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EXPERIMENTAL ARTICLES

Isolations of α-Glucosidase-Producing Thermophilic Bacilli from Hot Springs of Turkey[1]

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Abstract—From 42 different hot springs in six provinces belonging to distinct geographical regions of Turkey, 451 thermophilic bacilli were isolated and 67 isolates with a high amylase activity were selected to determine the α-glucosidase production capacities by using *p*NPG as a substrate. α-Glucosidase production capacities of the isolates varied within the range from 77.18 to 0.001 U/g. Eleven of our thermophilic bacilli produced α-glucosidase at significant levels comparable with that of the reference strains tested; thus, five strains, F84b (77.18 U/g), A333 (48.64 U/g), F84a (36.64 U/g), E134 (32.09 U/g), and A343 (10.79 U/g), were selected for further experiments. 16S rDNA sequence analysis revealed that these selected isolates all belonged to thermophilic bacilli 16S rDNA genetic group 5, four of them representing the genus *Geobacillus*, while strain A343 had an uncultured bacterium as the closest relative. Changes in α-glucosidase levels in the intracellular and extracellular fractions were determined during 48-h cultivation of A333, A343, F84a, F84b, E134, and the reference strain *G. stearothermophilus* ATCC 12980. According to α-glucosidase production type and enzyme levels in intracellular and extracellular fractions, *Geobacillus* spp. A333, F84a, and F84b were defined as extracellular enzyme producers, whereas the thermophilic bacterium A343 was found to be an intracellular α-glucosidase producer, similar to ATCC 12980 strain. *Geobacillus* sp. E134 differed in α-glucosidase production type from all tested isolates and the reference strain; it was described as a membrane-associated cell-bound enzyme producer. In this study, apart from screening a great number of new thermophilic bacilli from the hot springs of Turkey, which have not yet been thoroughly studied, five new thermostable α-1,4-glucosidase-producing bacilli that have biotechnological potential with α-glucosidases located at different cell positions were obtained.

Key words: isolation, thermophilic bacilli, 16S rDNA, α-glucosidase.

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INTRODUCTION

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) occur in microorganisms as intracellular, extracellular, or cell-bound enzymes and hydrolyze terminal, nonreducing α-1,4-linked-D-glucose residues in short-chain oligosaccharides including maltose, which are formed by other amylolytic enzymes, like α-amylase (EC 3.2.1.1) or glucoamylase (EC 3.2.1.3) [1]. They have a number of potential applications in fundamental research, in industrial starch processes, in synthesis of oligo-, di-, and trisaccharides, as an indicator for sterilization control, and in α-amylase assay in clinical laboratories. They are usually found in association with other amylolytic enzymes which accomplish complete degradation of starch, and are widely distributed throughout the three major kingdoms [2, 3].

The industrial demand for enzymes that can withstand harsh conditions has greatly increased over the past decade. Therefore, thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability. Advances in this area have been possible with the isolation of a large number of thermophilic microorganisms from different ecological niches and subsequent production of valuable enzymes [4, 5].

Typically, enzyme production in the course of *Bacillus* fermentation processes occurs during a relatively short period of time, with very low cost carbon and nitrogen sources. Members of the genus *Bacillus* have been used in multiple research works aimed on screening and purification of thermostable enzymes [6, 7]. α-Glucosidases from *B. caldovelax* DSM411 [8], *B. flavocaldarius* KP1228 (FERM-P9542) [9], *B. thermoamyloliquefaciens* KP1071 (FERM-P84776) [10], *G. stearotherothermophilus* ATCC 7953 [11], *G. stearothermophilus* ATCC 12016 [12], *G. thermodenitrificans* HR010 [13], *Geobacillus* HTA-462 [14], and *Bacillus* sp. DG0303 [15] have been well characterized.

The aim of this study was to isolate and screen α-glucosidase-producing thermophilic microorganisms from Turkish hot springs in order to determine their

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enzyme production capacities and to define the α-glucosidase production types in some of the new isolates by measuring the change in the enzyme levels in intracellular and extracellular fractions.

MATERIALS AND METHODS

Bacterial Isolates and Standard Strains

One hundred and ninety-one samples of water, soil, and sediments were collected from 42 different hot springs and high-temperature well pipelines of geographically widespread locations in the provinces of Ankara, Aydin, Denizli, Izmir, Manisa, and Nevsehir in Turkey. The water temperature and pH of these geothermal regions were in the range 60–90°C and 6.0–9.0, respectively. α-Glucosidase-producing strains: A333 and A343 were isolated from soil and sediment samples of Germencik (Aydin) and Salavatli (Aydin), respectively. F84a and F84b were both obtained from sediment samples of Kizilcahamam (Ankara) high-temperature well pipelines. E134 was isolated from a water sample of Kozakli (Nevsehir) hot spring. *Geobacillus stearothermophilus* ATCC 12980, *G. stearothermophilus* ATCC 43223, *Anoxybacillus flavothermus* DSM 2641, *Anoxybacillus kestanbolensis* NCIB 13971, *Anoxybacillus gonensis* NCIB 13933, and *Anoxybacillus ayderensis* NCIB 13972 were kindly provided by Prof. Dr. Ali Osman Beldüz (Karadeniz Technical University, Turkey) and *G. stearothermophilus* ATCC 7953 (DSM 5934), *G. thermoglucosidasius* DSM 2542, and *Bacillus amyloliquefaciens* DSM 7 were provided by DSMZ (the German Collection of Microorganisms and Cell Cultures).

