



Danger of exposure to keratinophilic fungi and other dermatophytes in recreational place in the northeast region of India

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Abstract Keratinophilic fungi are a group of environmentally and epidemiologically important fungi that cycle a recalcitrant animal protein known as keratin. A large group of keratinophilic fungi cause diseases in human and animal mycoses so called as dermatophytes. The present study was undertaken to evaluate the diversity and activity for the group of keratinophilic fungi from the humus of “Phumdis” in Loktak Lake. Keratin-degrading keratinophilic fungus was isolated from the humus of “Phumdis” collected from fresh water Loktak Lake. The isolated fungal strains were initially identified on the basis of macro- and microscopic characteristics and confirmed using molecular approach. This study indicates first time that this Loktak Lake is a significant reservoir of keratinophilic fungi which can pose a health risk to its users, particularly the tourists and the employees. The keratinolytic activity of these fungi is crucial to cause

superficial infections in human and animals, and recycling of keratinic material of this atmosphere.

Keywords Keratinophilic fungi · Dermatophytes · Keratinases · Biodegradation · Human hair

1 Introduction

Dermatophytes are keratinophilic fungi come under Arthrodermataceae family. Keratinophilic fungal species are spread worldwide and have the ability to occupy keratinised tissues (skin, hair, and nails) resulting in a situation called dermatophytosis or tinea. In India, the occurrence of soil-associated dermatophyte, *Microsporum gypseum* was reported for the first time in the Dibrugarh district of Assam in 1955 by Dey and Kakoti (Dey & Kakoti, 1955). Later a widespread distribution of keratinophilic fungi in Indian soils and Lakes were reported by various workers (Deshmukh & Agrawal, 1983; Garg, 1966; Garg et al., 1985; Randhawa & Sandhu, 1965; Sharma et al., 2015; Singh et al., 2009). However, till date, there were no report about the occurrence and molecular characterization of keratinophilic fungi in Loktak Lake, Manipur, India. Loktak Lake of Manipur is the largest freshwater lake (26 km long and 13 km wide with 980 km² catchment area), a Ramsar site, (93° 46′–93° 55′E & 24° 25′–24° 42′N) in North East

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India (Fig. 1). The lake is the floating island called “Phumdis” a unique feature which form the Kaibul Lamjao National Park—the main home of *Rucervus eldii eldii*. Phumdis, are a heterogeneous mass of soil, vegetation and organic matter at different stages of decomposition (Takhelmayum & Gupta, 2011) (Fig. 2). The current investigation is carried out in the hitherto unexplored habitats of North-East India, under Indo-Burma Biodiversity hot spot. This communication aimed to document the diversity of specialized keratinophilic fungi communities in Loktak Lake using cultured-based and sequencing

approaches. We further screened these fungal isolates for proteolytic and keratinolytic potentials.

2 Methodology

2.1 Collection of samples

Sample collection was carried out in the month of November 2017. Different samples of “Phumdis” around 1 m thicker along with its “humus” were collected in sterile polyethylene bags from 8 different



Fig. 1 Map of Manipur showing the location of Loktak Lake



Fig. 2 Unique Loktak Lake and its Phumdi ecosystem

sites/ locations in Loktak Lake, Manipur (India) and stored at 4 °C until further use.

2.2 Collection and processing of human hair

Human hair were obtained from a local hair salon and were surface sterilized with ethanol (3%) (Călin et al., 2017), washed thoroughly under running water, and then dried at 60 °C. The hair strands were cut into pieces of about 2 cm in length and autoclaved at 121 °C for 20 min.

