DOI 10.12905/0380.sydowia72-2020-0001 Published online 28 January 2020

Bioprospecting of hot springs and compost in West Anatolia regarding phytase producing thermophilic fungi

Sennur Çalışkan Özdemir* & Atac Uzel

Ege University, Faculty of Science, Department of Biology, Basic and Industrial Microbiology Section, 35100, Bornova, İzmir, Turkey

* e-mail: caliskansennur@gmail.com

Özdemir S.Ç. & Uzel A. (2020) Bioprospecting of some hot springs and compost in West Anatolia regarding phytase producing thermophilic fungi. – Sydowia 72: 1–11.

Phytase is commonly used as feed supplement for poultry and catalyses the hydrolysis of phytate into inorganic phosphates and myo-inositol phosphates. Extreme environments, especially warm habitats constitute an important resource for the discovery of microorganisms with unique enzymes. Therefore, we aimed to investigate the culturable thermophilic and thermotolerant fungal biodiversity of hot springs and compost samples in Western Anatolia and their extracellular phytase production capacities for the first time. A total of 43 environmental samples (26 soils and 17 sediments) were collected from 17 different hot springs and 1 compost sample was taken from a mushroom farm. A total of 48 filamentous fungal strains were isolated. Fourteen (29 %) strains were classified as thermophilic and 34 (71 %) strains as thermotolerant regarding to their heat requirements. Of the 48 isolates, 33 (69 %) were *Aspergillus* species. All isolates were quantitatively screened for their extracellular phytase activities and 42 (88 %) of the 48 isolates produces phytase in a range of 8.82 – 331.22 (U/mg). This study demonstrates that hot springs in West Anatolia harbour a rich thermophilic/thermotolerant fungal diversity possessing phytase producing potential and mushroom farming selectively enhances thermophilic fungi.

Keywords: fungal diversity, *Aspergillus,* enzyme production, molecular phylogeny.

Phytases are enzymes belonging to the histidine acid phosphatases that can hydrolyse phosphomonoester bonds in phytic acid and release myo-inositol and inorganic phosphates through a series of myo-inositol phosphate intermediates (Singh & Satyanarayana 2015, Gaind & Singh 2015). Monogastric animals such as poultry, pigs and fish, are unable to utilize phytic acid and release inorganic phosphorus because they have little or no phytase enzyme in their gastrointestinal tracts (Reddy et al. 1982, Mittal et al. 2011, Dahiya 2016). Therefore, phytase is added to the monogastric animal feeds in order to increase the utilization of phytate-P in the feed and to prevent environmental phosphor pollution. One of the most desired properties sought in commercial phytases is their stability at high temperatures for pelleting process (Singh & Satyanarayana 2015). Currently most of the commercial phytase enzymes are produced mainly from filamentous fungi such as *Aspergillus niger*, *A. ficuum, A. fumigatus* (Zhang & Kim 2010) and some *Penicillium* strains (Zhao et al. 2010). However, since the global phytase market is rapidly developing in many areas, new and thermostable phytases are still needed.

Microorganisms have developed various genetic or physiological adaptations to survive under extreme conditions such as high temperature, pH and high pressure (Cooney & Emerson 1964, Aguilar 1996, Mouchacca 1997, Stetter 1999, Pan et al. 2010). Among others, temperature is the most important abiotic factor for microbial ecosystems. Thermophilic microorganisms are generally located in bacteria and archaea, while some fungi have the ability to survive in environments at high temperatures although not as much as these. Heat-tolerant fungi are characterized according to maximum and minimum growth temperatures as thermophilic or thermotolerant (Cooney & Emerson 1964, de Oliveira et al. 2015). Thermophilic fungi have an optimal growth between 40–50 °C and do not grow at or below 20 °C. Thermotolerant fungi have a maximum growth at or below 50 °C and grow well at and below 20 °C (Crous et al. 2019).

Research on heat-tolerant fungi has attracted attention because of the secretion of new enzymes

with heat stability, organic solvent tolerance and a long shelf-life, all of which are desirable properties of enzymes for industrial and biotechnological applications (Singh & Satyanarayana 2011). Thermophilic/thermotolerant fungi are found in different sources in nature such as nests of birds, decomposing litter, soils from furnace area, cattle dung, zoo dump, industrial waste, vegetable market compost, mushroom compost, horse dung, municipal waste, chicken manure and coal mine soils and other environments providing organic matter, and warm, humid and aerobic conditions (Redman et al. 1999, Chen et al. 2003, Ryckeboer et al. 2003, Kumar & Sujath 2013, Welday et al. 2014).