Isolation of Thermophilic Amylolytic Bacilli

For enrichment, the water, sediment, and soil samples were incubated with shaking at 60°C and 250 rpm for 24 h in 5 ml of the *G. thermoglucosidasius* medium (MI) containing 1% soluble starch (Sigma S2004), 0.5% pepton, 0.3% yeast extract, 0.3% meat extract, 0.3% K_2HPO_4, and 0.1% KH_2PO_4 (pH 7.0) [16]. Pure cultures showing different colony morphology were isolated by streaking on plates of the same medium containing 3% agar. All the isolates were tested for their Gram reaction, cell and colony morphology, motility, sporulation, thermophilic growth, and catalase and amylase activity [17]. Cell morphology, sporulation, and motility were determined by phase-contrast microscopy of freshly prepared wet mounts [17, 18].

After cultivation of new isolates on MI agar plates at 60°C for 24 h, the plates were treated with iodine solution (0.2% I_2 in 2% KI) in order to determine amylolytic activity. The thermophilic amylolytic isolates with starch digestion zones around their colonies were selected for further investigations on α-glucosidase production capacities [7, 16]. All the isolates were maintained on nutrient agar slants at 4°C and stored in nutrient broth containing glycerol at –80°C.

16S rDNA Amplification and Sequencing

Genomic DNA was extracted according to Marmur [19] from the cultures growing in nutrient broth for 18 h at 60°C. The gene encoding 16S rDNA was amplified by PCR with the 16S bacteria-specific 27Fa forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and the 1492R reverse primer (5'-GGTTACCTTGTTACGACTT-3') using the method of Bond et al. [20]. The amplification products were purified from agarose gel using a Qiagen QiaexII Gel Extraction kit. These products were cloned into PCR2.1 vector with TOPO TA Cloning kit (Invitrogen) and sequenced by using M13 (–21) and M13R primers peculiar to PCR2.1 vector.

Phylogenetic Analysis

A homology search was carried out by using the basic BLASTN search program at the NCBI web site. 16S rDNA sequences were aligned by using Clustal W [21]. The tree distance matrix was calculated on the basis of the algorithm of Jukes and Cantor [22]. A phylogenetic tree was constructed by the neighbor-joining method and evaluated by bootstrap sampling (1000 replicates) using the MEGA 4 program [23].

Enzyme Assay

α-Glucosidase activity was determined spectrophotometrically by measuring the hydrolysis of *para*-nitrophenol α-D-glucopyranoside (*p*NPG, Sigma N1377) as substrate, by the modified methods [16, 24, 25]. The standard reaction mixture in a total volume of 4 ml contained 33.3 mM potassium phosphate (pH 6.8), 2 mM *p*NPG, and 500 μl of an appropriately diluted enzyme solution. The reaction was carried out at 37°C for 10 min and stopped by adding 1 M Na_2CO_3. The release of *p*NP (*para*-nitrophenol) was measured by absorption at 400 nm, and one unit of enzyme was defined as the amount of enzyme needed for hydrolysis of 1 μmol *p*NPG per minute at 37°C, pH 6.8. The millimolar extinction coefficient of *p*NP at 400 nm and pH 6.8 was measured as 18.5 L mM^{-1} cm^{-1} and was used to calculate the product yield. All the enzyme assays were performed at least three times

Growth Conditions for α-Glucosidase Production

In order to determine intracellular or extracellular α-glucosidase production capacities of thermophilic, amylolytic isolates, and enzyme-producing reference strains (*G. stearothermophilus* ATCC 12980, *G. stearothermophilus* ATCC 43223, *G. stearothermophilus* ATCC 7953, and *G. thermoglucosidasius* DSM 2542), the organisms were cultured on MI agar plates at 60°C for 24 h. Actively growing cells were then resuspended in 0.85% NaCl to an absorbance value of 0.16–0.3 at 660 nm. For each of the isolates, 0.5 ml of the resulting suspension was inoculated into three test tubes of 5 ml enzyme production medium (MII) containing 2% solu-

Table 1. General characteristics of the isolated thermophilic bacilli from geothermal regions

Property	Characteristic	Positive results (%)
Gram reaction		100
Cell shape	Rod	100
Motility	+	100
Catalase activity	+	100
Amylase activity	+	17
Endospore location	Central	7
	Subterminal	48
	Terminal	45
Swollen endospore	+	61
Spore shape	Circular	0.6
	Oval	11.4
	Elipsoidal	88
Temperature requirement	Mesophilic	13
	Thermophilic	87

ble starch, 2% pepton, 0.2% yeast extract, 0.5% meat extract, 0.3% K_2HPO_4, and 0.1% KH_2PO_4 (pH 7.0) [16, 26]. Liquid cultures were incubated at 60°C, 250 rpm for 15 h, and cells and supernatants were obtained by centrifugation at 10 000 rpm for 15 min at 4°C [9].

Extraction of Intracellular and Extracellular α-Glucosidase

After centrifugation of the culture, the cells were washed twice with 5 ml 0.85% NaCl. The wet weight of the pellets and final pH of the supernatant were measured, and the pellets were stored at –20°C prior to sonication. The supernatant was mixed with the washings, and three-times diluted solution was used for the determination of extracellular α-glucosidase activity [16, 26].