2.3 Isolation and identification of keratinophilic fungi

The hair baiting method described by Vanbreuseghem, 1952 (Vanbreuseghem, 1952) was used for the isolation of keratinophilic fungi. For this purpose, sterile Petri dishes were half filled with the soil samples and then, pieces of sterile hair fragments were dispersed over the surface of the soil samples and moistened with sterile distilled water supplemented with antibiotic solutions, chloramphenicol (50 mg/l), and streptomycin-penicillin (1000 IU/ml). The plates were incubated at room temperature (25 ± 2 °C). To prevent from dryness, the plates were occasionally moistened with sterile distilled water. The soil samples were assessed regularly for fungal growths on the hair baits under a stereoscopic binocular microscope. Portions of the growth were aseptically transferred to SDA (Sabouraud's dextrose agar) plates amended

with chloramphenicol (50 mg/l) and plates were incubated at room temperature for five to ten days till the fungus grows. The fungal colonies were sub-cultured in order to obtain the pure cultures. These fungi were initially identified based on the morphological characterization following the protocol of Sigler and Carmichael (Sigler & Carmichael, 1976), Oorschot (Van Oorschot, 1980), Currah (Doveri et al., 2012) and (Domsch et al., 1980). Afterward they were subjected for DNA based identification.

2.4 Molecular identification of keratinophilic fungi

Fungi were cultured in Sabouraud's dextrose broth (SDB) for one week. Fungal genomic DNA was isolated as described by (Agrawal et al., 2018; Hermosa et al., 2000) with minor modifications. The ITS region of the nuclear small-subunit rRNA gene was amplified in an automated thermocycler (Bio-rad-C1000 Thermal Cycler) using the primer pairs ITS1/ITS4 (5'-TCC GTA GGT GAA CCT GCG G-3'/ 5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS1F/ITS4B (5'-CTTGGTCATTTAGAGGAAGTAA-3'/ 5'-CAGGAGACTTGTCACGGTCCAG-3') as described by (Innis et al., 2012; White et al., 1990). Final PCR reaction mix volume was adjusted to 25 µl containing 2.5 µl of 10 × buffer, 1.5 µl of MgCl₂ (25 mM), 0.5 µl of dNTP (10 mM), 1 µl each of ITS1 and ITS4 primers (5 pm), 0.2 µl of Taq Polymerase (2.5 U), 3 µl of DNA sample (5 µg/ ml), and

remaining volume was adjusted using sterile Milli Q Water. The PCR reaction was carried out in C1000 Touch™ Thermal Cycler (Bio-Rad, U.S.A) with the PCR cycle of 5 min denaturation at 94 °C, 33 elongation cycles (50 s at 95°C, 50 s at 56°C, 1 min at 72°C) with final extension period for 10 min at 72°C and finally hold at 4°C. The final PCR products were analyzed by running in the gel electrophoresis on 1% Agarose (Sigma-Aldrich, St. Louis, U.S.A). Finally, the PCR products were visualized under UV light using Gel imaging system (BioRad, Chemi Doc, MP). The amplified PCR-DNA products were purified and sequenced using the same primers. The sequences were analyzed by Finch TV 1.4.0 (Mishra et al., 2010). The sequences were then compared with the NCBI GenBank database by the BLASTN program. Phylogenetic relationships were estimated using MEGA7.0.18 (Kumar et al., 1994).

2.5 Screening of fungal isolates for proteolytic ability using skim milk agar medium

The proteolytic activity exhibited by the isolated fungi was evaluated as per method discussed by Nwadiaro, 2015 (Nwadiaro et al., 2015). The 6 mm discs of actively growing 7 days old mycelia of the test fungi were inoculated on the skim milk agar plates and incubated for 5 days at 25 ± 2 °C. After incubation the plates were observed for a clear zone of hydrolysis around the inoculated culture. Mean diameters of triplicate were recorded for each fungal species. The fungus that had the noteworthy diameter of zone of clearance was chosen for additional work.

2.6 Fungal cultivation for crude keratinase production using submerged fermentation (SmF)

Discs of pure cultures of actively growing fungi from Sabouraud's dextrose agar plate was inoculated into sterile production medium and allow to grow through submerged fermentation (SmF) (Wawrzekiewicz et al., 1987). The 100 ml production medium contained the following components per liter (g/l) in 250 ml Erlenmeyer flasks: ground human hair (1%), The human hairs were thoroughly washed, defatted by soaking in chloroform for 24 h and then rinsed in three times in autoclaved distilled water. After washing the sample was completely dried before it got converted into

powdered form and it serves as a sole carbon and nitrogen source, NaCl (0.5 g), KH_2PO_4 (1.5 g), K_2HPO_4 (1.0 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g). The flasks were incubated at 28 ± 2 °C for 15 days and extra-cellular keratinases was tested from the supernatant. Changes in the pH of the medium were checked/recorded additionally before and after the assay.

