POTENTIAL OF PSYCHROTROPHIC FUNGI ISOLATED FROM SIACHEN GLACIER, PAKISTAN, TO PRODUCE ANTIMICROBIAL METABOLITES

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Abstract. This is the first study of recovery and characterization of fungi from ice, sediments and water samples collected from Siachen glacier, Himalaya Range, Pakistan. The isolation and Total Viable count (CFU/ml or g) was carried out by spread plate technique at 4°C and 15°C. Seventeen fungal isolates were obtained and identified by analysis of 18S rRNA ITS region. Most frequently isolated fungal isolates were *Leotiomycetes* sp., followed by *Thelebolus*, *Penicillium*, *Cladosporium*, *Trichoderma*, *Periconia*, *Geomyces*, *Cryptococcus* and *Pueraria*. All isolates were found halophilic and they were able to tolerate NaCl concentration up to 10-20%. Some isolates showed viability at 45°C and most of the isolates were able to grow at pH 1- 13. All isolates were screened for their antimicrobial activity against clinically isolated bacterial and fungal strains but they showed good antimicrobial activity against Gram (+) bacteria. None of the fungal isolates inhibited Gram negative clinically isolated *Escherichia coli* and *Klebsiella pneumonia* but few were able to inhibit Gram positive bacterial and fungal strains. Fungal isolates were also screened for production of extracellular enzymes (amylase, cellulase, deoxyribonuclease, lipase, phosphatase and protease). Various isolates were good producers of cellulase, lipase and protease whereas only 2 out of 17 produced DNase and 4 produced phosphatase.
Keywords: *non-polar glacier, psychrophilic fungi, enzymes, halophilic*

Introduction

Psychrophilic fungi grow optimally at 15°C or lower but can also grow at temperature around 20°C or below, while psychrotrophic fungi grow well at temperature above 20°C (Maheswari, 2005; Robinson, 2001). Such type of fungi have been found and investigated in all major cold habitats, such as Antarctica (Blanchette et al., 2010), Arctic regions (Sonjak et al., 2006) and cold deep sea environments (Damare et al., 2006). Various fungi representing different genera and species e.g. *Thelebolus microspores*, *Lemonniera*, *Tetracladium*, have been isolated from different regions of Himalaya, India (Sati et al., 2014; Anupama et al., 2011).

The fungi in cold environments are facing numerous extreme limiting factors, including frequent freeze-thaw cycles, high salt concentration, low moisture content, extreme UV radiation and low nutrient availability (Robinson, 2001, McKenzie et al., 2003). To face such harsh conditions, fungi adapt themselves through various physiological and ecological mechanisms (Anupama et al., 2011). Although, several cold adaptive mechanisms of psychrophilic fungi have been described but it is assumed that a mixture of such mechanisms are employed by psychrophiles including production

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of antifreeze proteins, compatible solutes, trehalose and other freeze tolerance mechanisms (Robinson, 2001; Ruisi et al., 2007).

Psychrophilic and psychrotrophic fungi are capable of providing a large number of biotechnological and pharmaceutical applications. Psychrophilic fungi are capable of synthesizing secondary metabolites that are very unique to cold ecosystems (Rosa et al., 2008). Psychrophilic fungi are producers of cold shock and cold-acclimation proteins and enzymes (e.g. proteases, lipases and cellulases) that are widely used in various biotechnological fields (Gounot, 1991). These include cold-water detergents, food additives, flavor modifying agents and biosensors. The psychrophilic fungi can also be central to astrobiology as other psychrophiles are (Montes-Hugo et al., 2009).

Antibiotic resistant pathogens emerge faster than the rate of discovery of new antibiotics. Extended spectrum beta-lactamase (ESBL) producing Enterobacteriaceae, vancomycin resistant *Enterococci* sp. and methicillin-resistant *Staphylococcus aureus* (MRSA) are all examples of the pathogens that are difficult to treat due to lack of effective antibiotics. It is important to look for new antibiotics from extreme sources that have not yet been explored for this purpose. We need to investigate antibiotics production from new extreme and unexplored sites against both multi-drug resistant bacteria and fungi.

