et al.*, 2003), *Geobacillus zalihae* (Rahman *et al.*, 2007), *Geobacillus* sp. TW1 (Li and Zhang, 2005), and *Anoxybacillus kamchatkensis* KW 12 (Olusesan *et al.*, 2009). In this study, we represented the data of lipase production capacities of totally 32 thermophilic, endospore-forming bacilli by comparing the results with 20 reference strains tested. Furthermore, high amounts of thermostable lipase producing isolates were determined for further studies in addition with displaying their genotypic diversity by representing the ITS-PCR and *Rep*-PCR DNA fingerprints.

Materials and Methods

Bacterial isolates and reference strains

Totally 32 thermophilic bacterial isolates and 20 reference strains were used for the screening of lipase production. The 16S rRNA gene sequence analyses of these thermophilic isolates were formerly determined from our previous studies and their gene sequences were found in GenBank databases (Cihan *et al.*, 2011). These thermophilic bacilli were found to belong to totally four different genera from *Anoxybacillus* (15 isolates), *Geobacillus* (11), *Aeribacillus* (4) and *Thermolongibacillus* (2). Twenty one samples of water (2), soil (13), sediments (8) or tree branch (1) were collected from different hot springs and high-temperature well pipelines of geographically widespread locations in Turkey. These regions are located in two geographically separated areas in Turkey: Aegean Region and Middle Anatolian Region. Of those geothermal regions, Aydin (Region A; 27°51' E, 37°51' N), Denizli (Region C; 29°06' E, 37°46' N) and Izmir (Region D; 27°09' E, 38°25' N) provinces are in the Aegean Region, whereas Nevsehir (Region E; 34°43' E, 38°38' N) and Ankara (Region F; 32°52' E, 39°56' N) provinces are located in the Middle Anatolian Region of Turkey. The isolates were designated according to their isolated provinces and the sample numbers. The designation and origin of the 32 isolates, their detailed taxonomic position and the reference strains used in this study are presented below in Table 1.

PCR based fingerprinting analyses of *Rep* elements and Intergenic 16S-23S rRNA genes

Repetitive Elements-PCR (*Rep*-PCR) genomic fingerprintings were performed with the (GTG)₅ and

BOXA1R primers using the PCR conditions that were described by Versalovic *et al.* (1994). Primer sets S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 were used for the amplification of Intergenic Transcribed Spacers (ITS) between 16S and 23S rRNA genes and PCR conditions were adjusted according to Daffonchio *et al.* (2003). The PCR products were electrophoresed in a 1.5% agarose gel, using 1 X TBE buffer at 120 V for 4 h. In the statistical analysis, the individual ITS-PCR, BOXA1 and (GTG)₅-PCR fingerprintings were analyzed by the GelCompar II software packages (Applied Maths, Belgium) according to the software instructions. Similarities of the digitized profiles were calculated using Dice correlation and an average linkage (UPGMA) dendrogram was obtained.

At the final stage, all of the individually examined ITS-, (GTG)₅- and BOX-PCR fingerprintings were taken into a cumulative cluster analysis which combined all these tests in a single dendrogram by using the GelCompar II software packages. This paper, thus, presents these combined results containing dendrogram rather than showing all these three individual fingerprinting tests. In clustering analyses which were constructed by using the presence or absence of DNA bands, their sizes and also their densities, the similarity limits of 16.7, 33.34, 50.0, 66.7, and 83.4% values were used with GelCompar II software. In these contexts, the bacteria displaying 0-16.7% similarities were denoted as having unique distinctive profiles, the ones having similarities between 75.0% and 99.9% were determined as showing similar profiles, and the ones with 100% similarity were implied as displaying the same profiles.

Lipase assay

The lipase activity was determined spectrophotometrically by measuring the hydrolysis of *para*-nitrophenol butyrate (*p*NPB, Sigma N9876) as substrate with modified methods of Lee *et al.* (1999). The reaction solution was prepared by mixing 100 mM potassium phosphate buffer (pH 7.0), ethanol and 50 μM *p*NPB at a concentration of 95:4:1. The standard reaction mixture in a total volume of 1.2 mL contained: 0.9 mL freshly prepared reaction solution and 0.3 mL enzyme. The enzymatic reaction was carried out at 60 °C for 15 min and stopped by storing the solution at -20 °C for 8 min in order to cool down. The release of *p*NP (*para*-nitrophenol) at 400 nm was measured by using a UV visible Elisa reader (BioTek), and one unit of enzyme was defined as the amount of enzyme needed for the hydrolysis of 1 μmol *p*NPB per minute at 60 °C, pH 7.0. The millimolar extinction coefficient of *p*NP at 400 nm and pH 7.0 was measured as 5.081 L.mM⁻¹.cm⁻¹, and it was used to calculate the amount of product yielded. All the enzyme assays were performed at least three-times.

Table 1 - Bacterial isolates and reference strains used in this study.

Taxonomic positions of the isolates	Designations of the isolates	Number of bacteria
Genus <i>Anoxybacillus</i>		
<i>Anoxybacillus calidus</i>	C161ab ^{so}	1
<i>Anoxybacillus caldiproteolyticus</i>	A142 ^{se} , A146 ^{se} , A335 ^{so} , A392b ^{so} , A394 ^{so} , A403 ^{so} , A404 ^{so} , A412b ^{so} , A413 ^{so} , C226 ^{so} , D494 ^{se} , D504 ^{se} , D621 ^{so} , D623 ^{so}	14
Genus <i>Geobacillus</i>		
<i>Geobacillus</i> sp. (Mix group)	A353 ^{se} , C304 ^{se} , D413 ^{so} , E173b ^{so}	4
<i>Geobacillus stearothermophilus</i>	A113 ^{so}	1
<i>Geobacillus thermodenitrificans</i>	A333 ^{so} , D195 ^{se}	2
<i>Geobacillus thermodenitrificans</i> subsp. <i>calidus</i>	F84a ^{se} , F84b ^{se}	2
<i>Geobacillus thermoglucosidasius</i>	B84a ^w	1
<i>Geobacillus toebii</i>	E134 ^w	1
Genus <i>Aeribacillus</i>		
<i>Aeribacillus pallidus</i>	A364 ^{so} , C196 ^{so} , D642 ^{so} , E334 ^t	4
Genus <i>Thermolongibacillus</i>		
<i>Thermolongibacillus altinsuensis</i>	E265 ^{se}	1
<i>Thermolongibacillus kozakliensis</i>	E173a ^{so}	1
	Total number of the bacterial isolates	32
Reference strains		
	<i>Geobacillus stearothermophilus</i> ATCC 12980 ^T (=DSM 22 ^T), <i>Geobacillus stearothermophilus</i> ATCC 43223, <i>Geobacillus stearothermophilus</i> ATCC 7953 (=DSM 5934), <i>Geobacillus thermoglucosidasius</i> DSM 2542 ^T , <i>Geobacillus toebii</i> DSM14590 ^T , <i>Geobacillus thermodenitrificans</i> DSM 465 ^T , <i>Geobacillus thermodenitrificans</i> subsp. <i>calidus</i> DSM 22629 ^T , <i>Geobacillus vulcanii</i> DSM 13174 ^T , <i>Geobacillus thermoleovorans</i> DSM 5366 ^T , <i>Geobacillus kaustophilus</i> DSM 7263 ^T , <i>Aeribacillus pallidus</i> DSM 3670 ^T , <i>Anoxybacillus caldiproteolyticus</i> DSM 15730 ^T , <i>Anoxybacillus flavithermus</i> DSM 2641 ^T , <i>Anoxybacillus ayderensis</i> NCIMB 13972 ^T , <i>Anoxybacillus thermarum</i> DSM 17141 ^T , <i>Anoxybacillus kamchatkensis</i> DSM 14988 ^T , <i>Anoxybacillus amylolyticus</i> DSM 15939 ^T , <i>Anoxybacillus rupiensis</i> DSM 17127 ^T , <i>Anoxybacillus voinovskiensis</i> DSM 17075 ^T , <i>Anoxybacillus tepidamans</i> DSM 16325 ^T , <i>Anoxybacillus thermarum</i> DSM 17141 ^T and <i>Bacillus licheniformis</i> DSM 13 ^T	20
	Total number of the reference strains	20