2.7 Assay of Keratinolytic Protease

The keratinolytic protease assay was performed according to the method described by Ramnani, 2004., (Ramnani & Gupta, 2004). Equal volume of 1 ml crude supernatant, 1% ground hair and 1 ml of 0.05 M Glycine–NaOH buffer combination was incubated for 1 h at 37 °C and the pH was adjusted at 9.0. The keratinase activity was measured from the supernatant at 520 nm. One unit of keratinase activity was defined as an increase in absorbance of 0.01 at 520 nm under standard assay conditions.

3 Results

3.1 Isolation and identification of keratinophilic Fungi

Among the samples collected from the eight different sites, samples of only three sites yielded fungal colonies on the human hair (Fig. 3) which were isolated and purified for further study. Initially, we observe many morphologically different fungal growths on hairs, however we further observed that after transferring them to Sabouraud's dextrose agar medica plates many of them lost their growth. Only ten fungal isolates were found able to grow on Sabouraud's dextrose agar medium (Fig. 4) and were identified by 18S rRNA gene sequence analysis. These eight species belong to six genera: *Trichoderma harzianum*, *Curvularia lunata*, *Curvularia affinis*, *Poitrasia circinans*, *Fusarium oxysporum*, *Chrysosporium mephiticum*, *Chrysosporium lucknowense*, *Westerdykella dispersa* (Fig. 5). All these fungal strains have been deposited in IBSD Microbial Repository Centre with reference numbers as MRCF.115-K1, MRCF.116-K2, MRCF.117-K3, MRCF.118-K4, MRCF 22,401-K5, MRCF.119-K7, MRCF 22,445-K8, MRCF.120-K9.