This study was commenced to investigate the presence of psychrophilic fungi from from Siachen glacier, Pakistan, as well as to evaluate various physiological parameters, antimicrobial activity and extracellular enzyme production.

Materials and methods

Sampling

Siachen glacier is the second longest (70 km) non-polar glacier in the world, located in the Himalaya Range. Total width of the glacier is between 2-8 km and the total area is less than 1,000 km^2. Three different forms of samples (glacier ice, sediments and water) were collected from Siachen glacier, Pakistan, using sterile bottles following standard microbiological protocols and were transported on ice to Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad, within 24 h of sampling for further processing. pH of all the samples was neutral (7.0), whereas, temperature of sediments and water was 1°C and ice at -3°C.

Isolation of fungi

The general purpose fungal medium, Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) were used for the isolation of fungi. Isolation was carried out at two temperatures, 4°C and 15°C. After 4 weeks of incubation, colony forming units (CFU) were counted and expressed as CFU/mL for ice and melt water samples as well as CFU/g for the sediment sample.

Morphology

The colony morphology of pure fungal cultures was observed on SDA, with respect to their colony color, texture, shape etc. (front and reverse of the colony). Microscopy of the fungal isolates was done using lacto-phenol cotton blue staining method (40x).

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DNA extraction, sequencing and phylogenetic analysis

Fungal DNA extraction was performed according to protocol described earlier (Rosa et al., 2009). The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for amplification of ITS regions (ITS1-5.8S ITS2). The PCR conditions were: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, followed by 10 min final extension at 72°C. PCR products were run on agarose gel with DNA ladder to confirm the correct size of the gene. The PCR products were sent to Macrogen (Macrogen Inc. Seoul, Korea) for sequencing of 18S rRNA gene. The obtained sequences were analysed through Chromas Lite and were further examined by comparing the nucleotide sequences available in NCBI database (Thompson et al., 1997). The evolutionary history was inferred via the Maximum Likelihood method based on Tamura-Nei model (Tamura and Nei, 1993). The phylogenetic tree was created in MEGA software using Maximum Likelihood method (Tamura and Nei, 1993) at the bootstrap value 1000.

Evaluation of physiological parameters

Growth tolerance of all the fungal isolates to varying temperature (4 to 50°C), pH (pH 1 to 13) and salt concentration (NaCl up to 26%), was checked on SDA using 10 day old colonies, following incubation at 4°C and 15°C for 10 days.

Antimicrobial activity evaluation

Clinically isolated human pathogens (multi-drug resistant) such as *E. coli* (MDR), *Klebsiella pneumonia* (MDR), *Staphylococcus aureus* (MDR), *Staphylococcus* sp., *Enterococcus* sp. (VRE), *Candida albicans* and *Aspergillus niger* were used as target subjects. Point inoculation method was used for evaluation of antimicrobial activity. Using a sterile wire loop, a pure test microbial colony was transferred into the test tube containing normal saline and adjusted the turbidity with 0.5 McFarland solution as the standard. A sterile cotton swab was used to prepare homogenous lawn on Potato Dextrose Agar and Tryptic Soy Agar. A small portion of each fungal mycelium was inoculated on plates containing bacterial lawn.

Extracellular enzymes

Fungal isolates were screened for the production of extracellular enzymes including amylase, deoxyribonuclease, lipase, cellulose, protease and phosphatase according to protocols described by Hankin and Anagnostakis (1975) and Pikovskaya (1948). The isolates were screened for cellulolytic activity by using carboxymethylcellulose (CMC) as a substrate. For cellulolytic activity, the plates were flooded with 0.5% Congo red solution for 10 minutes, then washed with distilled water and flooded with 1 M NaCl. The clearing zone around the colony was observed. All qualitative extracellular enzyme activities were assayed at 4 and 15°C.

Results

In the current study, 17 fungal strains were isolated from all the three samples (glacial ice, water and sediments) of the Siachen glacier, Pakistan, by culturing at two

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temperatures 4°C and 15°C. The fungal CFU/g/mL in sediments was highest at both temperatures followed by water and ice (*Table 1*).