Thermophily in fungi is not considered very extreme as found in some members of eubacteria or archaea. Perhaps because of their moderate degree of thermophily and because their habitats are not exotic, thermophilic fungi are generally overlooked in comparison with thermophilic prokaryotes. However, most of the eukaryotes cannot survive above 50 °C and thermophilic and thermotolerant fungi are prolific sources of many enzymes with scientific and industrial interests. In the Aegean region of the Anatolian peninsula, there are many hot springs located parallel to the fault lines. Most of these hot springs are directly falling into soils and rivers and so they produce suitable habitats for thermophilic fungi. Besides, there are a few mushroom farms in the same region which can select and enrich the natural thermophilic funga during the composting process. These natural habitats provide untapped sources for bioprospecting studies. Starting from this point we aimed to investigate the culturable thermophilic and thermotolerant fungi of some hot springs and a compost sample and their extracellular phytase production capacities.

Materials and methods

Soil, sediment and compost samples

A total of 43 soil and sediment samples were collected from 17 different hot springs in 5 different provinces in the Aegean region (Tab.1). One compost sample was also collected from a mushroom farm in the same region. Samples were taken into sterile container after removal from 5–10 cm surface layer and brought to the laboratory in a cold box and analysed at the same day.

Isolation of heat tolerant fungi

Three different media were used in isolation studies: (i) Potato dextrose agar, PDA (Merck), (ii) Rose Bengal Chloramphenicol agar, RBCA (Merck) (iii) Malt Extract agar, MEA (Merck). All media were prepared according to the manufacturers' instructions. PDA and MEA agars were supplemented with a final concentration of 100 µg/ml streptomycin.

Ten grams of wet samples were added to 90 ml physiological saline solution (PSS) and homogenized by shaking (180 rpm) at room temperature for 30 min. After homogenisation, serial dilutions were made using sterile PSS and isolation plates were inoculated with 100 µl solution. Each experiment was carried out in triplicate. Plates were incubated at 45 °C up to 2–3 weeks in a humidified atmosphere. After incubation growing fungal colonies were purified and stored for further experiments.

Determination of thermotolerance

All isolates were plated on MEA and PDA plates and incubated for 5–7 days at 18 °C in order to determine the thermophilic/thermotolerant character of the isolates. The isolates that were growing at 18 °C were classified as thermotolerant and those that failed to grow were classified as thermophilic (Chen et al. 2000).

Identification of the isolates

All isolates were identified using a polyphasic taxonomic approach (Cooney & Emerson 1964, Barnett & Hunter 1999). Colony characteristics and microscopic features of the isolates are determined after incubation on PDA and MEA for 7 days (Pitt 2000, Klich 2002). Total genomic DNA was extracted from the isolates by High Pure PCR Template preparation kit (Roche). Genomic DNA quantity and purity were checked using Nanodrop 2000c UV-Vis Spectrophotometer (Thermoscientific). All PCRs were performed with the HelixAmpTM Taq DNA polymerase kit (NanoHelix). PCR reactions were carried out in 50 µl mixtures containing; 10 mM PCR buffer, 0.2 mM dNTP, 0.2 mM primers, 1.25 U of Taq DNA polymerase and 20–50 ng of genomic DNA template. The primers used for the amplification of the ITS rDNA were ITS1 and ITS4 (White et al. 1990) and for calmodulin region Cmd5 and Cmd6 (Hong et al. 2006). PCR conditions for ITS and calmodulin sequences were as follows: 2 min of denaturation at 95 °C, 35 cycles of 20 s at 95 °C, 58 °C/56 °C for 40 s/50 s, and 50 s/35 s of extension at 72 °C. All amplicons were bidirectionally sequenced on an ABI 3730xl automated sequencer (GATC-Biotech, Germany). The nucleotide sequences of reference species were downloaded from NCBI

Tab. 1. Location, isolation material and temperature of samples.