Frozen cells were thawed in 50 mM potassium phosphate–5 mM EDTA (pH 7.0) buffer; final cell concentration was adjusted to 20% wet weight/volume and then disrupted by sonication (Vibracell Sonics). The cell debris was removed by centrifugation and washed twice with 0.85% NaCl. Both the cell-free fractions and the washings were combined and assayed for intracellular α-glucosidase activity [3, 9, 16, 27].

Statistical analysis determining the mean, standard deviation, and standard error was carried out for triplicate enzyme assays in three parallel experiments of both intracellular and extracellular fractions. The mean values of the intracellular and extracellular enzyme activities in each fraction were expressed as the total amount of α-glucosidase per cell yield. Furthermore, for the comparison of enzyme production levels in both fractions, t-test analyses were applied to the values of intracellular and extracellular enzyme activity per cell yield.

Determining the Growth Curve and α-Glucosidase Production Levels of High-Enzyme-Producing Isolates

Five isolates with the highest α-glucosidase activity (A333, A343, E134, F84a, F84b) and the reference strain (*G. stearothermophilus* ATCC 12980) were incubated on MI agar at 60°C for 24 h. The cells were resuspended in 30 ml MII broth, and these suspensions (15 ml) were used to inoculate 150 ml MII in 500 ml flasks. The cultures were incubated at 60°C with shaking at 250 rpm for 48 h, and the samples (5 ml) were taken at 4-h intervals. Intracellular and extracellular enzyme fractions were obtained as mentioned before and assayed for α-glucosidase activity. The bacterial growth curve was obtained by measuring the absorbance of the culture at 660 nm; the pH changes of the culture medium were also followed [16].

RESULTS AND DISCUSSION

Thermophilic Amylolytic Isolates

A total of 516 isolates with different colony morphologies were obtained from the samples from different geothermal regions. All isolates were rod-shaped, gram-positive, motile, endospore-forming, catalase-positive bacteria. General characteristics of the isolated bacilli are shown in Table 1. Isolates which could grow on nutrient agar between 50 and 75°C were defined as thermophilic, whereas the ones growing between 30 and 45°C were taken as mesophilic. Although 451 of 516 isolates exhibited thermophilic growth, 65 were found to be mesophilic. The distribution of the thermophilic isolates according to the provinces was as follows: 82 from Aydin (A), 41 from Manisa (B), 40 from Denizli (C), 178 from Izmir (D), 98 from Nevşehir (E), and 12 from Ankara (F) as shown in Table 2.

As α-glucosidase activity is closely associated with the presence of other amylolytic enzymes, amylolytic activity of the isolates was tested. Out of 451 thermophilic bacilli capable of high amylase activity, 67 were selected for the determination of the intracellular and extracellular α-glucosidase production capacities. Since the presence of starch or cellulose is not typical of hot springs, the number of microorganisms having amylolytic activity is quite low in such environments [28]. In order to obtain the α-glucosidase-producing thermophilic *Bacillus* species, Suzuki and colleagues [16] studied 523 thermophilic isolates and only 25% of them showed amylolytic activity. In another study of α-glucosidases from thermophilic *Bacillus* species, the screening for amylolytic strains by Arellano-Carbajal and Olmos-Soto [7] resulted in 27 *Bacillus* isolates. In our work, like those mentioned above, only 15% of our

Table 2. Diversity of thermophilic bacilli found in different geothermal regions of Turkey

Province/Region code	Number of:					
	Hot springs	Samples	Isolates	Mesophilic isolates	Thermophilic isolates	Thermophilic amylolytic isolates
Aydin/(A)	9	39	92	10	82	19
Manisa/(B)	3	10	43	2	41	0
Denizli/(C)	6	32	49	9	40	4
Izmir/(D)	15	67	204	26	178	28
Nevsehir/(E)	5	33	114	16	98	14
Ankara/(F)	4	10	14	2	12	2
Total	42	191	516	65	451	67

thermophilic isolates possessed amylolytic activity. Thus, we can conclude that the bacterial communities found in hot springs of Turkey in the majority are not able to hydrolyze starch because of its rare presence in such environments.

α-*Glucosidase Production Capacities of 67 Amylolytic and Thermophilic Bacilli*

Intracellular, extracellular, and total α-glucosidase production levels of 67 amylolytic isolates and four reference strains after 15-h cultivation were determined. Figure 1 demonstrates high levels of enzyme production. The enzyme locations, ratio of intracellular and extracellular activities, and t-test results of some of the high-level α-glucosidase-producing isolates and reference strains are given in Table 3.

The average extracellular and intracellular enzyme activities were used for the calculation of t-test analysis ratios of activity in cell fractions, as well as for comparison of the differences in the enzyme production levels