Table 1. *Total viable count (CFU/mL/g) of fungal isolates at 15°C and 4°C*

Temperature (°C)	Samples	CFU/mL/g
15	Glacier ice	3.0×10^{1}
	Glacier water	4.0×10^{1}
	Glacier sediment	3.75×10^{2}
4	Glacier ice	1.5×10^{1}
	Glacier water	2.0×10^{1}
	Glacier sediment	4.5×10^{2}

Morphological evaluation

Fungal isolates had different colony morphology, mostly were of tough and mucoid texture while powdery and cottony texture was also observed. The microscopic features of the fungal isolates were observed in terms of fruiting bodies, hyphal structure (i.e. branched or single hyphae, septate or aseptate), spore, spore shape (circular, oval, rod or others). The macroscopic and microscopic characteristics of different fungal isolates on the SDA are given in *Supplementary Table 1*.

Molecular characterization

Based on sequencing of the ITS regions (ITS1 – ITS4), all the fungal isolates were found to belong to varied taxonomic groups. The phylogenetic tree, describing evolutionary relationships among all fungal isolates is given in *Figure 1* and the resemblance index of strains with respective homology of the isolates is summarized in *Table 2*. Majority of the fungal isolates showed close similarity with the respective homologous species between 99-100% and only LS3 showed 97% similarity with *Thelebolus microspores*.

Physiological parameters

Optimum temperature for all the isolates was between 4 and 15°C but many fungal isolates showed growth up to 37°C while few were able to grow at 45°C as well but none of them displayed growth at 50°C (*Table 3*). However, there was less growth at 37 and 45°C. Fungal isolates exhibited growth at wide range of pH. Optimum pH for all fungal isolates was observed between 5 and 8. Most of the isolates were able to grow at pH 2-13, while 6 isolates could also grow at pH 1. Towards alkaline range, all the isolates tolerated pH up to 13. Salt tolerance of the fungal isolates was between 2 and 20% of NaCl. Based on these results, the isolates were considered as cold, pH and salt tolerants.

Antimicrobial activity evaluation

Fungal isolates exhibited good antibacterial activity as compared to antifungal activity (*Table 4*). Mostly, they exhibited antimicrobial activity against Gram positive bacteria (6 showed activity against *Staphylococcus* sp., 5 against *Staphylococcus aureus*, and 1 against

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Enterococcus sp.), while only 1 fungal isolates showed antifungal activity against *Candida albicans* and *Aspergillus niger*, respectively. None of the isolates exhibited antibacterial activity against Gram negative bacteria (*E. coli* and *Klebsiella pneumoniae*).

Table 2. *The resemblance index of strains with respective homology of the fungal isolates*

Isolates	Accession No.	Homologous species [accession number]	ID (%)	No of analysed bp
HS_1	KR676355	*Geomyces pannorum* |HQ703417.1|	100	488
HS_2	KR676356	*Leotiomycetes* sp |KC514892.1|	99	480
HS_3	KR676357	*Pueraria montana* |EF432795.1|	100	539
HS_4	KR676358	*Thelebolus microspores* |KM822751.1|	100	481
HS_5	KR676359	*Penicillium brevicompactum* |KF990149.1|	100	517
HS_6	KR676360	*Cladosporium uredinicola* |KM513616.1|	99	491
HS_7	KR676361	*Trichoderma viride* |DQ093772.1|	100	500
HS_8	KR676362	*Pueraria montana* |EF432796.1|	100	537
HS_9	KR676363	*Leotiomycetes* sp |KC514892.1|	99	480
LS_1	KR676364	*Leotiomycetes* sp |KC514892.1|	99	480
LS_2	KR676365	*Pueraria montana* |EF432796.1|	100	539
LS_3	KR676366	*Thelebolus microspores* |KM822751.1|	97	520
LS_4	KR676367	*Periconia* sp |KF907244.1|	99	492
LS_5	KR676368	*Thelebolus microspores* |KM822751.1|	99	512
LS_6	KR676369	*Leotiomycetes* sp |KC514892.1|	100	481
LS_7	KR676370	*Cryptococcus albidus* |KP131887.1|	99	532
LS_8	KR676371	*Thelebolus ellipsoideus* |KM816688.1|	100	490