Location of the samples		Coordinates	Material	Temperature
İzmir	Nebiler Hot Spring	39°09′31.0″N 26°54′12.1″E	Soil	45 °C
	Nebiler Hot Spring	39°09′31.0″N 26°54′12.1″E	Sediment	45 °C
	Zeytindalı Hot Spring	39°00′52.2″N 26°55′34.3″E	Soil	40 °C
	Çamur Hot Spring	39°03′35.3″N 26°55′22.5″E	Sediment	45 °C
	Çamur Hot Spring	39°03′35.3″N 26°55′22.5″E	Soil	40 °C
	Çamur Hot Spring	39°03′35.3″N 26°55′22.5″E	Soil	40 °C
	Çamur Hot Spring	39°03′35.3″N 26°55′22.5″E	Soil	40 °C
	Karakoç Hot Spring	38°05′23.0″N 26°55′04.7″E	Soil	60 °C
	Karakoç Hot Spring	38°05′23.0″N 26°55′04.7″E	Soil	65 °C
	Karakoç Hot Spring	38°05′23.0″N 26°55′04.7″E	Sediment	60 °C
	Karakoç Hot Spring	38°05′23.0″N 26°55′04.7″E	Soil	55 °C
	Mushroom Farm	38°40′51.3″N 26°56′48.9″E	Compost	~70 °C
Denizli	Buharkent	37°56′48.7″N 28°49′45.2″E	Soil	43 °C
	Buharkent	37°56′48.7″N 28°49′45.2″E	Soil	55 °C
	Buharkent	37°56′48.7″N 28°49′45.2″E	Soil	58 °C
	Buharkent	37°56′48.7″N 28°49′45.2″E	Soil	48 °C
	Buharkent/Kabaağaç	37°56′05.5″N 28°45′40.1″E	Sediment	50 °C
	Buharkent/Tekkeköy	37°36′25.1″N 29°10′08.8″E	Soil	55 °C
Aydın	Çamköy	37°57′23.2″N 27°35′17.9″E	Sediment	58 °C
	Çamköy	37°57′23.2″N 27°35′17.9″E	Sediment	48 °C
	Çamköy	37°57′23.2″N 27°35′17.9″E	Soil	50 °C
	Alangüllü/Bozköy	37°56′00.5″N 27°37′36.4″E	Soil	50 °C
	Alangüllü/Bozköy	37°56′00.5″N 27°37′36.4″E	Sediment	60 °C
Kütahya	Çitgöl	39°07′58.0″N 28°58′01.9″E	Sediment	60 °C
	Çitgöl	39°07′58.0″N 28°58′01.9″E	Soil	50 °C
	Eynal /Simav	39°07′39.9″N 28°59′48.0″E	Sediment	60 °C
	Eynal /Simav	39°07′39.9″N 28°59′48.0″E	Sediment	48 °C
	Esire / Hisarcık	39°12′13.0″N 29°16′36.5″E	Soil	48 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Sediment	43 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Sediment	51 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Sediment	48 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Soil	45 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Sediment	50 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Soil	40 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Soil	40 °C
	Ilıcasu/Gediz	38°57′32.4″N 29°16′31.3″E	Soil	40 °C
Manisa	Emir /Kula	38°33′06.2″N 28°38′52.88″E	Soil	35 °C
	Acısu /Kula	38°36′57.61″N 28°45′22.28″E	Soil	35 °C
	Sart/Salihli	38°27′22.6″N 28°02′50.1″E	Soil	50 °C
	Urganlı	38°34′17.5″N 27°50′31.0″E	Sediment	41 °C
	Urganlı	38°34′17.5″N 27°50′31.0″E	Sediment	60 °C
	Urganlı	38°34′17.5″N 27°50′31.0″E	Soil	45 °C
	Urganlı	38°34′17.5″N 27°50′31.0″E	Sediment	65 °C
	Urganlı	38°34′17.5″N 27°50′31.0″E	Soil	60 °C

GenBank. Sequences were subjected to BLAST analysis in the GenBank and phylogenetic analysis was conducted for the selected strain using MEGA v.7.

Phytase production capacities of the isolates

Extracellular phytase activities of the isolates were determined using a quantitative enzyme assay. All fungal strains were activated in MEA for 7 days at 45 °C. Fermentations were performed in 250 ml flasks containing 50 ml fermentation media (pH 5.5) with the following composition (g/l): malt extract 3.0; yeast extract 3.0; peptone 5.0; glucose 10.0, and Na-phytate 1.0 (Kjer et al. 2010). Flasks were incubated for 7 days at 45 °C and 150 rpm. Fermentation broths were separated from the cells by centrifugation at 5000 rpm for 30 min followed by filtration. The resulting supernatant was used as the crude enzyme preparation for the determination of enzyme activity, protein content and specific activity.