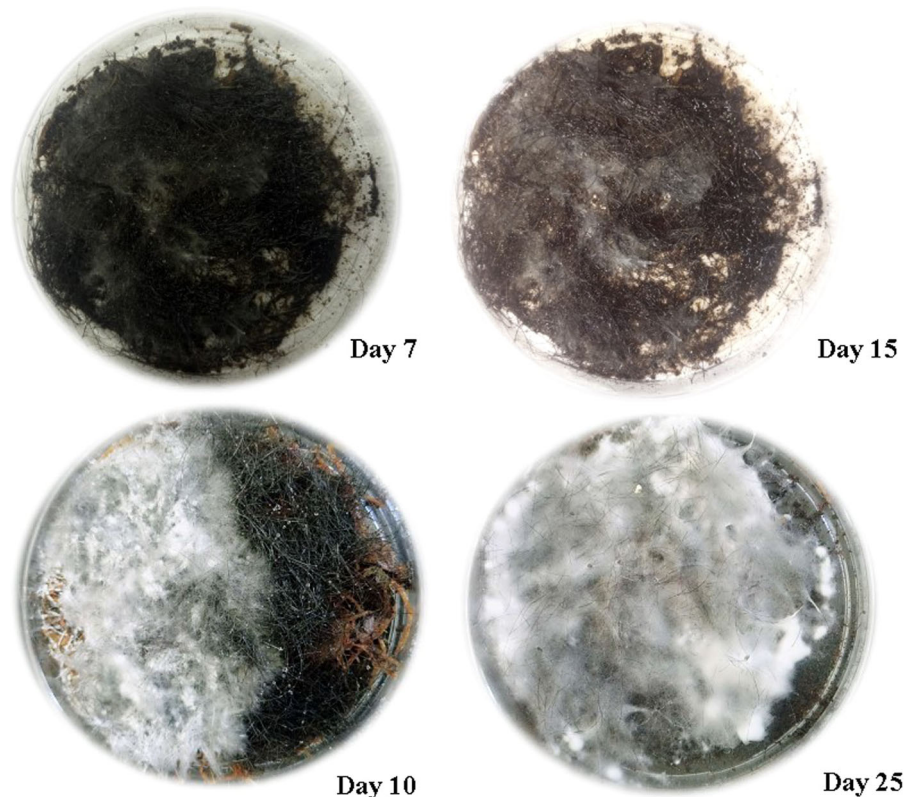


Fig. 3 Keratinophilic fungal colonization on hair

3.2 Screening of protease producing ability on skim milk agar medium

The protease producing ability of fungi were determined using skim milk agar plate assay. All isolates grew on the milk powder plates and produced a clear zone around or under the mycelia, indicating protease activity. Among all the fungi, only three isolates viz., *Poitrasia circinans* > *Curvularia affinis* > *Curvularia lunata* exhibits clear zone around the mycelial front. considerably, while rest of the fungi could not hydrolyze the skim milk agar.. Out of the three fungi, *Poitrasia circinans* showed the maximum activity (Fig. 6).

3.3 Keratinolytic activity of the test fungus using 1% human hair

The keratinase activity was tested from the cell free supernatant of the three fungi, viz., *Curvularia lunata*, *Poitrasia circinans* and *Curvularia affinis*, grown on human hair. The results showed that there was enzyme

activity on keratinous substrates used as the sole carbon and nitrogen sources. The maximum activity of 17.0 U/ml was observed in *Poitrasia circinans*, followed by *Curvularia affinis* and *Curvularia lunata* with activity of 15.0 U/ml and 11.0 U/ml, respectively (Fig. 7). This result is an indication that these fungi degraded the human hair. It is attention-grabbing to notice that these fungi possibly biodegrade keratin in natural setting. It is also observed that pH of the medium was found to be increased from 5.5 to 6.3 during crude enzyme synthesis and is believed to be as a result of the discharge of cysteine, keratinase, and proteins by the fungus. It has been instructed that the premise of keratinolysis is because of elevated level of deamination and eventual alkalization of the medium, although deamination and alkalization surely play a serious role in keratinolysis (Fernandes et al., 2017).



Fig. 4 Pure colony of isolated on SDA medium after 15 days of incubation

4 Discussion

Keratinophilic fungi are an important group of fungi with ecological importance. Some groups are responsible for cutaneous fungal infections and other for saprophytic fungi mainly represent hyalohyphomycosis (Garg et al., 1985). Many fungal species of the

genus *Trichophyton* are keratinophilic in nature (Anbu et al., 2006; Ciesielska et al., 2021; Weary & Canby, 1967) and causes infection in humans (Robati et al., 2018; Shen et al., 2021; Zhang et al., 2019). Another class of fungi belongs to genus *Microsporum* are pathogenic and most prevalent dermatophytes of domestic animals with a zoonotic potential that may