Abbreviations: se; hot spring sediment sample, so; soil sample, t; tree branch sample in hot spring, w; hot spring water sample.

Qualitative screening of lipase production

In order to determine the extracellular lipase production capacities of thermophilic isolates and reference strains quantitatively, media containing a pH indicator such as phenol red was used. For this purpose, Tributyrin agar medium (2.5 g meat extract, 3 g yeast extract, 1 g CaCl₂, 10 mL tributyrin (Sigma W222305), 30 g agar, 0.1 g phenol red/L, pH 7.0), (Abdel-Fattah *et al.*, 2008), and Olive oil agar plates (3.25 g Nutrient Broth, 1 g CaCl₂, 10 g gum arabic (Sigma-51198), 10 mL olive oil (Sigma O1514), 30 g agar, 0.1 g phenol red/L, pH 7.0), (Castro-Ochoa *et al.*, 2005) were used. All the isolates and reference strains were incubated in 5 mL broth media of Tributyrin and Olive oil which did not contain any phenol red or agar at 60 °C for 24-48 h (Shellab shaking incubator). After centrifugation (Hettich), supernatant was used as enzyme source. Then 100 µL of these enzyme sources were loaded into 6 mm sterile Oxoid discs, which were previously put on phenol red containing Tributyrin and Olive oil agar plates. The yellow zone occurred around the enzyme containing discs af-

ter 2 h of incubation at 60 °C was determined as a positive result for lipase activity (Yadav *et al.*, 1998).

Quantitative screening of the extracellular lipase production

When determining the amount of lipase production of the isolates in accordance with the enzyme production time, lipase activities were measured from the samples, taken from the same medium at the incubation hours of 24, 48 and 72. As in the case of qualitative measurements, two different kinds of media were used containing various substrates. Isolates were initially cultured in both Tributyrin and Olive oil plates without phenol red for 18 h at 60 °C. After incubation, actively growing cells were suspended in 0.85% NaCl which gave an absorbance of 0.16-0.3 at 660 nm. For each of the isolates, 0.5 mL starting enrichment suspension was inoculated into 3 different tubes of 5 mL enzyme production broths containing Tributyrin and Olive oil without phenol red, so as to triplicate the assay. Broth cultures were shaken at 60 °C, 250 rpm for 18-72 h, and both cells and cell-free supernatants were separated by centrifugation at

10,000 rpm for 15 min at +4 °C at the time of 24, 48 and 72 h. The bacterial growth curve was determined not only by measuring the absorbance of the culture at 660 nm, but also determined by measuring the wet-weight of the bacterial biomass (mg), during 72 h. The change in pH of the medium was also observed (Li *et al.*, 2005; Abdel-Fattah *et al.*, 2008).

Statistical analysis including the mean, standard deviation and standard error were calculated from the triplicate enzyme assays, which were carried on with three parallel experiments for the extracellular fractions. The mean values of the extracellular enzyme activities (U) in each triplicate were calculated, then divided by the cell yield (mg), and expressed as the total amount of lipase per cell yield (U/mg) in order to arrange the isolates in an index indicating the enzyme production levels. Furthermore, for the comparison of enzyme production levels, t-test analysis was applied.

Results and Discussion

The phylogenetic diversity of the thermophilic isolates according to *Rep*- and ITS-PCR fingerprintings

16S rRNA gene sequencing is a widely used standard technique in modern bacterial taxonomy by forming the basis of the bacterial phylogeny, and used to apply the rRNA gene technology as a part of 'polyphasic approach' (Rosello-Mora, 2005; Ludwig and Schleifer, 1999). However, there are some limitations when comparing the 16S rRNA gene sequences of phylogenetically homogeneous groups of bacteria as the structurally conserved sequences found in 16S rRNA gene might not allow strains identify up to species level in closely related microorganisms (Rodas *et al.*, 2003). Therefore, the DNA of the isolates showing more than 97.0% 16S rRNA gene sequence similarities with their closest relatives need to be hybridized in cases of species descriptions (Stackebrandt *et al.*, 2002; Logan *et al.*, 2009).

The 32 thermophilic endospore-forming bacilli used in this study were formerly grouped into genera *Anoxybacillus* (15 isolates), *Geobacillus* (11), *Aeribacillus* (4) and *Thermolongibacillus* (2) according to their 16S rRNA gene sequences (Cihan *et al.*, 2011). In the previous studies, 11 of the isolates were found to belong to genus *Geobacillus*. As isolates of *Geobacillus* sp. E173b, *Geobacillus* sp. D413, *Geobacillus* sp. C304 and *Geobacillus* sp. A353 showed high sequence similarities (> 99%) to more than one closest relative of *G. vulcanii*, *G. thermoleovorans* and *G. kaustophilus*, they could not be identified up to species level without DNA-DNA hybridization analyses. The rest of the isolates, with the exception of these four, could be identified up to species level by means of their comparative sequence analyses and their taxonomic positions were determined according to their closest relatives. The rest of the

isolates were found to belong to species from *G. stearothermophilus* (A113), *G. thermodenitrificans* (D195, A333), *G. thermodenitrificans* subsp. *calidus* (F84a, F84b), *G. toebii* (E134), *G. thermoglucosidasius* (B84a), *T. kozakliensis* (E173a), *T. altinsuensis* (E265), *A. pallidus* (A364, C196, D642, E334), *A. calidus* (C161ab) and *A. caldiproteolyticus* (A413, A404, D504, A403, A394, A412b, C226, D621, D623, A146, A142, D494, A392b, A335) (Table 1).

