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Isolation and identification of keratinophilic fungi in soil samples from excavation area of ancient city of Stratonikeia, Turkey and determination of its enzyme potentials

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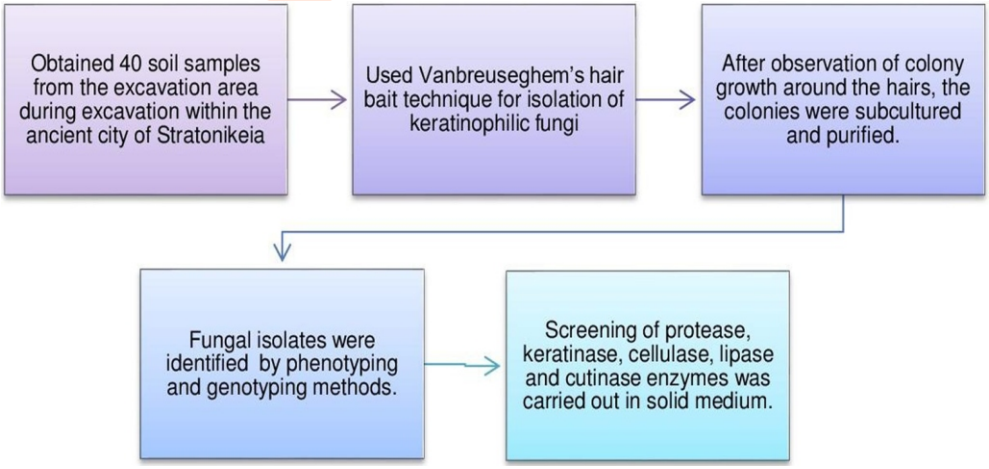
Abstract

Aim: To isolate and identyfy keratinophilic fungi from soil samples excavated excavation area within the ancient city of Stratonikeia, Turkey and determination of their enzyme potentials. Stratonikeia, a city in the interior of Caria, located at Eskihisar Village, in the Yatagan district of Mugla province of Turkey

Methodology: Keratin bating technique was applied for isolating of dermatophytes and keratinophilic fungi. Fungal isolate were identified by phenotyping and genotyping methods. Screening of protease, keratinase, cellulose, lipase and cutinase enzyme was carried at solid medium.



Results: Non-dermatophyte species, viz., *Aspergillus fumigatus, Engyodontium album, Chrysosporium keratinophilum, Lecanicillium lecani* and *Purpureocillium lilacinum* were identified. Protease, keratinase and cellulase were determined at moderate and high levels, while lipase and cutinase were not recorded.

Interpretation: Non-dermatophyte strains having high keratinase, cellulase and protease activities are not only involved in pathogenesis, but also have a great ecological significance due to keratin degrading potential.

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Introduction

Keratinophilic fungi are an important group of fungi that live in soil. Dermatophytes within keratinophilic fungi cause human and animal mycoses (Narula and Sareen, 2011; Soleymani *et al.,* 2015). Non-dermatophyte genera such as *Chrysosporium, Aspergillus, Alternaria, Trichurus, Curvularia, Cladosporium, Fusarium, Geomyces, Gleomastis, Monodictys, Myrothecium, Paecilomyces, Stachybotrys, Ulocladium, Scopulariopsis, Sepedonium, Penicillium* and *Doratomyces* (Gupta and Rammani, 2006) play an important role in decomposition of natural keratin in soil, and also cause other types of mycoses in humans and animals(Narula and Sareen, 2011; Soleymani *et al.,* 2015). Archaeological recreation workshops are generally indoor locations within archaeological excavation sites that are actively used in summer and autumn seasons, where sunlight is less and humidity is higher than outdoors. People working during the processing of findings are in close contact with the soil (Poirier, 2001).