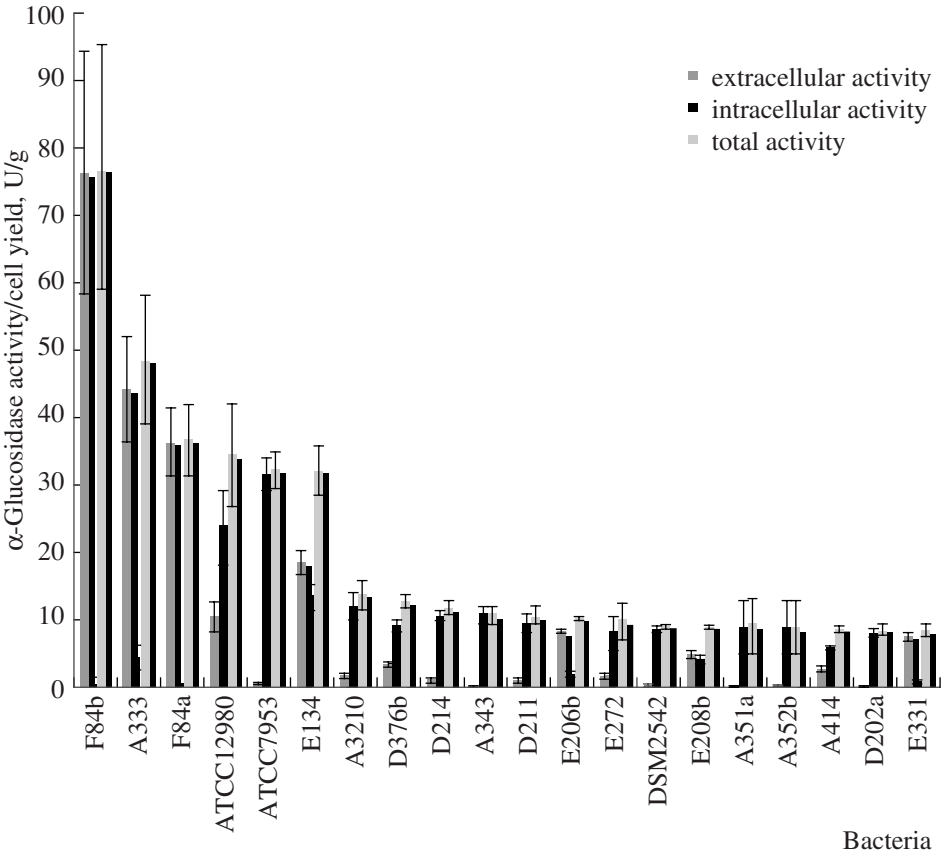

Fig. 1. Intracellular, extracellular, and total α-glucosidase activity/cell yield levels of thermophilic bacilli and reference strains after cultivation for 15 h at 60°C in MII medium.

Table 3. Extracellular, intracellular and total enzyme production levels of some high α-glucosidase-producing amylolytic isolates and reference strains after cultivation of 15 hours in MII medium

Bacteria	Location of enzyme	Extracellular α-Glucosidase			Intracellular α-Glucosidase			Total α-Glucosidase		*t*-Test results[a]
		U/ml	U/g	Percent (%)	U/ml	U/g	Percent (%)	U/ml	U/g	
F84b	Extracellular	0.57	76.52	99	0.005	0.66	1	0.57	77.18	0.007
A333	Extracellular	0.66	44.11	91	0.06	4.53	9	0.72	48.64	0.004
F84a	Extracellular	0.57	36.42	99	0.003	0.22	1	0.57	36.64	9E-04
ATCC 12980	Intracellular	0.28	10.49	31	0.63	23.77	69	0.91	34.26	0.049
ATCC 7953	Intracellular	0.01	0.47	1	0.86	31.63	99	0.87	32.10	9E-05
E134	Cell Bound	0.63	18.6	58	0.45	13.49	42	1.08	32.09	0.081
A3210	Intracellular	0.04	1.79	13	0.27	12.04	87	0.31	13.83	0.004
D376b	Intracellular	0.07	3.67	29	0.17	9.11	71	0.24	12.78	0.002
D214	Intracellular	0.02	1.15	10	0.22	10.75	90	0.24	11.90	2E-04
A343	Intracellular	<0.001	<0.001	1	0.45	10.79	100	0.45	10.79	7E-04
D211	Intracellular	0.02	1.17	11	0.18	9.57	89	0.20	10.74	0.001
E206b	Extracellular	0.25	8.34	80	0.06	2.06	20	0.31	10.40	2E-05
E272	Intracellular	0.04	1.67	17	0.19	8.19	83	0.23	9.86	0.035
DSM 2542	Intracellular	0.02	0.54	6	0.29	8.69	94	0.31	9.23	9E-06

[a] *t*-Test indicates the difference between intracellular and extracellular α-glucosidase activity.

measured for different enzyme locations within the cells. According to the difference in t-test results and ratios of 15-h activity in intracellular and extracellular fractions, the location of α-glucosidase was defined as intracellular, extracellular, or cell-bound.

The total enzyme activities in fractions were found to be between <0.001–1.08 and 0.01–0.91 U/ml for the new isolates and reference strains, respectively. Strain E134 showed the highest α-glucosidase activity (1.08 U/ml), whereas *G. stearothermophilus* ATCC 12980 had the highest α-glucosidase activity among the reference strains (0.91 U/ml). The total α-glucosidase activities per pellet wet weight were found to be 0.001–77.18 U/g for the isolates and 0.001–34.26 U/g for the reference strains. In general, there was a correlation between the total amount of enzyme activity and the total amount of activity per pellet wet weight. Strains F84b (77.18 U/g), A333, (48.64 U/g), F84a (36.64 U/g), *G. strearothermophilus* ATCC 12980 (34.26 U/g), *G. strearothermophilus* ATCC 7953 (32.10 U/g), E134 (32.09 U/g), A3210 (13.83 U/g), D376b (12.78 U/g), D214 (11.9 U/g), A343 (10.79 U/g), D211 (10.74 U/g), E206b (10.4 U/g), and E272 (9.86 U/g) were found to have the highest α-glucosidase activity. Thus, some of our thermophilic isolates had higher α-glucosidase activity than the reference strains tested.

