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

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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 pNPB 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 tributyrin. 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 465T (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 tributyrin. 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), (Vaneechoutte 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 thermozyymes, 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 thermoleovarans ID-1 (Lee et al., 1999), Geobacillus stearothermophilus L1 (Kambourouva 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 thermostable, 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 Geobacillus (15 isolates), Geobacillus (11), Aeribacillus (4) and Thermolongi bacillus (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 fingerprints were performed with the (GTG)3 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)3-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)3- 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 (pNPB, 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 pNPB 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 pNP (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 pNPB per minute at 60 °C, pH 7.0. The millimolar extinction coefficient of pNP 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.
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 after 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’ (Roselló-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. thermodenitricans* and *G. kaustophilus*, they could not be identified up to species level without DNA-DNA hybridization analyses. The rest of the isolates were found to belong to species from *G. stearothermophilus* (A113), *G. thermodenitricans* (D195, A333), *G. thermodenitricans* subsp. *calidus* (F84a, F84b), *G. toebii* (E134), *G. thermoglucosidasius* (B84a), *T. kozakiakensis* (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 (Ver-salovic 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. thermoeovorans* and *G. kaustophilus*, but also the isolates (E173b, D413, C304, A353) from mix group (G1-G-4), having high RNA 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. stearo-thermophilus* 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 Geobacillus** | | | | | | | |
| G. vulcanii 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 |
| Geobacillus sp. E173b | | | G-1 | ITS-2 | GTG-1 | BOX-1 | 5 |
| Geobacillus sp. D413 | | | G-1 | ITS-3 | GTG-2 | BOX-2 | 5 |
| Geobacillus sp. C304 | | | G-1 | ITS-4 | GTG-2 | BOX-2 | 5 |
| Geobacillus sp. A353 | | | G-1 | ITS-5 | GTG-3 | BOX-3 | 15 |
| G. thermoleovorans DSM 5366$^T$ | | | G-3 | ITS-6 | GTG-1 | BOX-4 | 5 |
| G. kaustophilus DSM 7263$^T$ | | | G-4 | ITS-7 | GTG-4 | BOX-5 | 5 |
| G. stearothermophilus | | | | | | | |
| A113 | | | G-5 | ITS-8 | GTG-5 | BOX-6 | 2 |
| G. stearothermophilus ATCC 43223$^T$ | | | G-5 | ITS-8 | GTG-6 | BOX-7 | 2 |
| G. stearothermophilus ATCC 12980$^T$ | | | G-5 | ITS-8 | GTG-6 | BOX-7 | 2 |
| G. stearothermophilus ATCC 7953 | | | G-5 | ITS-8 | GTG-6 | BOX-7 | 2 |
| G. thermodenitrificans D195 | | 98.7-99.2% to DSM 465$^T$ | G-6 | ITS-9 | GTG-7 | BOX-8 | 14 |
| G. thermodenitrificans A333 | | | G-6 | ITS-10 | GTG-7 | BOX-8 | 14 |
| G. thermodenitrificans DSM 465$^T$ | | | G-6 | ITS-11 | GTG-7 | BOX-8 | 14 |
| G. thermodenitrificans subsp. callidus F84a | | 98.3% to DSM 22629$^T$ | G-7 | ITS-12 | GTG-8 | BOX-9 | 14 |
| G. thermodenitrificans subsp. callidus F84b DSM 22629$^T$ | | | G-7 | ITS-12 | GTG-8 | BOX-9 | 14 |
| Genus Thermolongibacillus | | | | | | | |
| T. kozakliensis E173a | | 97.5% to each other, 94.2-95.0% to DSM 14590$^T$ | T-1 | ITS-17 | GTG-12 | BOX-13 | 17 |
| T. altinsuensis E265 | | | T-2 | ITS-17 | GTG-13 | BOX-14 | 4 |
| Genus Aeribacillus | | | | | | | |
| Aeribacillus sp. A364 | | 98.0-99.2% to DSM 3670$^T$ | Ae-1 | ITS-18 | GTG-14 | BOX-15 | 18 |
| Aeribacillus sp. C196 | | | Ae-1 | ITS-18 | GTG-15 | BOX-15 | 8 |
| Aeribacillus sp. D642 | | | Ae-1 | ITS-18 | GTG-16 | BOX-15 | 13 |
| Aeribacillus sp. E334 | | | Ae-1 | ITS-18 | GTG-16 | BOX-15 | 13 |
| A. pallidus DSM 3670$^T$ | | | Ae-1 | ITS-18 | GTG-16 | BOX-15 | 13 |
| Genus Anoxybacillus | | | | | | | |
| A. ayderensis NCIMB 13972$^T$ | | 99.7-94.3% to each other | An-1 | ITS-19 | GTG-17 | BOX-16 | 7 |
| A. flavithermus DSM 2641$^T$ | | | An-2 | ITS-20 | GTG-18 | BOX-17 | 3 |
| A. kamchatkensis DSM 14988$^T$ | | | An-3 | ITS-21 | GTG-19 | BOX-18 | 1 |
| A. amylolyticus DSM 15930$^T$ | | | An-4 | ITS-22 | GTG-20 | BOX-19 | 7 |
| A. rupiensis DSM 17127$^T$ | | | An-5 | ITS-23 | GTG-21 | BOX-20 | 19 |
| A. voionovskiiensis DSM 17075$^T$ | | | An-6 | ITS-24 | GTG-22 | BOX-21 | 16 |
| A. thermarum DSM 17141$^T$ | | | An-7 | ITS-25 | GTG-23 | BOX-22 | 1 |
| A. caldus C161ab | | 96.8% to DSM 17127$^T$ and DSM 17075$^T$ | An-8 | ITS-26 | GTG-24 | BOX-23 | 12 |
| A. caldiproteolyticus A413 | | 97.9-99.9% to DSM 15730$^T$ | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| A. caldiproteolyticus A404 | | | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
their DNA banding patterns of BOX and GTG elements. Strains of *G. stearothermophilus* ATCC 12980T 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 465T 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 differed from each other according to their unique ITS bandings.