Keratinophilic fungi having naturally a great role in the degradation of the keratin residues are ecologically significant. Therefore, the interest towards keratinophilic fungi increases day by day. The archeological excavation workshops are the areas where keratinophilic fungi are present. The aim of this study is to determine the keratinophilic fungi found in ancient Stratonikeia excavations by phenotypic and genotypic methods and to characterize the production potential of some enzymes that play a role in the pathogenesis of identified fungi

Materials and Methods

Isolation: Forty soil samples were collected from the excavated sites during the excavation within the ancient city of Stratonikeia after seeking permission from the excavation directorate. Vanbreuseghem's hair bait technique was followed for isolating of keratinophilic fungi (Ergin *et al.,* 2008). After observating the colony growth around the hair baits, the colonies were subcultured on Sabouraud's dextrose agar (SDA) with and without chloramphenicol (50 mgl^{-1}) and cycloheximide (500 mgl^{-1}). Pure cultures of isolates were then inoculated into tubes containing Potato dextrose agar and stored at + 4 ° C.

Phenotypic identification: The fungi were identified by using macro- and micro-morphological characters of these cultures according to the key described by Humber (1997); Pitt (1985); Hasenekoğlu (1991); Barnett and Hunter (1998) and Samson *et al.* (2010). Isolated *Aspergillus* colonies were identified on the basis of their micro and macro-morphological characteristics using standard taxonomic key used prevoiusly (Raper and Fennel, 1965; Samson *et al.,* 2010).

Molecular identification: DNA was isolated following the method of Liu *et al.* (2000). ITS1- 5.8-ITS2 region (ITS) of nuclear ribosomal DNA was amplified with ITS1 and ITS4 universal primers (Aveskamp *et al.,* 2009). Sequencing of ITS regions were fulfilled in Genmar Laboratory (İzmir, Turkey). After DNA sequencing analyses, fungi were identified by comparing with other ITS1- 5.8-ITS2 region (ITS) sequences in the public database. ITS1- 5.8-ITS2 region (ITS) sequences acquired were submitted to Gen Bank database and accession numbers for fungi were obtained.

Screening of enzyme activity : After identification of 19 fungal isolates, they were examined for production of protease (Yavuz, 2013), lipase (Kotagan *et al.,* 2014), cutinase (Saima and Roohi, 2013), cellulase (Topuz *et al.,* 2007) and keratinase (Kim, 2003). Enzyme activity (Pz) was measured by dividing the diameter of colony by the diameter of the colony plus precipitation zone. According to the values of their Pz coefficient, the tested strains were grouped in to four classes: Pz between 0.9 and 1 (+), very high Pz group (very low activity); 0.89–0.80 (++) high Pz group (low activity); 0.79–0.70 (+++) low Pz group (high activity) and Pz\0.69 (++++) very low Pz group (very high activity) ((Arslan and Findik, 2003).

Results and Discussion

Recent studies have shown that non-dermatophytic filamentous fungi are the causative agents of skin infection that produce clinically similar lesions caused by dermatophytes in humans and animals (Aksu, 2009; Narula and Sareen, 2011; Turhan, 2011). Studies have also shown that similar to dermatophytes, non- dermatophytic filamentous fungi can degrade keratin *in-vitro* and produce proteolytic enzymes, including keratinase (Gugnani, 2000). Keratinophylic fungi often cause disease in people in areas open to external factors such as hands, feet and nails. Persons working in close contact with the soil are exposed to environmental keratinophilic fungi (Poirier, 2001).

Studies on isolation of dermatophyte and keratinophilic fungi from soil has been reported from several countries including Egypt, Pakistan, Spain, Australia, Palestine, Kuwait, Jamaica, Poland, Korea, India, Iran and Malaysia (Kim, 2003; Gugnani *et al.,* 2012; Irum *et al.,* 2007; Ganaie *et al.,* 2010; Kornıllowıcz *et al.,* 2011; Mini *et al.,* 2012; Gugnanı *et al.,* 2014; Pakshir *et al.,* 2013; Soleymani *et al.,* 2015; Tambekar *et al.,* 2007; Geetanjali and Kumar, 2014) However, reports on isolation of dermatophyte and keratinophilic fungi from Turkey is meagre. Ergin *et al.* (2008) isolated keratinophilic fungi from soil samples obtained from an archaeological workshop in Denizli. Yavuz (2013) used molecular methods and identified dermatophytes from soil and humans. Our study constitutes a data set concerning environmental keratinophilic fungi in Turkey. It is required to examine the keratinophilic fungal flora of Turkey at different sites and environmental conditions that are considered to constitute risk groups.