The intracellular, extracellular, and cell-bound α-glucosidase-producing isolates were found to comprise 53, 30, and 17% of the total number, respectively. Isolates of strains A3210, D376b, D214, A343, D211, and E272 were found to be intracellular enzyme producers like reference strains ATCC 12980 and ATCC 7953, whereas F84b, A333, F84a and E206b isolates were classified as extracellular enzyme producers.

Strain E134 was found to be a cell-bound enzyme producer.

Up to now, most of the α-glucosidase-producing thermophilic *Bacillus* strains have been reported as intracellular enzyme producers [14]. Although a more concentrated enzyme could be obtained by using intracellular enzyme fractions, for enzyme purification processes, it is an advantage to use the strains producing extracellular enzymes in order to avoid cell sonication treatment and to facilitate enzyme purification. So far, only α-glucosidase-I of the thermophilic *B. thermoamyloliquefaciens* KP1071 strain has been reported as an extracellular enzyme producer by Suzuki and colleagues [29]. Furthermore, *G. thermodenitrificans* HR010 was defined as a cell-bound α-glucosidase-producing thermophilic strain [13]. Except for these two reports, there is no information on extracellular and cell-bound α-glucosidases. Thus, our newly obtained thermophilic isolates are of significant value as they possess a broad variety of intracellular, extracellular, or cell-bound α-glucosidases, the latter two enzymes being scarcely reported.

16S rDNA Sequencing Results

According to the partial 16S rDNA sequences, five of the selected α-glucosidase-producing isolates belonged to the thermophilic bacilli of 16S rDNA genetic group 5 [30–32]. 16S rDNA sequence data of the isolates A333, A343, F84a, F84b, and E134 reported in this article were submitted to GenBank and the assigned nucleotide accession numbers for the 16S rDNA sequences are EU326497, EU326496, EU47771, EU477772, and EU47773, respectively. The evolutionary distance phylogenetic tree based on 16S rRNA gene sequences of A333, A343, E134, F84a, F84b, and representative members of related genera of the family '*Bacillaceae*' is presented in Fig. 2. The 16S rDNA sequence comparison results of A333, F84a, and F84b indicated that these isolates were closely related to *Geobacillus tropicalis* CS4 (DQ925489) and *Geobacillus thermodenitrificans* DSM 465^T (Z26928) exhibiting the rDNA sequence homologies of 99.6 and 99.4%, 99.4 and 99.3%, and 99.9 and 99.6% for both of the strains, respectively. The 16S rDNA sequence of strain E134 most closely resembled *G. toebii* DSM 14590^T (AF326278) with the sequence homology of 99.4% and *G. thermoglucosidasius* DSM 2542^T (X60641) with 98% homology. Thus, A333, E134, F84a, and F84b were all identified as the members of genus *Geobacillus*. Isolate A343 was phylogenetically remote from genera *Geobacillus*, *Anoxybacillus*, and *Ureibacillus* and demonstrated 100% 16S rDNA sequence similarity only to an uncultured, low G+C gram-positive bacterium (AY699384) [33].

The Growth Curve and α-Glucosidase Activity in Intracellular and Extracellular Fractions of Selected Strains

Active α-glucosidase-producing strains *G. stearothermophilus* ATCC 12980, thermophilic bacterium A343, *Geobacillus* sp. A333, *Geobacillus* sp. F84a, *Geobacillus* sp. F84b, and *Geobacillus* sp. E134 were selected for determination of the change in α-glucosidase levels in both intracellular and extracellular fractions and concurrent growth and changes in pH of the culture medium during 48 h of growth. These results are presented in Fig. 3. It was found that there was a significant correlation between α-glucosidase production and bacterial growth. The sporulation of all the isolates occurred in the 20th hour of cultivation in the stationary phase, during 48 h in MII broth containing 2% soluble starch.

G. strearothermophilus ATCC 12980 (Fig. 3a) and thermophilic bacterium A343 (Fig. 3b) had similar growth curves and type of enzyme production. After a short lag phase, both of them reached the logarithmic phase. The stationary phase started after 12 h of growth and pH of the medium decreased from 7.2 to 5.5. α-Glucosidase production started quickly and reached a maximum during the logarithmic phase in the fourth hour of cultivation. However, a sudden decrease in the intracellular enzyme level was observed after 8 h. This moment corresponded to the midpoint of the logarithmic phase; no accumulation of α-glucosidase was observed throughout the remaining 40 h of cultivation in either intracellular and extracellular fractions. Suzuki et al. [16] reported *G. stearthermophilus* ATCC 12980 as an intracellular α-glucosidase producer. So, depending on the high amount of sudden enzyme accumulation within 4 h in the intracellular fraction and low release of enzyme to the culture liquid, thermophilic bacterium A343 was also defined as an intracellular α-glucosidase producer.