Phytase activity was determined using modified ammonium-molybdate-blue method and inorganic phosphate as standard. The enzyme reaction was performed in 250 µl 0.1 M sodium acetate buffer (pH 5.5) using sodium phytate (5.0 mM, Sigma) as a substrate and 250 µl crude enzyme preparations. After incubation at 45 °C for 15 min, the reaction was stopped by adding an equal volume of 10 % (w/v) trichloroacetic acid. The released phosphate ions were quantified by 1 ml colouring solution (1 % (w/v) ammonium-molybdate, 5.5 % (v/v) H_2SO_4 and 2.5 % (w/v) ferrous sulfate solution) and the absorbance was read at 700 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of inorganic phosphate from the substrate in 1 min under the assay conditions. Protein concentration in the crude enzyme preparation was determined according to the Bradford method using bovine serum albumin as a standard (Bradford 1976). All assays were done in triplicate and the mean absorbance values were used in the activity calculation.

Results

A total of 48 filamentous fungal strains were isolated from soil, sediment and a compost sample. The temperatures of sediments and soils were differing between 35–60 °C and the compost was at 70 °C at the sampling time. Thirty-four (71 %) of the isolates were cultivated from hot springs in five different provinces and 14 (29 %) were cultivated from the compost sample (Tab. 1). According to the heat requirements, 14 (29 %) of the isolates were thermophilic and the 34 (71 %) were thermotolerant (Tab. 2). However, almost all of the thermophilic strains 93 % (13 strains) were isolated from the compost sample.

Kütahya samples were the most prolific sources among the hot springs comprising 20 of the isolates (42 %) followed by İzmir Dikili region (13 isolates, 27 %) and Manisa Urganlı (1 isolate, 2 %). However, thermophilic or termotolerant fungal strains could not be isolated from Aydin, Denizli, Kula, Salihli and Seferihisar hot springs (Tab. 2). Moreover, a total of 14 isolates were obtained from the mushroom farm (30 %), 13 of which were thermophilic.

Identification of the isolates was primarily based on ITS sequences, and culture and microscopic data (not shown). For isolates which could not be adequately discriminated at species level by ITS sequences, calmodulin gene sequences were used (Raja et al. 2017). GenBank accession numbers are presented in Tab. 2. Of the 48 isolates, 33 (69 %) were *Aspergillus* species. The remaining 16 isolates are distributed in seven genera including *Thermomyces, Penicillium, Humicola, Scytalidium, Lichtheimia, Acrophialophora* and *Myceliophthora* (Fig. 1a).

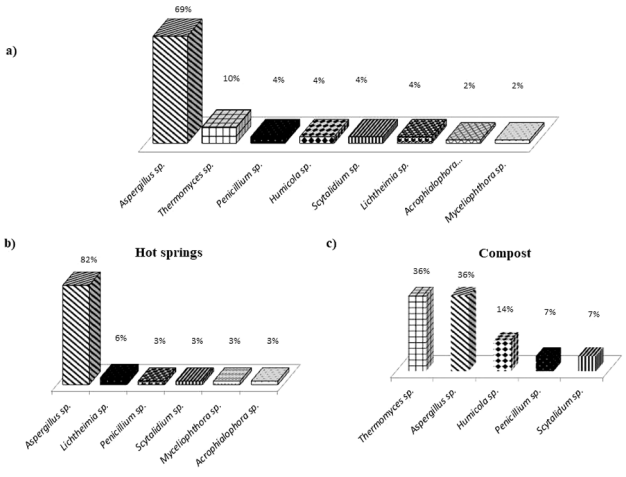

Fig. 1. Distribution and percentage of genera of thermophilic and thermotolerant fungal strains isolated from Anatolia. **a.** total, **b.** hot springs, **c.** compost.