The fungal species identified by phenotypic and molecular methods were: *Aspergillus fumigatus, Purpureocillium lilacinum, Chrysosporium keratinophilum, Lecanicillium lecani* and *Engyodontium album* (Table 1). Jain and Sharma (2011)

Table 1: Isolate code, GenBank accession number for Nuclear ribosomal DNA ITS1- 5.8-ITS2 (ITS) region, and protease, cellulase, cutinase and keratinase activities for fungal species identified by phenotypic and genotypic identification

Isolate code	Species	Accession number	Protease	Cellulase Pz*	Lipase	Cutinase Pz*	Keratinase Pz*
D1	*Aspergillus fumigatus*	KY801312	++++	++++	-	-	++++
D2	*Engyodontium album*	KY801311	++++	++++	-	-	++++
CK1	*Aspergillus fumigatus*	KY801316	++++	-	-	-	++++
S1	*Chrysosporium keratinophilum*	KY801300	++++	-	-	-	++++
D3	*Chrysosporium keratinophilum*	KY801310	+	-	-	-	+++
CK2	*Aspergillus fumigatus*	KY801315	++++	+++	-	-	+++
S2	*Lecanicillium lecani*	KY801299	++++	++++	-	-	++++
D4	*Purpureocillium lilacinum*	KY801309	++++	++++	-	-	++++
D5	*Purpureocillium lilacinum*	KY801308	++++	++	-	-	++++
D6	*Aspergillus fumigatus*	KY801307	+++	++++	-	-	++++
D7	*Chrysosporium keratinophilum*	KY801306	+	-	-	-	++
D8	*Purpureocillium lilacinum*	KY801305	++++	++++	-	-	++++
CK3	*Purpureocillium lilacinum*	KY801314	++++	++++	-	-	++++
D9	*Purpureocillium lilacinum*	KY801304	++++	+++	-	-	++++
D10	*Purpureocillium lilacinum*	KY801303	+++	++++	-	-	++++
D11	*Purpureocillium lilacinum*	KY801302	++++	++	-	-	+++
CK4	*Purpureocillium lilacinum*	KY801313	++++	+++	-	-	++++
S3	*Purpureocillium lilacinum*	KY801298	++++	++++	-	-	++++
L1	*Purpureocillium lilacinum*	KY801301	++++	++++	-	-	++++

isolated dermatophytes from soil samples collected from different areas; in addition to dermatophytes, fungi belonging to *Chrysosporium, Paecilomyces, Fusarium* and *Scopulariopsis* genera were also isolated. In Nigeria, keratinophilic fungi like *Aspergillus flavus, Fusarium* sp. and *Chrysosporium indicum* and dermatophytes such as *Microsporum gypseum* and *Trichophyton mentagrophytes* (Oyeka and Okoli, 2002) were isolated from soil. Similarly,Sharma and Sharma (2010) collected soil samples from the lands of school and college parks in Jaipur, India were studied for the prevalence of keratinophilic fungi and related dermatophytes. Thus, they isolated *Chrysosporium, Trichophyton, Microsporum, Paecilomyces* genus from the parks. This is known as the first report on the isolation of *Trichophyton* sp. and *Paecilomyces* sp. as keratinolytic fungi from soil samples of Jaipur (India).