Moreover, DNA fingerprinting methods containing the Repetitive Extragenic Palindromic (*REP*) elements such as BOX and (GTG)₅ elements and the Intergenic Transcribed Spacers (ITS) between the 16S and 23S rRNA genetic loci are frequently used in PCR fingerprinting to discriminate at the species and intraspecies levels (Versalovic *et al.*, 1994; Daffonchio *et al.*, 2003). The polymorphisms in these analyses were used for discriminating closely related endospore-forming bacilli in the previous studies of Daffonchio *et al.* (2003), Kuisiene *et al.* (2008) and Manachini *et al.* (2000), Cihan *et al.* (2011b). As described before, the limitations on 16S rRNA gene sequencing directed us to determine the genetic diversity of our lipase producing isolates by using *Rep*-PCR and ITS-PCR techniques having resolution power up to subspecies. Thus, all the thermophilic isolates and their related type species were subjected to the *Rep*-PCR and ITS fingerprinting analyses. The three fingerprinting analyses were initially taken into cluster analyses individually. The numbers of the individual clusters and their contents are presented in Table 2. Then a cumulative phylogenetic tree was obtained by combining both the *Rep*-PCR and ITS-PCR methods which is displayed in Figure 1.

According to the individual cluster analyses of the ITS-, (GTG)₅- and BOX-PCR fingerprintings, totally 28, 28 and 25 clusters were obtained. When these three fingerprintings were combined in a cumulative cluster analysis, totally 20 clusters were obtained as presented in Figure 1 and Table 2. All of the reference strains formed unique distinctive patterns which could easily be differentiated from each other (Figure 1 and Table 2). In addition, 16 of 32 isolates showed unique patterns different from all the other isolates and reference strains at least one to three of these fingerprinting analyses used. In this context, isolates and references from genus *Geobacillus* were grouped into 9 different 16S rRNA gene clusters form G-1 to G-9. Not only the reference strains of *G. vulcanii*, *G. thermoleovorans* and *G. kaustophilus*, but also the isolates (E173b, D413, C304, A353) from mix group (G1-G-4), having high rRNA gene similarities to more than one closest relative differed in their ITS, BOX or (GTG)₅ fingerprinting profiles. It is, in fact, significant to note that all these genetically homogeneous group of bacteria showed different ITS profiles. 16S rRNA gene group G-5 contained species from *G. stearothermophilus* which could be differentiated by means of

Table 2 - Phylogenetic clusters of the thermophilic isolates and reference strains derived from their 16S rRNA genes, BOX- and (GTG)₅ elements and also ITS regions.

Bacteria	Phylogenetic groups					
	16S rRNA gene identities	16S rRNA gene	ITS region	(GTG) ₅ element	BOX element	Cumulative clusters
Genus <i>Geobacillus</i>						
<i>G. vulcanii</i> DSM 13174 ^T	99.0-99.8% to DSM 13174 ^T , DSM 5366 ^T and DSM 7263 ^T	G-2	ITS-1	GTG-1	BOX-1	5
<i>Geobacillus</i> sp. E173b		G-1	ITS-2	GTG-1	BOX-1	5
<i>Geobacillus</i> sp. D413		G-1	ITS-3	GTG-2	BOX-2	5
<i>Geobacillus</i> sp. C304		G-1	ITS-4	GTG-2	BOX-2	5
<i>Geobacillus</i> sp. A353		G-1	ITS-5	GTG-3	BOX-3	15
<i>G. thermoleovorans</i> DSM 5366 ^T		G-3	ITS-6	GTG-1	BOX-4	5
<i>G. kaustophilus</i> DSM 7263 ^T		G-4	ITS-7	GTG-4	BOX-5	5
<i>G. stearothermophilus</i> A113	99.8% to DSM 22 ^T	G-5	ITS-8	GTG-5	BOX-6	2
<i>G. stearothermophilus</i> ATCC 43223 ^T		G-5	ITS-8	GTG-5	BOX-6	2
<i>G. stearothermophilus</i> ATCC 12980 ^T		G-5	ITS-8	GTG-6	BOX-7	2
<i>G. stearothermophilus</i> ATCC 7953		G-5	ITS-8	GTG-6	BOX-7	2
<i>G. thermodenitrificans</i> D195	98.7-99.2% to DSM 465 ^T	G-6	ITS-9	GTG-7	BOX-8	14
<i>G. thermodenitrificans</i> A333		G-6	ITS-10	GTG-7	BOX-8	14
<i>G. thermodenitrificans</i> DSM 465 ^T		G-6	ITS-11	GTG-7	BOX-8	14
<i>G. thermodenitrificans</i> subsp. <i>callidus</i> F84a	98.3% to DSM 22629 ^T	G-7	ITS-12	GTG-8	BOX-9	14
<i>G. thermodenitrificans</i> subsp. <i>callidus</i> F84b DSM 22629 ^T		G-7	ITS-12	GTG-8	BOX-9	14
<i>G. thermoglucosidasius</i> B84a	99.2% to DSM 2542 ^T	G-8	ITS-13	GTG-9	BOX-10	11
<i>G. thermoglucosidasius</i> DSM 2542 ^T		G-8	ITS-14	GTG-10	BOX-11	11
<i>G. toebii</i> E134	99.2% to DSM 14590 ^T	G-9	ITS-15	GTG-11	BOX-12	9
<i>G. toebii</i> DSM 14590 ^T		G-9	ITS-16	GTG-11	BOX-12	9
Genus <i>Thermolongibacillus</i>						
<i>T. kozakliensis</i> E173a	97.5% to each other, 94.2-95.0% to DSM 14590 ^T	T-1	ITS-17	GTG-12	BOX-13	17
<i>T. altinsuensis</i> E265		T-2	ITS-17	GTG-13	BOX-14	4
Genus <i>Aeribacillus</i>						
<i>Aeribacillus</i> sp. A364	98.0-99.2% to DSM 3670 ^T	Ae-1	ITS-18	GTG-14	BOX-15	18
<i>Aeribacillus</i> sp. C196		Ae-1	ITS-18	GTG-15	BOX-15	8
<i>Aeribacillus</i> sp. D642		Ae-1	ITS-18	GTG-16	BOX-15	13
<i>Aeribacillus</i> sp. E334		Ae-1	ITS-18	GTG-16	BOX-15	13
<i>A. pallidus</i> DSM 3670 ^T		Ae-1	ITS-18	GTG-16	BOX-15	10
Genus <i>Anoxybacillus</i>						
<i>A. ayderensis</i> NCIMB 13972 ^T	99.7-94.3% to each other	An-1	ITS-19	GTG-17	BOX-16	7
<i>A. flavithermus</i> DSM 2641 ^T		An-2	ITS-20	GTG-18	BOX-17	3
<i>A. kamchatkensis</i> DSM 14988 ^T		An-3	ITS-21	GTG-19	BOX-18	1
<i>A. amylolyticus</i> DSM 15939 ^T		An-4	ITS-22	GTG-20	BOX-19	7
<i>A. rupiensis</i> DSM 17127 ^T		An-5	ITS-23	GTG-21	BOX-20	19
<i>A. voinovskiensis</i> DSM 17075 ^T		An-6	ITS-24	GTG-22	BOX-21	16
<i>A. thermarum</i> DSM 17141 ^T		An-7	ITS-25	GTG-23	BOX-22	1
<i>A. calidus</i> C161ab	96.8% to DSM 17127 ^T and DSM 17075 ^T	An-8	ITS-26	GTG-24	BOX-23	12
<i>A. caldiproteolyticus</i> A413	99.9-99.9% to DSM 15730 ^T	An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A404		An-9	ITS-5	GTG-25	BOX-3	6

Table 2 (cont.)