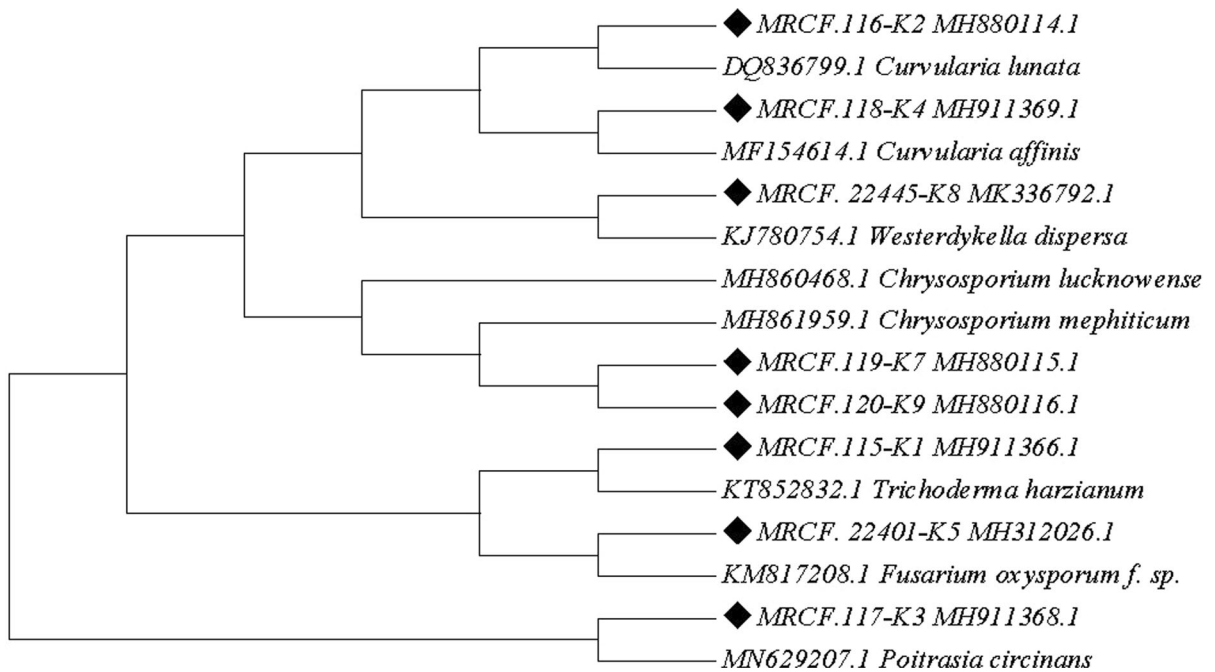
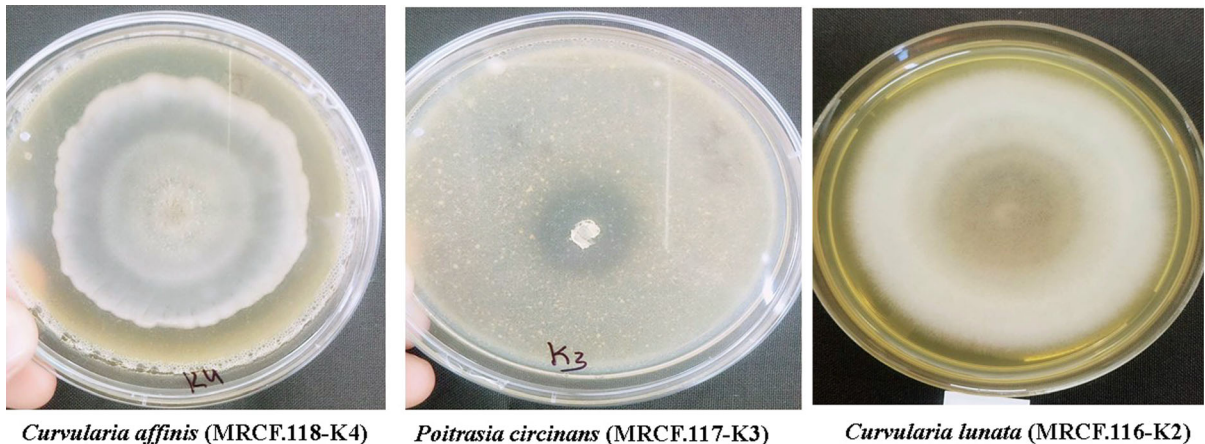


Fig. 5 Phylogenetic tree based on ITS1-5.8S-ITS4 region sequences for keratinophilic fungi with reference strains. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA6



Curvularia affinis (MRCF.118-K4)

Poitrasia circinans (MRCF.117-K3)

Curvularia lunata (MRCF.116-K2)

Fig. 6 Clearing zone formation of the fungi in Skim milk agar plate

cause tinea capitis and tinea corporis in humans (Aly et al., 2000; Patel & Schwartz, 2011). Clinical presentation of this infection is characterized by multifocal alopecia and scaly, circular lesions (usually referred to as “ringworm”), similar to that caused by other dermatophytes or other skin diseases. The pathogenesis of this infection is usually associated with the secretion of virulence factors like keratinase or activation of genes involved in keratinolytic

activity (Descamps et al., 2003; Monod, 2008; Weary et al., 1965). Further the keratinolytic activity of strains of the genus *Epidermophyton* was also observed which causes infections in humans (Cabañes et al., 1987; Gnat et al., 2021).