Hot spring samples harbour six fungal genera (Fig. 1b). Distribution of the species according to the isolation sources revealed that thermotolerant *Aspergillus* species are very prevalent in Kütahya region comprising 18 of the 20 (90 %) *Aspergillus* strains among hot spring samples. In contrast to the hot spring isolates, *Thermomyces* was the most

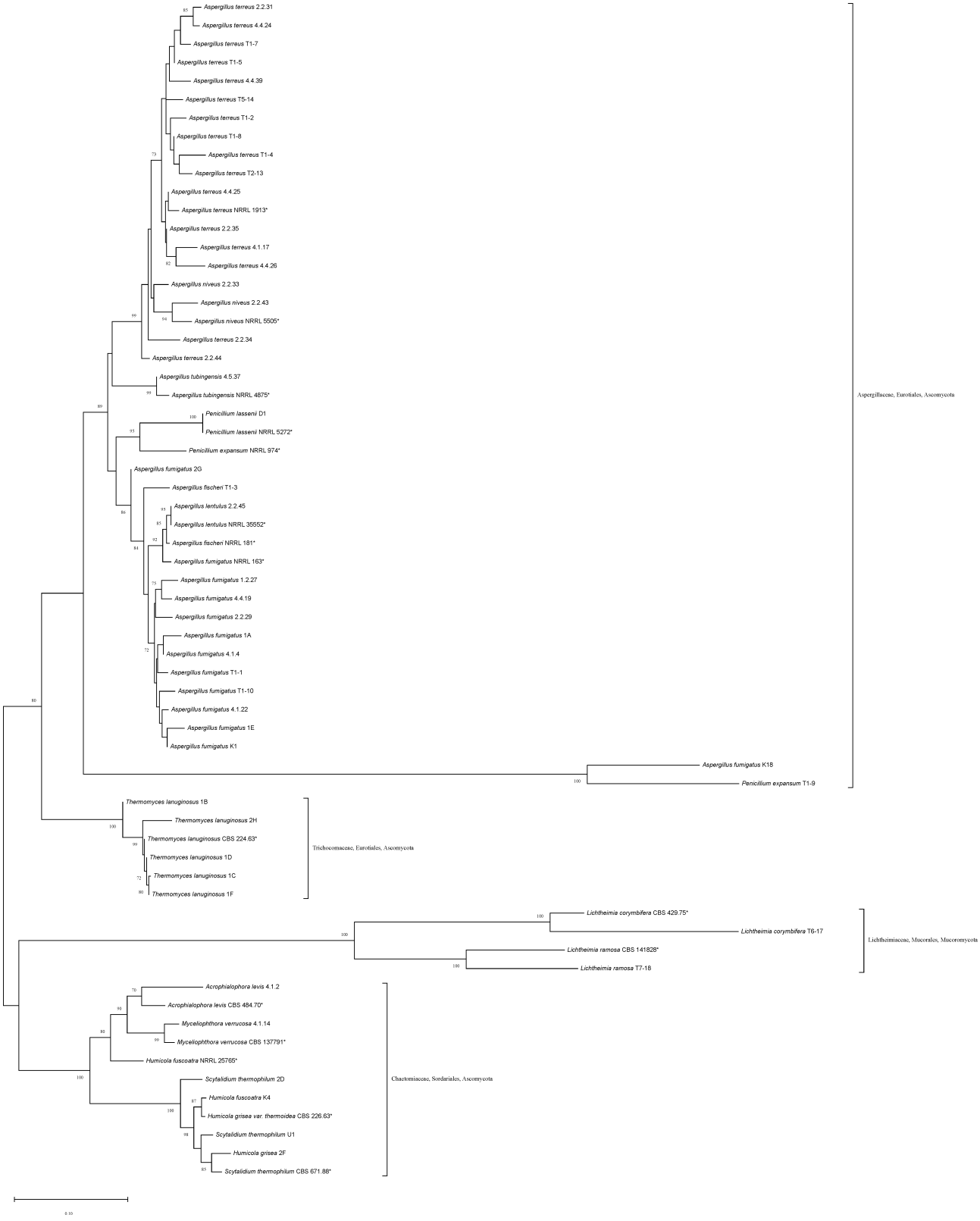

Fig. 2. Phylogenetic analysis of the strains isolated based on ITS gene sequences of the isolates and type strain sequences from NCBI database; neighbour joining method based on the Tamura-Nei model (MEGA7.0)

Tab. 2. Polyphasic identification results of the isolates from hot springs and compost in West Anatolia, Turkey and their extracellular quantitative phytase activities.