Bacteria	Phylogenetic groups					
	16S rRNA gene identities	16S rRNA gene	ITS region	(GTG) ₅ element	BOX element	Cumulative clusters
<i>A. caldiproteolyticus</i> D504		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> DSM 15730 ^T		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A403		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A394		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A412b		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> C226		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> D621		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> D623		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A146		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A142		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> D494		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A392b		An-9	ITS-5	GTG-25	BOX-3	6
<i>A. caldiproteolyticus</i> A335		An-9	ITS-5	GTG-26	BOX-3	6
<i>A. tepidamans</i> DSM 16325 ^T	96.0-98.1% to DSM 16325 ^T	An-10	ITS-27	GTG-27	BOX-24	19
Genus <i>Bacillus</i>						
<i>B. licheniformis</i> DSM 13 ^T	-	B-1	ITS-28	GTG-28	BOX-25	20

Abbreviations: The unique and distinctive fingerprinting profiles (% similarities) were written in bold character. The cumulative clusters obtained from three of these analyses were indicated in the right column. 16S rRNA gene groups: G-; *Geobacillus*, Ae-; *Aeribacillus*, An-; *Anoxybacillus*, B-; *Bacillus*, T-; *Thermolongibacillus*, BOX-; groups for BOX element, GTG-; groups for (GTG)₅ element, ITS-; groups for ITS region.

their DNA banding patterns of BOX and GTG elements. Strains of *G. stearothermophilus* ATCC 12980^T and ATCC 7953 displayed similar ITS, BOX and (GTG)₅ profiles, A113 isolate showed the same fingerprinting patterns with *G. stearothermophilus* ATCC 43223 strain. Group G-6 and G-7 contains species from *G. thermodenitrificans* and *G. thermodenitrificans* subsp. *calidus*. D195, A333 isolates and *G. thermodenitrificans* DSM 465^T had identical *Rep*-PCR profiles, whereas they could be differed from each other according to their unique ITS bandings.

Furthermore, although the isolates of F84a and F84b belonging to *G. thermodenitrificans* subsp. *calidus* displayed similar fingerprinting patterns, they could be diverged from the *G. thermodenitrificans* type strain with all their three fingerprinting profiles. In addition, *G. toebii* E134 strain in G-8 group (with *Rep*-PCR and ITS-PCR fingerprintings) and *G. thermoglucosidasius* B84a from G-9 group (with ITS-PCR profile) displayed different banding patterns from their type species.

E137a and E265 isolates from genus *Thermolongibacillus* (T-1) showed differences in their *Rep*-PCR fingerprintings. The isolates A364, C196 and D642 from *Aeribacillus* Ae-1 group differed from their type species *A. pallidus* and E334 isolate by means of their (GTG)₅ element. In addition, all the reference strains from genus *Anoxybacillus* from An-1 to An-10 16S rRNA gene group clearly differentiate from each other by displaying com-

pletely unique banding patterns in all these fingerprinting analyses. Their cluster analyses grouped the *Anoxybacillus* isolates into two different *Anoxybacillus* species: one of which was only composed of C161ab isolate (An-8) belonging to *A. calidus*, which completely differed in all the *Anoxybacillus* isolates and references by representing distinctive profiles. The other 14 isolates including A413, A404, D504, A403, A394, A412b, C226, D621, D623, A146, A142, D494, A392b and A335 (An-9) all belonged to *A. caldiproteolyticus* species. Of those isolates, all of them showed the same fingerprinting patterns with *A. caldiproteolyticus* type species, except A335 having unique (GTG)₅ profile. Finally, *B. licheniformis* (B-1), which is a facultative thermophilic and endospore-forming member of genus *Bacillus*, was also studied as an out group. This type species completely diverged from the other thermophilic isolates and reference strains according to all its fingerprinting profiles by branching as an out group in the cluster analyses (Figure 1).

In conclusion, it is obvious that the ITS-PCR, (GTG)₅PCR and BOX-PCR products generated a high number of bands giving discriminative information below species and subspecies level between these thermophilic isolates and strains studied. Moreover, the cluster analyses of the *Rep*- and ITS-PCR fingerprintings allowed us to differentiate these isolates and reference strains genetically from each other, and also to group them in numbers accord-