The availability of keratin, pH, and geographical location are the factors responsible for the incidence of such fungi. Some of these fungi could be transmitted from soil and water to humans. In general, the

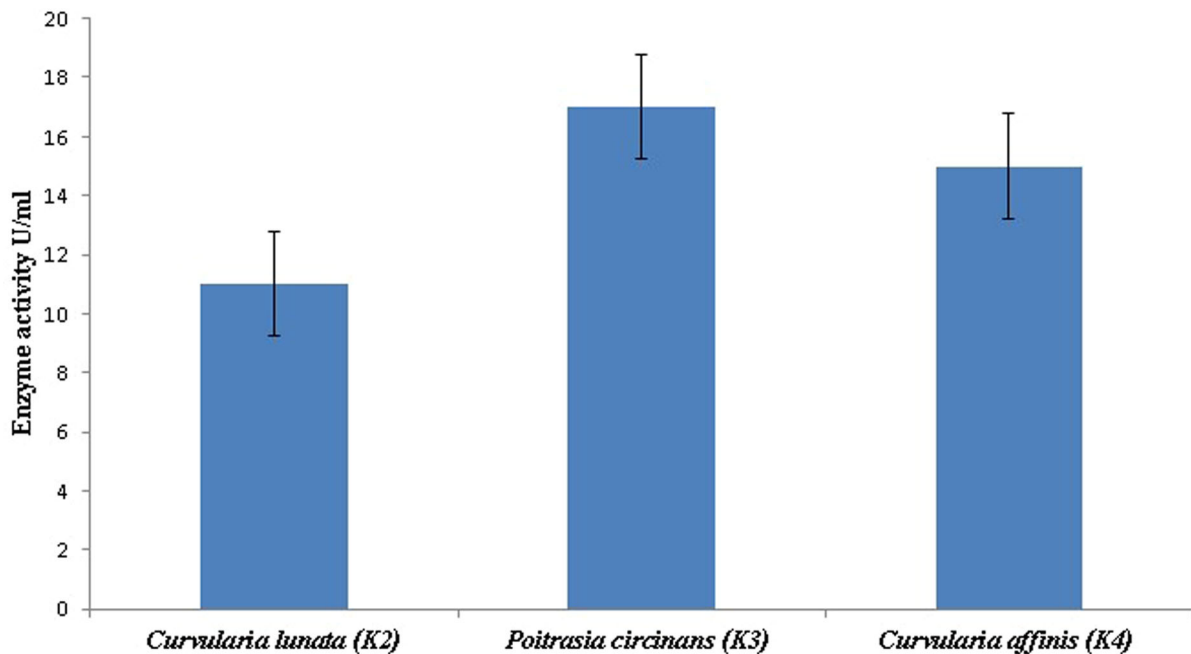


Fig. 7 Keratinase activity using 1% human hair as substrate

presence of humus and organic materials in soil and water bodies leads to the growth of keratinolytic and saprophytic fungi (Marchisio, 2000). In the last decade, there are reports on the occurrence of geophilic dermatophytes and keratinophilic fungi from the soil and water in many parts of the country (Deshmukh et al., 2017, 2018; Randhawa & Sandhu, 1965), and also reported to have industrial applications (Călin, 2016; Verekar & Deshmukh, 2017). (Sharma & Sharma, 2009) reported that the temperature 25 °C–35°C and 50–95% relative humidity is the best for growth and sporulation of keratinophilic fungi and the environmental data shows the relatively similar around Loktak Lake. This higher sporulation plays significant role in the disperse of fungal spores in the air and this further leads the disease in animals and humans, such as allergies, poisoning and infections. Since it is a tourist spot, many tourists are visiting this lake and many families staying in and around the lake, are fully depended on the lake resources for their day today living and various activities have been carried out in Loktak Lake, which can potentially lead at risk for being exposed to keratinophilic fungi.