Table 3. *Growth responses of the fungal isolates to temperature, pH and the salt*

Isolates	Temperature (°C) range	pH range	Salt range (%)
HS_1	4−37	2−13	2−16
HS_2	4−37	2−13	2−16
HS_3	4−37	2−13	2−18
HS_4	4−37	1−13	2−16
HS_5	4−37	1−13	2−20
HS_6	4−37	2−13	2−18
HS_7	4−37	2−13	2−18
HS_8	4−45	1−13	2−16
HS_9	4−37	2−13	2−16
LS_1	4−37	2−13	2−16
LS_2	4−37	1−13	2−16
LS_3	4−45	1−13	2−18
LS_4	4−37	1−13	2−10
LS_5	4−45	2−13	2−14
LS_6	4−37	2−13	2−18
LS_7	4−45	2−13	2−16
LS_8	4−37	2−13	2−14

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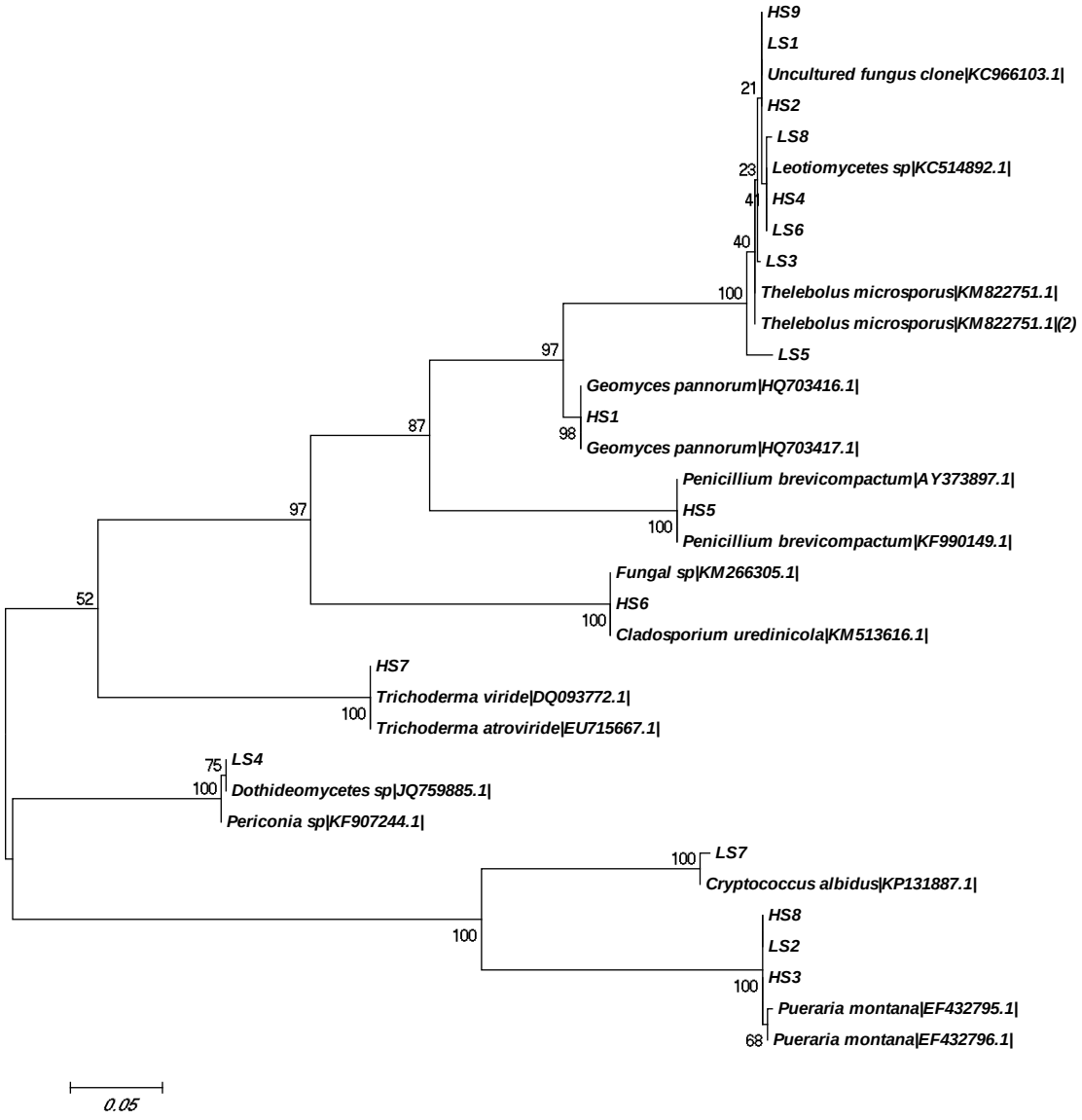