GenBank accession no.	Isolate number	Species	Identity (%)	Heat requirements	Isolation Materials	Specific phytase activity (U/mg)
MG458679	4.1.2	*Acrophialophora levis**•	99	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	81.72
MG458690	T1-3	*Aspergillus fischeri*	98	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	8.82
MG458685	K1	*Aspergillus fumigatus*•	100	Thermophilic	Pe-Ma Mushroom Farm Compost	84.81
MG458687	K18	*Aspergillus fumigatus*•	100	Thermophilic	Pe-Ma Mushroom Farm Compost	28.86
MG458688	T1-1	*Aspergillus fumigatus*•	100	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	21.09
MG279199	4.1.4	*Aspergillus fumigatus*•	100	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	19.54
MG279198	4.4.19	*Aspergillus fumigatus*•	97	Thermotolerant	Gediz Hot Spring/Kütahya Soil	14.48
MG249970	1.2.27	*Aspergillus fumigatus*•	98	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	230.56
MG279202	1A	*Aspergillus fumigatus*•	99	Thermophilic	Pe-Ma Mushroom Farm Compost	116.28
MG321620	T1-10	*Aspergillus fumigatus*•	99	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	13.56
MG321615	2.2.29	*Aspergillus fumigatus*•	97	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	n. d.
MG279206	1E	*Aspergillus fumigatus*•	98	Thermophilic	Pe-Ma Mushroom Farm Compost	19.68
MG321622	2G	*Aspergillus fumigatus*•	100	Thermophilic	Pe-Ma Mushroom Farm Compost	13.42
MG458682	4.1.22	*Aspergillus fumigatus*•	100	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	n. d.
MH458243	2.2.45	*Aspergillus lentulus*•	100	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	14.36
MG321617	2.2.33	*Aspergillus niveus*•	100	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	n. d.
MG321619	2.2.43	*Aspergillus niveus*•	99	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	246.45
MG458691	T1-4	*Aspergillus terreus*•	98	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	13.49
MG458692	T1-5	*Aspergillus terreus*•	100	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	11.70

Tab. 2. (continued)

GenBank accession no.	Isolate number	Species	Identity (%)	Heat requirements	Isolation Materials	Specific phytase activity (U/mg)
MG458693	T1-7	*Aspergillus terreus*•	99	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	66.92
MG458694	T1-8	*Aspergillus terreus*•	100	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	12.62
MG321616	2.2.31	*Aspergillus terreus*•	98	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	14.98
MG458695	T2-13	*Aspergillus terreus*•	99	Thermotolerant	Nebiler Hot Spring/İzmir Soil	15.32
MG458696	T5-14	*Aspergillus terreus*•	99	Thermotolerant	Çamur Hot Spring /İzmir Soil	10.10
MG470651	4.4.24	*Aspergillus terreus*•	100	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	59.22
MH458249	4.4.25	*Aspergillus terreus*•	100	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	13.88
MG279201	2.2.44	*Aspergillus terreus*•	97	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	129.98
MG458683	4.4.26	*Aspergillus terreus*•	97	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	80.52
MG458681	4.1.17	*Aspergillus terreus*•	97	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	64.78
MG458689	T1-2	*Aspergillus terreus*•	99	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	49.28
MG321618	2.2.34	*Aspergillus terreus*•	97	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	23.08
MH458244	2.2.35	*Aspergillus terreus*•	100	Thermotolerant	Eynal Hot Spring/Kütahya Sediment	89.00
MG458684	4.4.39	*Aspergillus terreus*•	99	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	n. d.
MH458242	4.5.37	*Aspergillus tubingensis*	99	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	331.32
MG458686	K4	*Humicola fuscoatra**	99	Thermophilic	Pe-Ma Mushroom Farm Compost	44.29
MG321621	2F	*Humicola grisea**	98	Thermophilic	Pe-Ma Mushroom Farm Compost	15.52
MG458698	T6-17	*Lichtheimia corymbifera**•	96	Thermotolerant	Çamur Hot Spring /İzmir Soil	17.06
MG458699	T7-18	*Lichtheimia ramosa**•	93	Thermotolerant	Çamur Hot Spring /İzmir Soil	45.71

Tab. 2. (continued)