In one of previous report the authors assessed the *Trichoderma* diversity associated with ecosystem, climate, and geographic location. They found that

Trichoderma afro-harzianum was the dominant species in lake ecosystems (38%) but represented less than 2% in forest ecosystems. *T. harzianum* had a higher relative abundance in river than other landscapes (21% versus 4%), and *T. virens* was more abundant in coastal ecosystems (16% versus 3%) (Hu et al., 2020). Usually, the fungi of the genus *Trichoderma* are classified as pathogen antagonistic. However its keratinolytic activity limited to specific species of animals and groups of people. (Gnat et al., 2019) carry out phenotypic analysis of the degree of keratinolytic activity of clinical isolates of *Trichophyton verrucosum* strains using hairs of humans and various animal species as substrates. They observed that the activity of keratinases is substrate dependent. The highest activity of keratinases was recorded in media containing keratin from cow (*Bos taurus*) and sheep (*Ovis aries*) hairs in comparison with that from other tested species. (Cao et al., 2008) reported a new keratinolytic *Trichoderma atroviride* strain F6 which completely degrades native chicken feather. It is also observed that this strain F6 is more suitable for feather degradation than its purified keratinase. (Ismail et al., 2012) reported *Trichoderma harzianum* MH-20 as a most potent keratinase and protease producer.

Curvularia lunata is an ascomycete filamentous fungus detailed as the etiological agent of keratomycosis, sinusitis, onychomycosis, eumycetoma, pneumonia, endocarditis, dialysis-associated peritonitis, cerebral abscess, and disseminated fungal infections (Narula et al., 2020; Wind & Polack, 1970). This species has also been reported to account for ocular infections, such as conjunctivitis, dacryocystitis, and endophthalmitis and also causes local and invasive phaeohyphomycoses in both immunocompromised and immunocompetent patients (Tóth et al., 2020). (Verma, 2013)., (Verma et al., 2013) reports occurrence of seven *Curvularia* species of from three Lakes of Bhopal, India including *Curvularia lunata*. (Pachade, 2014)., (Pachade et al., 2014) reported the incidences of mycotic infections caused by *Curvularia lunata* in *Channa punctatus* of Wadali Lake, Amravati, MS, India. (Kamalam et al., 1992) first time reported the keratolytic activity of the fungus *Curvularia lunata* in the nail and its complete clearance by topical clotrimazole. Keratinolytic activity of fungus *Curvularia lunata*, which was isolated from the feathers of living poultry birds (*Gallus domesticus*) also reported by (Vidyasagar et al., 2005). A *Curvularia* sp., was isolated from the lesion on foot from 74-year-old healthy Japanese man. Further it was observed that fungus grows well on keratotic materials (hairs, nails, and callus), which indicates that it might have the ability to infect the skin surface (Yanagihara et al., 2010).

Lee, (2018)., isolates *Poitrasia circinans* from the fresh water samples collected from the Geum River located in Gongju, Korea (Lee et al., 2018). *Fusarium* infection in humans is rare. However, *Fusarium oxysporum*, *F. solani*, are common causes of keratitis. They are also common causes of onychomycosis, endophthalmitis, and skin and musculoskeletal infections (Gupta et al., 2000; Nucci & Anaissie, 2007). (Edel, 2001)., assessed genetic diversity of soil-borne populations of *Fusarium oxysporum* using 350 isolates collected from six different French soils. All isolates were characterized by restriction fragment analysis of the PCR-amplified ribosomal intergenic spacer (IGS) (Edel et al., 2001). *Chrysosporium* is a kind of hyaline hyphomycetes fungi in the family Onygenaceae. Species of *Chrysosporium* are rarely isolated from skin and nail scrapings, especially from feet, but, because they are common soil saprotrophs, they are usually considered as dermatophyte related species