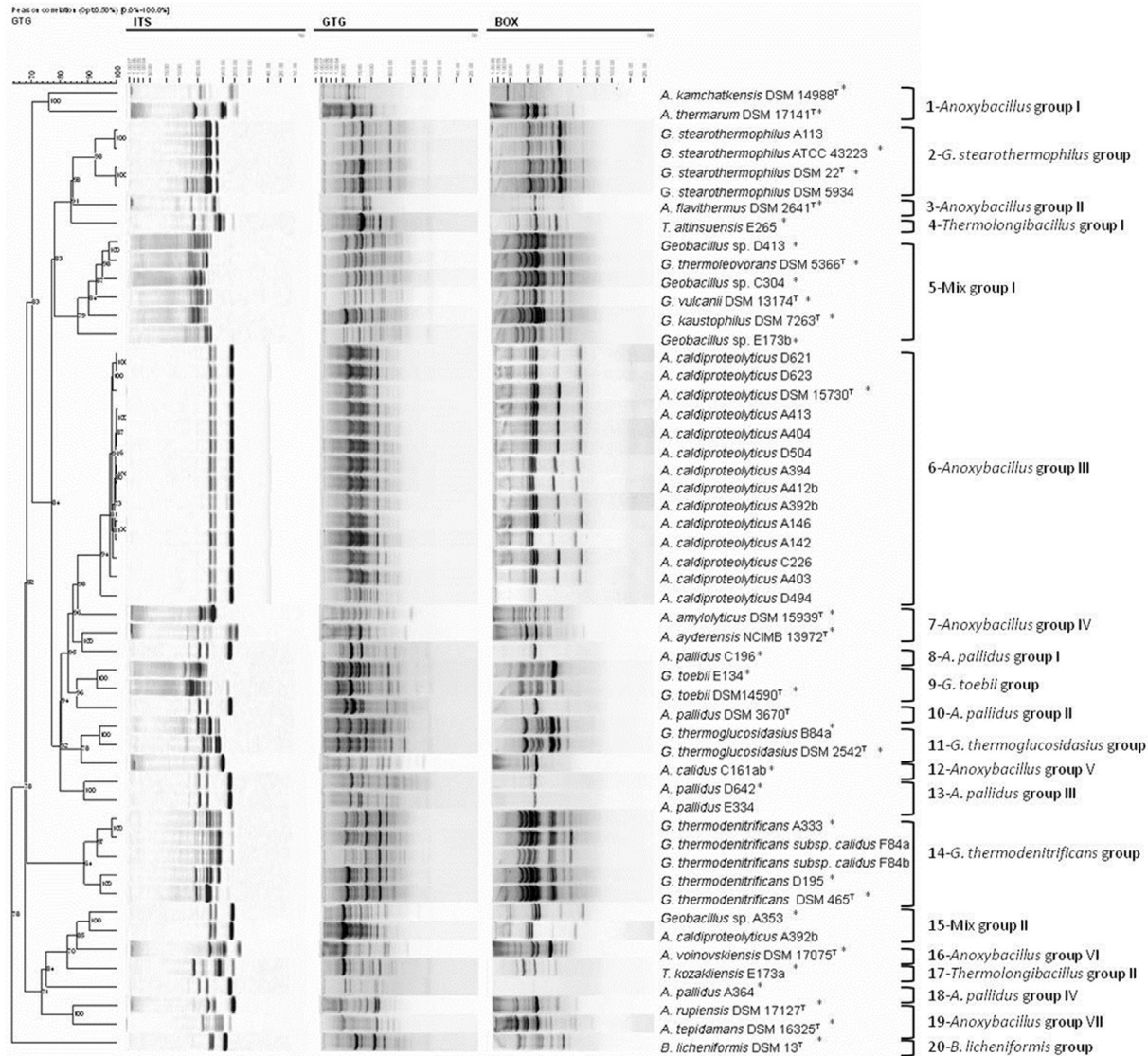


Figure 1 - The cumulative cluster analysis of some representative digitized banding patterns, generated by ITS-, BOX- and GTG-PCR profiles from isolates and reference strains belonging to family *Bacillaceae*. The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. The numbers of clusters obtained from three of the cumulative analyses were indicated in the right side of the figure. The isolates, having unique distinctive profiles, which displayed similarities less than 16.7% for one to three fingerprints patterns were indicated with “**”.

ing to their distinctive fingerprints. These easy, low-cost, discriminative fingerprinting techniques would be also very helpful, before deciding which relatives need to be hybridized according to the differences in their patterns when identifying the species of enzyme producing thermophilic isolates just before further enzymology researches. When screening the lipase producing isolates, investigating their genetic diversity as well as determining their species identifications is all important subjects in order to prevent studying with the same clones of a bacterial species. In addition, to determine the species of a biotechnologically valuable enzyme producing isolates before subsequent studies will not only help to develop their cultivation conditions, but

also give information about the degree of its pathogenity which is an undesirable characteristic for these kinds of enzyme producing valuable strains.

Results of qualitative screening for the lipase producing thermophilic bacilli

When measuring the qualitative lipase activity, in which two different substrates such as olive oil and tributyrin were used, the probable changes in the lipase production depending on the substrate specificities were minimized and a more proper screening for the lipase producing isolates could be determined. Furthermore, instead of incubating the cultures directly on the media containing phenol

red, the supernatant of the crude extracts were applied to the discs. Therefore, the likely variances in the pH value due to the presence of other cellular reactions could be prevented. The change in color from red to yellow, based upon the decrease in the pH was evaluated as positive result for the lipase screening analyses. Some of the positive lipase zones on petri dishes were showed in Figure 2.

According to the qualitative lipase production analyses, 11 of the 32 isolates and 7 reference strains were determined to be use both olive oil and tributyrin or only olive oil as substrate. It was found that all the *G. stearothermophilus* species; including A113 isolate and reference strains ATCC 12908^T, ATCC 43223^T and DSM 7953^T, *G. thermodenitrificans* DSM 465^T and isolates of F84a and F84b from *G. thermodenitrificans* subsp. *calidus* showed positive results on the media containing both olive oil and tributyrin. Furthermore, D195 isolate from *G. thermodenitrificans*; reference strains of *G. kaustophilus* 7263^T, *G. vulcani* 13174^T and isolates of *Geobacillus* sp. A353, D413 and E173b from genus *Geobacillus* mix group; *G. toebii* DSM 14590^T; isolates of D642 and C196 from genus *Aeribacillus*, and also isolates of A403 and D494 belonging to *A. caldoproteolyticus* were all found to be able to use only olive oil as a substrate. Beside these findings, any of the isolates or references could not solely use tributyrin.

Quantitative extracellular lipase production capacities of these thermophilic bacilli

Thermophilic 11 isolate and 7 standard strains showing qualitative lipase activity were incubated in two different broth media containing olive oil and tributyrin to determine their lipolytic activity during 72 h. Extracellular lipolytic activity of isolates and standard strains were in congruent with qualitative screening results which are shown in Table 3. In order to compare the extracellular lipase activities of these bacilli, an index was composed by

dividing the arithmetic lipolytic activities to wet weights of cells (U/mg). According to these results, the lipase activities (U/mg) of these bacilli in olive oil and tributyrin broths during 24, 48 and 72 h are also shown in Figures 3a, 3b, and 3c respectively.

The qualitative enzyme assays revealed that major of the isolates, using olive oil as substrate, showed maximum lipase activity after 72 h. On the other hand, the ones using tributyrin generally showed maximum activity at the end of 24 h. When olive oil was used as a substrate, it was observed that isolates of *G. stearothermophilus* A113, *G. thermodenitrificans* subsp. *calidus* F84a and F84b, and references of *G. thermodenitrificans* DSM 465^T and *G. stearothermophilus* ATCC 7953^T were the highest enzyme producing bacilli among all the bacteria used, but their maximum enzyme production intervals differed from each other. Different from other isolate and references, *G. stearothermophilus* A113 (0.011 U/mg), *G. thermodenitrificans* subsp. *calidus* F84b (0.0095 U/mg) and *G. thermodenitrificans* DSM 465^T (0.008 U/mg) produced the highest lipase at the 24 h of cultivation, whereas *G. thermodenitrificans* subsp. *calidus* F84a (0.0075 U/mg) and *G. stearothermophilus* ATCC 7953^T (0.006 U/mg) displayed their maximal activity at 72 h. When tributyrin was used as a substrate, *G. stearothermophilus* A113 (0.0045 U/mg) and *G. thermodenitrificans* subsp. *calidus* F84a (0.004 U/mg) also showed the maximum lipase activity in 24 and 72 h, respectively. In conclusion, the isolates of *G. stearothermophilus* A113 and *G. thermodenitrificans* subsp. *calidus* F84b in the case of olive oil and the isolates of *G. stearothermophilus* A113 and *G. thermodenitrificans* subsp. *calidus* F84a in the presence of tributyrin had higher lipolytic activity than the thermophilic standard lipase producing strains which might displaybiotechnological importance.