Geobacillus sp. A333 (Fig. 3c), F84a (Fig. 3d), and F84b (Fig. 3e) displayed almost identical growth curves and α-glucosidase production patterns. After a short lag phase, logarithmic growth of *Geobacillus* sp. A333 continued up to 12 h until the stationary phase, although in *Geobacillus* sp. F84a and F84b the lag phase continued up to 8 h. Then, the logarithmic growth phase began and it continued until 16 h. pH of the medium decreased from 7.4 to 6.0 at the end of incubation. α-Glucosidase production developed and reached a maximum level in the cell during the logarithmic phase of the three isolates, but the maximum accumulation time intervals differed in A333 from F84a and F84b. α-Glucosidase production of *Geobacillus* sp. A333 in the intracellular fraction reached its maximum level in the 12th hour of cultivation, throughout the end of the logarithmic phase. The maximum enzyme accumulation in F84a and F84b isolates developed at the beginning of the logarithmic phase of growth (8 h). After that, all isolates started secreting high amounts of

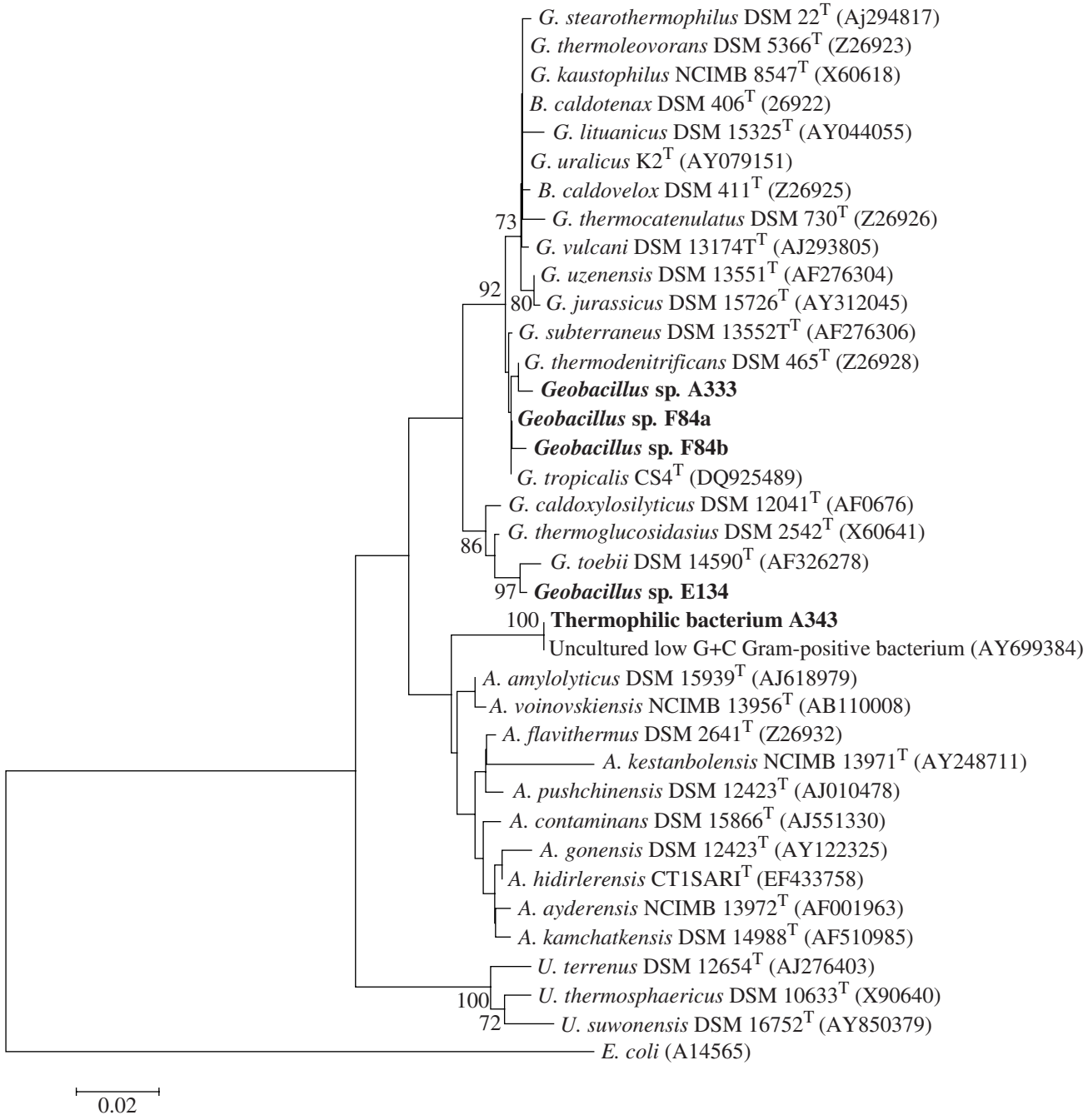

Fig. 2. Neighbor-joining evolutionary distance phylogenetic tree based on the 16S rRNA gene sequences of A333, A343, E134, F84a, F84b, and representative members of related genera of the family '*Bacillaceae*'. Bootstrap values (%) are based on 1000 replicates and shown for branches with more than 70% bootstrap support. Bar indicates 0.02 substitutions per nucleotide position. 16S rDNA sequence of *E. coli* was chosen arbitrarily as the outgroup.

enzyme to the culture liquid. α-Glucosidase activity in the extracellular fraction of A333 reached the maximum level at the stationary growth phase (16 h); for F84a and F84b, it occurred at the midpoint of the logarithmic phase (12 h). The amount of the extracellular enzyme gradually decreased until the end of the stationary growth phase. These data indicated that each of the isolates accumulated a high amount of α-glucosidase in the cell at the logarithmic growth phase and secreted the enzyme from this pool to the extracellular fraction owing to cell lysis at the stationary phase of growth, as reported before by Suzuki and colleagues [16]. Thus, *Geobacillus* sp. A333, F84a, and F84b were all defined as extracellular α-glucosidase producing strains.