Table 2 (cont.)

| Bacteria | Phylogenetic groups |
|----------|---------------------|
|          | 16S rRNA gene identities | 16S rRNA gene region | (GTG)₅ element | BOX element | Cumulative clusters |
| *A. caldiproteolyticus* D504 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* DSM 15730ᵀ | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* A403 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* A394 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* A412b | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* C226 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* D621 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* D623 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* A146 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* A142 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* D494 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* A392b | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. caldiproteolyticus* A335 | An-9 | ITS-5 | GTG-25 | BOX-3 | 6 |
| *A. tepidamans* DSM 16325ᵀ | 96.0-98.1% to DSM 16325ᵀ | An-10 | ITS-27 | GTG-27 | BOX-24 | 19 |

Genus Bacillus

| *B. licheniformis* DSM 13ᵀ | - | 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.

In conclusion, it is obvious that the ITS-PCR, (GTG)₅-PCR 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 fingerprints allowed us to differentiate these isolates and reference strains genetically from each other, and also to group them in numbers accord-
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 pathogenicity 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 tributyryrin 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 *Aerobicillus*, 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.
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* F84b, and *G. thermodenitrificans* subsp. *calidus* F84a 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 tributyrin 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

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**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) | Olive Oil   | Tributyrin |
|----------------------------------|------------------------|-------------|------------|
|                                  |                        | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| *G. stearothermophilus* A113     |                        | 0.011| 0.005| 0.0035| 0.0045| 0.0025| 0.001|
| *G. thermodenitrificans* subsp. *calidus* F84b |              | 0.0095| 0.009| 0.008| 0.002| 0.00| < 0.001|
| *G. thermodenitrificans* DSM 465T |              | 0.008| 0.007| 0.006| 0.0025| 0.00| < 0.001|
| *G. thermodenitrificans* subsp. *calidus* F84a |              | 0.006| 0.007| 0.0075| 0.0015| 0.002| 0.004|
| *G. stearothermophilus* ATCC 7953T |              | 0.0055| 0.006| 0.006| 0.002| 0.001| 0.001|
| Geobacillus sp. A353             |                        | 0.0045| 0.003| 0.0065| < 0.001| < 0.001| < 0.001|
| *G. thermodenitrificans* D195    |                        | 0.003| 0.0015| 0.0015| < 0.001| < 0.001| < 0.001|
| Geobacillus sp. E173b            |                        | 0.003| 0.003| 0.0035| < 0.001| < 0.001| < 0.001|
| *G. stearothermophilus* ATCC 43223T |              | 0.002| 0.003| 0.004| 0.001| 0.001| 0.002|
| *G. stearothermophilus* ATCC 12980T |             | 0.0015| 0.0015| 0.004| 0.003| 0.0025| 0.002|
| *A. caldiproteolyticus* A403     |                        | 0.0015| 0.0035| 0.002| < 0.001| < 0.001| < 0.001|
| *G. toebii* DSM 14590T           |                        | 0.001| 0.0025| 0.003| < 0.001| < 0.001| < 0.001|
| Aeribacillus sp. C196             |                        | < 0.001| 0.001| 0.0025| < 0.001| < 0.001| < 0.001|
| *A. caldiproteolyticus* D494     |                        | < 0.001| 0.002| 0.0025| < 0.001| < 0.001| < 0.001|
| Aeribacillus sp. D642             |                        | < 0.001| 0.0015| 0.002| < 0.001| < 0.001| < 0.001|
| *G. kaustophilus* DSM 7263T       |                        | < 0.001| 0.005| 0.0065| < 0.001| < 0.001| < 0.001|
| *G. vulcani* DSM 13174T           |                        | < 0.001| 0.003| 0.0045| < 0.001| < 0.001| < 0.001|
| Geobacillus sp. D413              |                        | < 0.001| 0.0025| 0.0025| < 0.001| < 0.001| < 0.001|
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 *A. kamchatkensis* 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.
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