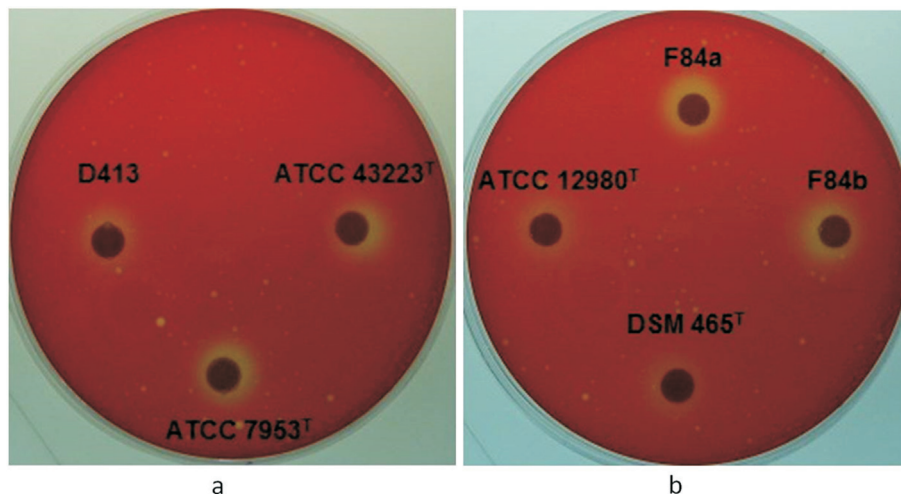


Figure 2 - Some qualitatively positive screening results for lipase on plates (a) with tributyrin (b) and olive oil.

Table 3 - The qualitative lipase activities of the isolates and reference strains in olive oil and tributyrin broths per cell yields (U/mg) during 24, 48 and 72 h (High activities were marked with grey).

Bacteria	Lipase activity (U/mg)					
	Substrate used in enzyme production media					
	Olive Oil			Tributyrin		
	24 h	48 h	72 h	24 h	48 h	72 h
<i>G. stearothermophilus</i> A113	0.011	0.005	0.0035	0.0045	0.0025	0.001
<i>G. thermodenitrificans</i> subsp. <i>calidus</i> F84b	0.0095	0.009	0.008	0.002	0.00	< 0.001
<i>G. thermodenitrificans</i> DSM 465 ^T	0.008	0.007	0.006	0.0025	0.00	< 0.001
<i>G. thermodenitrificans</i> subsp. <i>calidus</i> F84a	0.006	0.007	0.0075	0.0015	0.002	0.004
<i>G. stearothermophilus</i> ATCC 7953 ^T	0.0055	0.006	0.006	0.002	0.001	0.001
<i>Geobacillus</i> sp. A353	0.0045	0.003	0.0065	< 0.001	< 0.001	< 0.001
<i>G. thermodenitrificans</i> D195	0.003	0.0015	0.0015	< 0.001	< 0.001	< 0.001
<i>Geobacillus</i> sp. E173b	0.003	0.003	0.0035	< 0.001	< 0.001	< 0.001
<i>G. stearothermophilus</i> ATCC 43223 ^T	0.002	0.003	0.004	0.001	0.001	0.002
<i>G. stearothermophilus</i> ATCC 12980 ^T	0.0015	0.0015	0.004	0.003	0.0025	0.002
<i>A. caldiproteolyticus</i> A403	0.0015	0.0035	0.002	< 0.001	< 0.001	< 0.001
<i>G. toebii</i> DSM 14590 ^T	0.001	0.0025	0.003	< 0.001	< 0.001	< 0.001
<i>Aeribacillus</i> sp. C196	< 0.001	0.001	0.0025	< 0.001	< 0.001	< 0.001
<i>A. caldiproteolyticus</i> D494	< 0.001	0.002	0.0025	< 0.001	< 0.001	< 0.001
<i>Aeribacillus</i> sp. D642	< 0.001	0.0015	0.002	< 0.001	< 0.001	< 0.001
<i>G. kaustophilus</i> DSM 7263 ^T	< 0.001	0.005	0.0065	< 0.001	< 0.001	< 0.001
<i>G. vulcani</i> DSM 13174 ^T	< 0.001	0.003	0.0045	< 0.001	< 0.001	< 0.001
<i>Geobacillus</i> sp. D413	< 0.001	0.0025	0.0025	< 0.001	< 0.001	< 0.001

Growth characteristics in cultures containing olive oil and tributyrin

As explained in the qualitative lipase production capacities of the isolates, the highest enzyme producing *G. stearothermophilus* A113, *G. thermodenitrificans* subsp. *calidus* F84a, and *G. thermodenitrificans* subsp. *calidus* F84b were further selected for their enzyme production levels and their growth characteristics in media containing olive oil and tributyrin as substrates. The change in the enzyme activity, pH and the optic density of the *G. thermodenitrificans* subsp. *calidus* F84a during 72 h is presented in Figure 4a. *G. thermodenitrificans* subsp. *calidus* F84a showed maximum lipase activity (0,052 U/mL) in olive oil at the end of 72 h. The bacterial growth increased only up to 24 h and the pH values did not change significantly during 72 h. In contrast to the growth in olive oil medium, growth in medium containing tri butyrin was able to be achieved after 24 h. Maximum lipase activity (0,019 U/mL) was observed in tributyrin medium at the end of 72 h and the pH value decreased from 6.90 to 4.50. Therefore, F84a isolate preferred medium containing olive oil for both bacterial growth and lipase production.

As presented in Figure 4b, *G. thermodenitrificans* subsp. *calidus* F84b reached its maximum growth in the olive oil medium at the end of 24 h, but after 24 h, the growth

began to decrease which might be in relation with sporulation. In contrast to bacterial growth, the maximum lipase activity (0,052 U/mL) was observed within 72 h. This enzyme activity was one of the highest value measured in this study. The pH of the medium also showed a slow decrease through 48 h, then a little increase was observed. In the case of medium containing tributyrin, this strain showed a better growth rate, but the lipolytic activity was only observed within 24 h and in a low rate (0,007 U/mL).

In olive oil broth, the lipase activity of *G. stearothermophilus* A113 did not change significantly between 24-72 h, but maximum lipolytic activity was observed within 72 h (Figure 4c). The pH of this medium decreased through 72 h up to 4.70. A113 isolate showed the maximum growth between 24-48 h of cultivation, and then growth began to decrease throughout 72 h in olive oil media. In contrast, growth rate was increased when it was cultured in the broth including tributyrin during 72 h. The maximum lipase activity was observed within 24 h (0,008 U/mL) and the change in the pH value in the extracellular fraction decreased to 4.80 as in the case of olive oil broth.