(Ali-Shtayeh et al., 2003). Infection caused by this organism is rare in humans and typically occurs in immunocompromised patients. (Stebbins et al., 2004) reported a primary cutaneous infection with *Chrysosporium* spp., in an immunocompromised heart transplant patient. Similarly a primary cutaneous *Chrysosporium* infection following ear piercing in an immunocompetent patient was reported by (Suchonwanit et al., 2015). *Chrysophobia keratinophilum* may cause mild infections in humans and is sometimes responsible for onychomycosis (Hocquette et al., 2005). Fungus *chrysosporium keratinophilum* was isolated from the tissue on the neck and scales on the trunk, respectively in a 38-year-old woman (Mijiti et al., 2017). Previously researchers isolates various keratinophilic fungi by the hair baiting technique from the soil of potted plants of indoor environments in Kanpur, India, and their also evaluate their proteolytic ability (Singh et al., 2009).

Westerdykella dispersa was first time isolated in a critically burned patient. It is an important cause of Angioinvasive fungal infections (AFIs) among immunocompromised patients (Lipovy et al., 2018; Sue et al., 2014). In earlier studies, *Chrysosporium lucknowense* has been isolated, but at different frequencies than those reported here and from different types of sources (Abd El Hafez et al., 1991; Gherbawy, 1999). In 1999 in India (Madras), keratinophilic fungi were recovered from the soil of 30 primary schools and 15 public parks, isolating 31 species belonging to 15 genera (Ramesh & Hilda, 1998). *Chrysosporium tropicum* was the most isolated species (62.2%) in the soil of schools and parks in India.

Functional screening of largely unknown microbial diversity could provide important information regarding microbial ecology, but also constitutes the framework for searching useful microbial strains aiming technological applications in industrial biotechnology. In the present study the fungal isolates from Loktak Lake were screened for their proteolytic abilities on SMA plates. Out of all the isolate, three isolates exhibited significant proteolytic ability (Table 1). The fungi having strong keratinolytic activity could be employed for the bioconversion of hair, feathers, and other keratinous wastes to acquire biotechnologically relevant products and the characterization of the crude proteolytic enzyme is under way to postulate biocatalyst applications.

Table 1 Proteolytic abilities of the fungal isolates using skim milk agar

Fungal isolates	GenBank association number	Growth on Skim Milk agar	Clear zone
<i>Trichoderma harzianum</i> (MRCF.115-K1)	MH911366.1	+ + +	–
<i>Curvularia lunata</i> (MRCF.116-K2)	MH880114.1	+ + +	+
<i>Poitrasia circinans</i> (MRCF.117-K3)	MH911368.1	+	+ + +
<i>Curvularia affinis</i> (MRCF.118-K4)	MH911369.1	+ + +	+ +
<i>Fusarium oxysporum</i> (MRCF. 22,401-K5)	MH312026.1	+ + +	–
<i>Chrysosporium mephiticum</i> (MRCF.119-K7)	MH880115.1	+ +	–
<i>Westerdykella dispersa</i> (MRCF. 22,445-K8)	MK336792.1	+ + +	–
<i>Chrysosporium lucknowense</i> (MRCF.120-K9)	MH880116.1	+ +	–

The symbols + + +, + +, +, – indicates high, moderate, low, and no activity respectively

5 Conclusion

The occurrence of keratinophilic fungi was studied in many environments, but the present study is the first-time report that the water and humus associated with “Phumdis” of Loktak Lake, Imphal may be significant reservoirs of certain keratinophilic fungi. The keratinolytic activity of these fungi may be playing a significant role in superficial infections to humans and animals and may also decompose the keratinous substances added to the soil by human and animal activity. It is, therefore, important to investigate the presence of novel strains of keratinolytic fungi in varied environmental conditions and how many of them are pathogenic. It is fundamental that this information regarding isolated fungal agents be known by the municipal and state health departments of each geographic region evaluated, aiming at public health measures through sanitary and epidemiological surveillance.

Declaration

Conflicts of interest The authors would like to thank Department of Biotechnology, Government of India for the financial support in carrying out the research. The authors are also thankful to Dr. Chingkeihunba Akoijam for their contribution in sample collection. The authors declare that they have no conflicts of interest concerning this article.

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