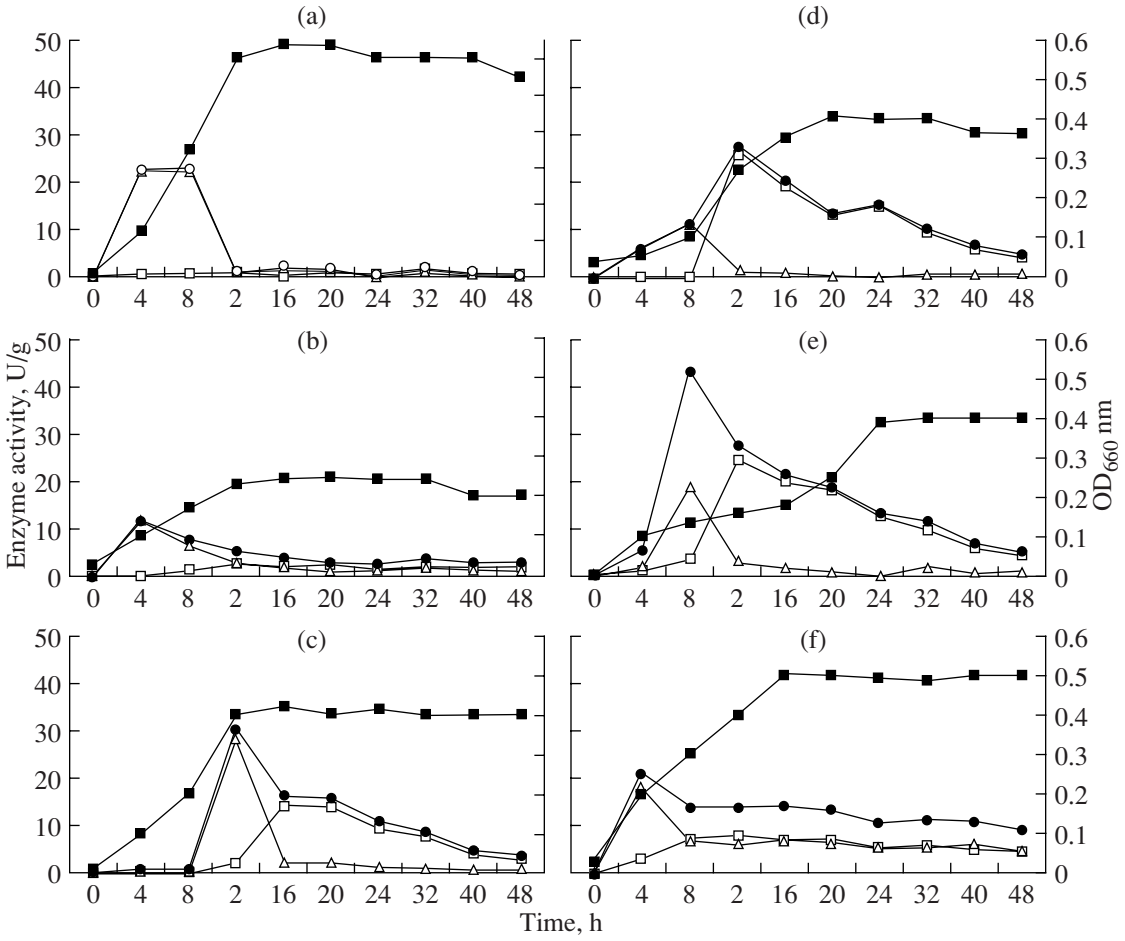

Fig. 3. The growth curve (■), change in α-glucosidase levels present in both intracellular (Δ) and extracellular (□) fractions, and total activity (•) during 48-h cultivation of *G. stearothermophilus* ATCC 12980 (a), thermophilic bacterium A343 (b), *Geobacillus* sp. A333 (c), *Geobacillus* sp. F84a (d), *Geobacillus* sp. F84b (e), and *Geobacillus* sp. E134 (f).

When compared with the other isolates and reference strain, *Geobacillus* sp. E134 is clearly different in terms of ways of α-glucosidase production (Fig. 3f). After a short lag phase, the logarithmic phase started and continued until 16 h of cultivation. The culture pH decreased from 7.4 to 5.9 by the end of cultivation. Synthesis of α-glucosidase started at the beginning of growth and reached its maximum level in the cell in the fourth hour of cultivation, and then the isolate started to secrete the intracellular enzyme to the culture liquid. In contrast to the other isolates, E134 continued to both synthesize α-glucosidase in the cell and release it to the culture medium simultaneously during the end of the stationary phase. The enzyme concentrations present in intracellular and extracellular fractions were concomitant to the growth, and, consequently, there was almost no decrease in the total enzyme amount throughout the stationary phase. This behavior is in agreement with previous reports which stated that α-glucosidase was cell-bound and both intracellular and extracellular cell-membrane-associated enzymes were produced [7, 13, 34]. Thus, α-glucosidase of *Geobacillus* sp. E134 was characterized as both an extracellular and membrane-associated intracellular enzyme.