In this study, not only our thermophilic isolates and their type species were screened for their lipase production capacities, but also their genetic diversities were investigated. Thus, by identifying the isolates up to subspecies

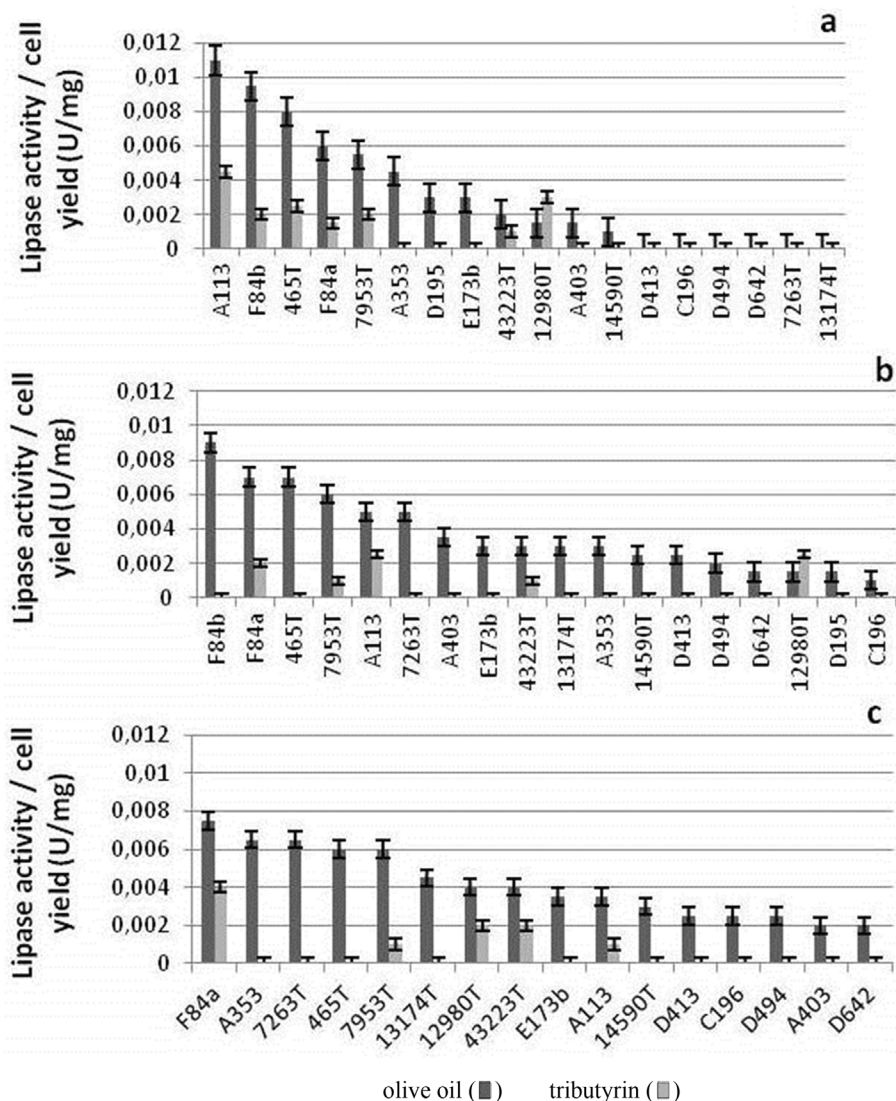


Figure 3 - The graphs of indexes showing the extracellular lipolytic activity/wet weight (U/mg) values of the thermophilic isolates and reference strains in olive oil and tributyrin broths at the hours of a) 24, b) 48, and c) 72.

level, studying with the same clones of a bacterial species was avoided before further studies. Only thermophilic species of *G. stearothermophilus* L1, *G. zalihae*, *G. thermoleovorans* ID-1, *Geobacillus* sp. TW1 and *A. kamchatkensis* KW 12 (Kim et al., 1998, Lee et al., 1999, Kambourova et al., 2003, Li and Zhang, 2005, Rahman et al., 2007, Olusesan et al., 2009) were reported as lipase producing isolates till now. In this study, when the type species of *G. stearothermophilus*, *G. thermoleovorans* and *A. kamchatkensis* were screened, only *G. stearothermophilus* was found to be a lipase producing strain. In this study, not only these thermophilic type species were screened for their lipase activities. In addition, the type species of *G. thermodenitrificans* and *G. thermodenitrificans* subsp. *calidus* were found to be valuable according to their lipase

production capacities. Of those from our isolates, *G. thermodenitrificans* subsp. *calidus* F84a and F84b in addition to *G. stearothermophilus* A113 were promising by means of their lipase production capacities for further applications as they displayed higher enzyme activities in comparison to the lipase producing references. When all these enzymatic and taxonomic studies were combined, it was revealed that the lipase production varies between the strains of a species and its subspecies. This also implies the importance of studying the taxonomic position of an enzyme producing isolate. To conclude, this research formed a base for further studies dealing with enzyme characterization and cloning studies by screening high levels of thermostable lipase producing isolates in addition with representing their genotypic diversity by applying DNA fingerprinting techniques.