GenBank accession no.	Isolate number	Species	Identity (%)	Heat requirements	Isolation Materials	Specific phytase activity (U/mg)
MG458680	4.1.14	*Myceliophthora verrucosa*•*	99	Thermotolerant	Gediz Hot Spring/Kütahya Sediment	97.78
MG458697	T1-9	*Penicillium expansum*	100	Thermotolerant	Zeytindalı Hot Spring/İzmir Sediment	n. d.
MH458250	D1	*Penicillium lassenii*	100	Thermotolerant	Pe-Ma Mushroom Farm Compost	18.17
MG458700	U1	*Scytalidium thermophilum**	99	Thermophilic	Urganlı Hot Spring/Manisa Sediment	18.90
MG458701	2D	Scytalidium thermophilum*	100	Thermophilic	Pe-Ma Mushroom Farm Compost	n. d.
MG279203	1B	*Thermomyces lanuginosus**	99	Thermophilic	Pe-Ma Mushroom Farm Compost	15.86
MG279204	1C	*Thermomyces lanuginosus**	100	Thermophilic	Pe-Ma Mushroom Farm Compost	42.05
MG279205	1D	*Thermomyces lanuginosus**	100	Thermophilic	Pe-Ma Mushroom Farm Compost	35.89
MG279207	1F	*Thermomyces lanuginosus**	100	Thermophilic	Pe-Ma Mushroom Farm Compost	142.80
MG321623	2H	*Thermomyces lanuginosus**	98	Thermophilic	Pe-Ma Mushroom Farm Compost	21.47

* Identification of these strains is only based on their ITS and calmodulin gene sequence analysis.
n. d. Phytase activity was not detectable.
• Opportunistic human pathogens in the BSL-2 category.

prevalent genus followed by *Aspergillus*, *Humicola*, *Penicillium* and *Scytalidium* in the compost sample (Fig. 1c). Phylogenetic analysis were performed based on the alignment of ITS gene sequences of the isolates and type strain sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/). Phylogenetic tree was inferred by using the neighbour joining method based on the Tamura-Nei model with MEGA7.0 (Fig. 2).

The extracellular phytase activities of the isolates were quantitatively determined from their cell free fermentation broths. Most of the isolates (88 %) showed phytase activity (Tab. 2). Specific phytase activities (U/mg) of the isolates ranged from 8.82–331.32. *Aspergillus tubingensis* strain isolated from Kütahya-Gediz sediment sample showed the highest activity.

Discussion

Since the phytase market grows continuously and there is not a single ideal phytase for all industrial applications we aimed to explore the heat tolerant fungal biodiversity of the hot springs located in West Anatolia and investigate their phytase production in order to obtain novel industrial strains. In this region there is only one study conducted by Berikten (2013) comprising just four hot springs at Kütahya region, however only hot water samples were used and sediment and soil samples were not included in this study.

In our study soil, sediment and a compost sample were collected from 17 different hot springs and one mushroom farm in five different provinces in the Aegean region. This region contains at least 58 hot springs and we almost sampled 30 % of them. Of the 48 fungi strains isolated from the samples, 71 % were obtained from hot springs and 29 % from compost. The most prolific hot springs were located at the Kütahya yielding 59 % of the all environmental isolates. Heat-tolerant fungi could not be isolated from nine hot springs located in İzmir, Manisa and Aydın provinces. Although it is not based on any analysis, it is thought that the poor vegetation in these regions and the quite high temperatures of some samples such as Buharkent could be the reason for the unfruitfulness. Berikten (2013) cultivated a total of 137 fungal strains from hot water samples taken from Afyon and Kütahya hot springs, while 132 isolates were from Kütahya only five came from Afyon. Both studies confirm that Kütahya is a prolific source regarding the thermotolerant fungal richness and abundance.

Forty-eight isolates were identified using polyphasic approach at the genus or species level. However, 16 isolates cannot be assigned to any taxon since the cultivation studies only yielded sterile mycelia. Therefore, identification of this 16 isolates was mainly based on ITS or when necessary calmodulin sequence similarities.