Kim (2003) reported 14 species of feather associated fungi belonging to 10 genera *Acremonium, Alternaria, Aspergillus, Cladosporium, Curvularia, Fusarium, Monascus, Mucor, Penicillum* and *Verticillium* from poultry soils in Korea. Cutaneous infections coused by *A. fumigatus, A. flavus, A. terreus* and *A. chevalieri* have been reported (Stevens *et al.,* 2000). Efuntoye and Fashanu (2001) determined that the most frequently observed species among keratinophilic fungi, isolated from feathers, beaks and nails of chickens, ducks, turkeys and doves, were *Chrysosporium keratinophilum.* Shadzi *et al.* (2002) also reported that *C. keratinophilum* was the dominant specie in 330 soil samples collected from primary schools and parks. *Chrysosporium* species also are important due to their potential pathogenic characteristics. For example, *C. zonatum* species caused systemic infection in a patient with chronic granulomatosis (Kumar *et al.,* 2013). *C. keratinophilum* is the most commonly isolated species in soil. Ali- Shtayeh and Jamous (2000) isolated fungi from different field soils irrigated with urban waste water and fresh water, and reported high keratinophilic activity in *C. keratinophilum, Rhizopus stolonifer* and *Trichophyton ajelloi.* Soleymani *et al.* (2015) reported the presence of dermatophyte fungi *Cunnighamella sp., Microsporum gypseum* and *Aspergillus niger* in soil samples from university hospitals in Iran. Ganaie *et al.* (2010) isolated keratinophilic fungi from different soil samples in Pakistan, and identified different species belonging to genera *Trichophyton, Chrysosporium, Microsporum, Aspergillus Fusarium, Alternaria, Trichoderma, Candida, Penicillium* and *Paecilomyces.*

The results of enzyme activity of different fungal species in solid medium are listed in Table 1. The perusal of data revealed high protease acitivty in *P. lilacinum* (9 strains), *A. fumigatus* (3 strains), *C. keratinophilum* (1 strain), *E. album* (1 strain) *and Lecanicillium lecanii* (1 strain); high cellulase activity was observed in *P. lilacinum* (6 strains) *A. fumigatus* (2 strains) and *L. lecanii* (1 strain); high keratinase activity in *P. lilacinum* (9 strains), *A. fumigatus* (3 strains), *E. album* (1 strain), *C. keratinophilum* (1 strain) and *L. lecanii* (1 strain). Cutinase and lipase activities were not observed in any fungal strains. It was observed that 4 strains (D4, D8, CK3, S3 and L1) belonging to five *P. lilacinum* species displayed high levels of protease, cellulase and keratinase activities. *P. lilacinum* is widely used as biocontrol agents, and is mainly considered to be a nematophagous, egg-parasitizing fungus, specifically against root-knot nematode, *Meloidogyne incognita* and several other nematodes including *Radopholus similis, Heterodera* spp. and *Globodeera* spp. (Lopez *et al.,*

2014). Also, strain S1 belonging to *C. keratinophilum* displayed high protease and keratinase activities.

Muhsin and Salih (2001) explored the production potentials of dermatophyte and non-dermatophyte species isolated from infected animals for lipase, amylase, protease and keratinase enzymes. They demonstrated that dermatophytes possessed protease activity in addition to high keratinase activity. Kim (2003) indicated that *Aspergillus fumigatus* was useful in microbial transformation of keratinous wastes and also that *A. flavus* produce keratinolytic enzymes. Soomra *et al.* (2007) indicated that *Aspergillus niger* was the most frequently observed species within keratinophilic fungi isolated from different soil samples in Pakistan, followed by *A. flavus* and *A. fumigatus.* Although *Aspergillus, Alternaria, Cochliobolus, Botrytis, Fusarium* and *Mucor* are non-keratinophilic fungi, however these fungi showed keratinolytic activity on keratine-rich substrates. Mini *et al.* (2012) found the highest keratinolytic activity in *A. flavus* species, followed by *C. keratinophylum, A. fumigatus, A. niger, A. nidulans M. gypseum* and *T. mentagrophytes* isolated from soil samples of poultry farms in the Kattayam region of India.

The European Union, which advocates environmentally-friendly technologies, encourages the use and improvement of keratinolytic microorganisms for effective processing of wastes containing keratin (Kornıllowıcz-Kowalska and Justyna, 2011). In this study, strains with high keratinase, protease and cellulase enzyme activities were isolated and the study achieved its objective. The keratinophilic fungi are not only known for pathogenesis but also have a ecological significance due to their keratin degrading nature.

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