Changes in α-glucosidase levels in the isolates and reference strain that took place in the intracellular or extracellular fractions over 48 h are shown in Table 4. While the maximum intracellular enzyme production time was 4 h (A343, E134, ATCC 12980), 8 h (F84a, F84b), and 12 h (A333), the maximum extracellular enzyme production time was 8 h (E134), 12 h (F84a, F84b), and 16 h (A333).

In industrial enzyme production, a desirable property of a strain is its ability to produce an enzyme rapidly during the early stages of growth. A343 accumulated a significant amount of the enzyme at the early growth phase, the same as the reference strain ATCC 12890. The majority of the reports on thermostable α-glucosidase-producing *Bacillus* strains concern intracellular enzyme-synthesizing species. *G. stearothermophilus* ATCC 12016 [12], *Bacillus* sp. SAM 1606 [35], *B. caldovelax* DSM 411 [8], *B. flavocaldarius* KP1228 (FERM-P9542) [9], *Geobacillus* sp. HTA-462 [14], and α-glucosidase II and III of *G. thermoamyloliquefaciens*

Table 4. Change of α-glucosidase levels (U/g) in intracellular and extracellular fractions during 48-hour cultivation of A333, A343, E134, F84a, F84b isolates and *G. stearothermophilus* ATCC 12980 in MII medium

Bacteria	Fractions	4 h	8 h	12 h	16 h	20 h	24 h	32 h	40 h	48 h
A333	Intracellular	0.87	0.86	**28.22**	2.29	2.23	1.50	1.09	0.84	0.96
	Extracellular	<0.01	<0.01	1.89	**14.14**	13.73	9.59	7.8	4.04	2.87
A343	Intracellular	**11.76**	6.4	2.66	1.80	0.78	1.27	1.84	1.13	1.01
	Extracellular	<0.01	1.19	2.64	1.93	1.9	0.98	1.6	1.41	1.64
E134	Intracellular	**17.96**	6.57	6.15	7.05	6.47	5.42	5.47	5.67	4.56
	Extracellular	2.75	**7.34**	7.68	6.99	6.89	5.41	5.7	5.01	4.44
F84a	Intracellular	5.71	**10.86**	1.44	1.06	0.20	0.10	0.57	0.47	0.61
	Extracellular	<0.01	<0.01	**26.06**	19.22	12.98	14.8	9.34	6.12	4.01
F84b	Intracellular	1.99	**18.83**	3.24	1.64	0.76	0.40	1.96	0.89	1.28
	Extracellular	0.85	3.61	**24.3**	19.98	18.1	12.89	9.57	5.97	4.34
ATCC 12980	Intracellular	**22.54**	22.27	0.74	1.40	0.84	0.10	1.04	0.15	0.11
	Extracellular	0.26	0.63	0.5	0.2	0.51	0.2	0.53	0.42	0.3

KP1071 [10] are examples of strains producing a thermostable intracellular enzyme. On the basis of these results, it can be concluded that (i) only intracellular and cell-bound α-glucosidase producers produce an enzyme in the early stages of growth, (ii) only extracellular enzyme producers secrete α-glucosidase from the cell during the late growth phase, and (iii) cell-bound enzyme producers can both pool and secrete α-glucosidase.

A study by Suzuki and colleagues [29] on a thermostable oligo-1,6 glucosidase isolated from *G. thermoglucosidasius* reported that the strain accumulated the enzyme in the medium as a pool during the logarithmic and stationary phases of growth. Enzyme synthesis was described as an exoenzyme production and appearance of oligo-1,6 glucosidase in the culture broth was associated with cell lysis. It is important for enzyme stability that an enzyme be active for a long time interval outside the cell. Oligo-1,6 glucosidases and α-glucosidases are both in the glucohydrolase family. As was shown for *G. thermoglucosidasius*, α-glucosidases of *Geobacillus* sp. A333, F84a, and F84b are extracellular enzymes. The previously isolated strain *B. thermoamyloliquefaciens* KP1071 was the only thermophilic strain of genus *Bacillus* that produced extracellular α-glucosidase [29]. α-Glucosidase II of strain KP1071 was purified from the culture liquid. Our isolates *Geobacillus* sp. A333, F84a, and F84b can be considered among the *Geobacillus* strains producing thermostable α-glucosidases.

Unlike other known extracellular or intracellular α-glucosidase-producing isolates, *Geobacillus* sp. E134 continuously produced α-glucosidase throughout the 48 h of cultivation, in both intracellular and extracellular fractions. This type of enzyme production was reported only in a study by Ezeji and colleagues [13], which indicated that α-glucosidase of *G. thermodenitrificans* HRO was produced as associated with the cell membrane and was termed a cell-bound α-glucosidase. Similarly to *G. thermodenitrificans* HRO, our isolate E134 also proved to be extracellular and a membrane-associated intracellular enzyme producer. Thus, strain E134 is one of the scarcely found *Geobacillus* species with a cell-bound α-glucosidase.

CONCLUSIONS

Thermophilic bacterial communities inhabiting the hot springs of Turkey have not been thoroughly studied yet. In this study, a great number of new thermophilic

bacilli were isolated from distinct geothermal regions of Turkey in order to screen their α-glucosidase production capacities. This work on thermophilic bacilli not only helps with further taxonomic and phylogenetic diversity studies, but also opens prospects for the discovery and description of new thermophilic species. Strains studied in this work were identified as *Geobacillus* sp. A333, E134, F84a, F84b, and bacterium A343 and were shown to have α-glucosidases of different location, thus making good candidates for biotechnological applications.

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