Among the 48 isolates 14 strains were found thermophilic and 34 strains thermotolerant according to their heat requirements. Thirteen of the thermophilic isolates were obtained from the compost sample and only one of them was from a hot spring (Urganlı sediment). While the 13 thermophilic compost strains were *Thermomyces lanuginosus*, *Aspergillus fumigatus*, *Scytalidium thermophilum*, *Humicola fuscoatra* and *Humicola grisea*, the sole thermophilic sediment strain was identified as *Scytalidium thermophilum*. Langarica-Fuentes et al. (2014) also reported that *Aspergillus fumigatus* and *Thermomyces lanuginosus* were the most common fungal species from two different commercial compost samples. Both studies confirm the prevalence of these species in compost samples. The composting process consists of four phases in total and the second one is the thermophilic phase which can be considered as a selective step for thermophilic fungi. The other strains were previously isolated from compost in another study (Langarica-Fuentes et al. 2014), where 16 fungal species were identified. Kütahya Gediz hot spring was the best location regarding to species richness with seven thermotolerant species comprising 44 % of the all species alone (Tab. 2). Studies on microbial diversity and biotechnological potential of the hot springs in this region are generally focused on bacteria and archaea and fungi have been neglected. Only, Berikten (2013) recently reported the isolation of 137 heat tolerant fungi from water samples of hot springs in Kütahya and Afyon provinces. On the other hand, many different groups have revealed the fungal diversity of the many hot springs around the world. Redman et al. (1999) reported 16 thermophilic/thermotolerant fungi from Yellowstone National Park and found that the most common genus was *Penicillium*. Chen et al. (2003) isolated 2.202 fungal strains from Yangmingshan National Park and assigned them to the genera *Aspergillus, Chrysosporium, Sporotrichum, Scytalidium, Papulaspora* and *Mycelia*. Sharma et al. (2013) reported *Myceliophthora thermophila* SH1 strain from Manikaran hot spring water samples (Sharma et al. 2013). Pan et al. (2010) demonstrated the presence of 102 strains from Tengchong Rehai National Park geothermal soil samples belonging to *Rhizomucor, Chaetomium, Talaromyces, Thermoascus, Thermomyces, Scytalidium, Malbranchea, Myceliophthora* and *Coprinopsis*.

Revealing the biodiversity of these extreme habitats is important since microorganisms in these environments have great biotechnological potentials. The industrial demand for enzymes that can withstand harsh conditions has greatly increased over in the last few decades. Therefore, industrial thermostable enzymes, which have been isolated mainly from thermophilic microorganisms, have found much commercial application due to their structural stability (Coleri et al. 2009). Especially heat tolerant fungi have enzymes with unique properties for many industrial processes (Singh & Satyanarayana 2011). Therefore, we also target potent thermostable phytase producing fungal strains. All isolates were screened for their extracellular phytase activity using a quantitative assay. It was determined that 42 of 48 isolates had more or less phytase activity (Tab. 2). Although specific activities of the 36 strains were below 100 U/mg, six strains were higher than this value. Four of these strains were isolated from Kütahya (Gediz and Eynal hot springs) region and two were isolated from compost. An *Aspergillus tubingensis* strain from Kütahya Gediz hot spring showed the best specific activity (331,22 U/mg). Our results agree with Berikten (2013) reports phytase activity in 44 % of the isolates in the Aegean region. The fact that the isolates with the highest specific phytase activity were obtained from the hot springs in the Kütahya region show that these hot springs have a great biotechnological potential.

Many researchers conducted similar bioprospecting studies in different soil samples and reported different ratios of phytase producing fungi. Chen (1998) determined that 71 % of the soil fungi isolates were phytase producers. Kumar et al. (2011) reported 161 fungi isolated from 40 soil samples and found that 33 of the 161 isolates were phytase producers. Gupta et al. (2014) similarly reported that phytase activity was found in 72 of 113 (64 %) fungal isolates from 58 different soil samples. If we compare the hot springs with the different soils regarding to the phytase producing fungi we may conclude that hot springs provide quite a rich habitat for such strains. The thermal springs in the Aegean region, in particular the Kütahya hot springs are a good source for the isolation of the new and powerful phytase producing microorganisms for industrial applications.

Acknowledgements

We gratefully acknowledge the support for this research by the Ege University Scientific Projects Foundations, Project No: 17-Bil-005. This work was also financed by the Scientific and Technological Research Council of Turkey (TUBITAK, Project No: 116Z114)

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(Manuscript accepted 4 November 2019; Corresponding Editor: I. Krisai-Greilhuber)