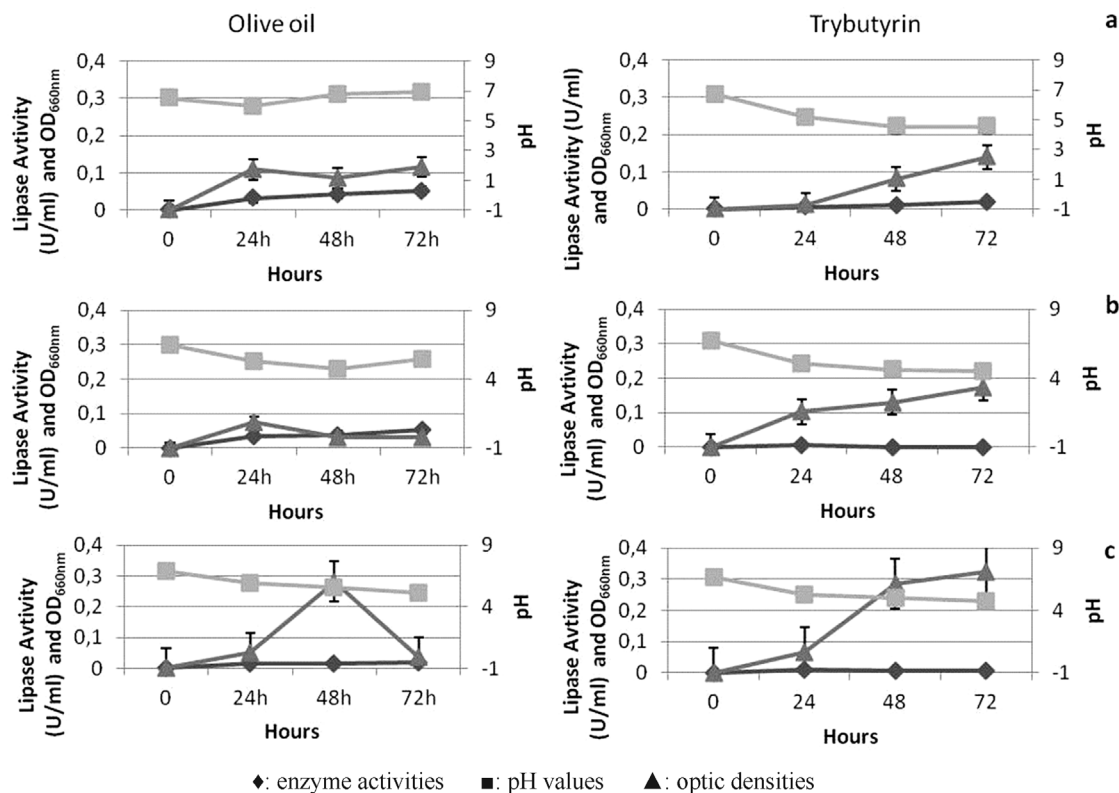


Figure 4 - The change of enzyme activities, pH values and optic densities of a) *G. thermodenitrificans* subsp. *calidus* F84a (DSM 22628), b) *G. thermodenitrificans* subsp. *calidus* F84b (DSM 22629^T), and c) *G. stearothermophilus* A113 in broth cultures containing olive oil (left) and trybutyryn (right) during the 72- h period of incubation.

Acknowledgments

This research was supported by the Scientific Research Project Office of Ankara University with the project number of 11B4240003.

References

- Abdel-Fattah SA, El-Sanhoury MH, El-Mednay NM *et al.* (2008) Thyroid activity, some blood constituents, organs morphology and performance of broiler chicks fed supplemental organic acids. *Int J Poultry Sci* 7:215-222.
- Bornscheuer U, Reif OW, Lausch R *et al.* (1994) Lipase of *Pseudomonas cepacia* for biotechnological purposes: purification, crystallization and characterization. *Biochim Biophys Acta* 1201:55-60.
- Castro-Ochoa LD, Rodriguez-Gomez C, Valerio-Alfaro G *et al.* (2005) Screening, purification and characterization of the thermoalkalophilic lipase produced by *Bacillus thermo-levorans* CCR11. *Enzyme Microb Technol* 37:648-654.
- Cihan AC, Ozcan B, Cokmus C (2011) *Anoxybacillus salavatliensis* sp. nov., an α -glucosidase producing, thermophilic bacterium isolated from Salavatli, Turkey. *J Basic Microbiol* 50:1-11.
- Cihan AC, Ozcan B, Tekin N *et al.* (2011b) *Geobacillus thermodenitrificans* subsp. *calidus*, subsp. nov., a thermophilic and α -glucosidase producing bacterium isolated from Kizilcahamam, Turkey. *J Gen Appl Microbiol* 57:83-92.
- Daffonchio D, Cherif A, Brusetti L *et al.* (2003) Nature of polymorphisms in 16S-23S rRNA gene intergenic transcribed spacer fingerprinting of *Bacillus* and related genera. *Appl Environ Microbiol* 69:5128-5137.
- Derekova A, Mandeva R, Kambourova M (2008) Phylogenetic diversity of thermophilic carbohydrate degrading bacilli from Bulgarian hot springs. *World J Microbiol Biotechnol* 24:1697-1702.
- Haki GD, Rakshit SK (2003) Developments in industrially important thermostable enzymes: a review. *Bioresour Technol* 89:17-34.
- Hough WD, Danson, MJ (1999) Extremozymes. *Curr Opin Chem Biol* 3: 39-46.
- Jaeger KE, Ransac S, Dijkstra BW *et al.* (1994) Bacterial lipases. *FEMS Microbiol Rev* 15:29-63.
- Joseph B, Ramteke PW, Thomas G (2008) Cold active microbial lipases: some hot issues and recent developments. *Biotechnol Adv* 26:457-470.
- Kambourova M, Kirilova N, Mandeva R *et al.* (2003) Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7. *J Mol Catal B Enzym* 22:307-313.
- Kim HK, Park SY, Lee JK *et al.* (1998) Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1. *Biosci Biotechnol Biochem* 62:66-71.
- Kuisiene N, Raugas J, Sproer C *et al.* (2008) *Bacillus butanolivorans* sp. nov., a species with industrial application for the remediation of n-butanol. *Int J Syst Evol Microbiol* 58:505-509.

- Lee DW, You-Seok K, Ki Jun K *et al.* (1999) Isolation and characterisation of thermophilic lipase from *Bacillus thermo-levorans* ID-1. *FEMS Microbiol Lett* 179:393-400.
- Li H, Zhang X (2005) Characterization of thermostable lipase thermophilic *Geobacillus* sp. TW1. *Protein Expression Purif* 42:153-159.
- Logan NAO, Berge AH, Bishop HJ *et al.* (2009) Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. *Int J Syst Evol Microbiol* 59: 2114-2121.
- Ludwig W, Schleifer KH (1999) Phylogeny of bacteria beyond the 16S rRNA standard. *ASM News* 65:752-757.
- Manachini PL, Mora D, Nicastro G *et al.* (2000) *Bacillus thermodenitrificans* sp. nov., nom. rev. *Int J Syst Evol Microbiol* 50:1331-1337.
- Mora D, Fortina MG, Nicastro G *et al.* (1998) Genotypic characterization of thermophilic bacilli: a study on new isolates and several reference strains. *Res Microbiol* 149:711-722.
- Olusesan AT, Azura LK, Abubakar NSA *et al.* (2009) Phenotypic and molecular identification of a novel thermophilic *Anoxybacillus* species: a lipase-producing bacterium isolated from a Malaysian hot spring. *World J Microbiol Biotechnol* 25: 1981-1988.
- Rahman RNZRA, Leow TC, Salleh AB *et al.* (2007) *Geobacillus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. *BMC Microbiol* 7:77 doi:10.1186/1471-2180-7-77
- Rodas AM, Ferrer S, Pardo I (2003) 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. *Syst Appl Microbiol* 26:412-422.
- Rosello-Mora R (2005) DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation. In: Stackebrandt E (ed.) *Molecular Identification, Systematics, and Population Structure of Prokaryotes*. Heidelberg, Springer, 23-50.
- Stackebrandt E, Fredericksen W, Garrity GM *et al.* (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* 52:1043-1047.
- Vaneechoutte M, Rossau R, De VP *et al.* (1992) Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS* 93:227-233.
- Versalovic J, Schneider M, De Bruijn FJ *et al.* (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth Mol Cell Biol* 5:25-40.
- White SDM, Navarro JF, Evrard AE *et al.* (1993) The baryon content of galaxy clusters: a challenge to cosmological orthodoxy. *Nature* 366:429-433.
- Yadav RP, Saxena RK, Gupta R *et al.* (1998) Purification and characterization of a regiospecific lipase from *Aspergillus terreus*. *Biotechnol Appl Biochem* 28:243-249.

Associate Editor: Jorge Gonzalo Farias Avendano

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.