Figure 1. *Molecular Phylogenetic analysis by Maximum Likelihood method*

Extracellular enzyme production

The fungal isolates were good producers of lipase and cellulase. Out of 17 fungal species, 5 exhibited positive amylolytic activity, 12 showed cellulosic activities and only 2 isolates showed positive production for DNase (*Table 5*). While, 14 fungal isolates exhibited lipolytic activity, 5 isolates were found positive by exhibiting phosphate solubilizing activity and only 8 isolates showed proteolytic activity. The studies clearly demonstrated that fungal isolates were capable of producing a wide range of cold-active extracellular enzymes.

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Table 4. *Antibacterial and antifungal activity of the fungal isolates by point inoculation*

Isolates	Bacteria					Fungi	
	E. coli	***Klebsiella pneumoniae***	***S. aureus***	***Staphylococcus* sp.**	***Enterococcus* sp.**	***Aspergillus niger***	***Candida albicans***
HS_1	−	−	+++	+++	++	−	−
HS_2	−	−	+	++	−	−	−
HS_3	−	−	−	−	−	−	−
HS_4	−	−	−	−	−	−	−
HS_5	−	−	−	−	−	++	++
HS_6	−	−	−	−	−	−	−
HS_7	−	−	−	−	−	−	−
HS_8	−	−	−	−	−	−	−
HS_9	−	−	+	++	−	−	−
LS_1	−	−	+	++	−	−	−
LS_2	−	−	−	−	−	−	−
LS_3	−	−	−	−	−	−	−
LS_4	−	−	−	++	−	−	−
LS_5	−	−	−	−	−	−	−
LS_6	−	−	−	−	−	−	−
LS_7	−	−	++	++	−	−	−
LS_8	−	−	−	−	−	−	−

Key: (+++) Zone up to 7 mm, (++) Zone up to 14 mm, (+) Zone above 14 mm and (-) No Zone

Table 5. *Production of various extracellular enzymes by fungal isolates*

Isolates	Enzymes					
	Amylase	**Cellulase**	**DNase**	**Lipase**	**Phosphatase**	**Protease**
HS_1	−	+	−	++	−	−
HS_2	−	+	−	++	−	+
HS_3	−	−	−	+	−	−
HS_4	+	+	−	+	+	−
HS_5	+	−	+	−	+	−
HS_6	−	++	−	−	−	−
HS_7	−	+	−	+	−	+
HS_8	+	+	+	+	−	−
HS_9	−	+	−	++	−	+
LS_1	−	+	−	++	−	+
LS_2	−	−	−	+	−	−
LS_3	+	++	−	−	−	++
LS_4	−	+	−	+	+	+
LS_5	−	−	−	++	−	−
LS_6	+	+	−	+	+	−
LS_7	−	+	−	++	−	++
LS_8	−	−	−	++	+	++

Key: (+++) Zone up to 6 mm, (++) Zone up to 12 mm, (+) Zone above 12 mm and (-) No Zone

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Discussion

The main purpose of our study was to isolate and characterize psychrophilic fungi from Siachen glacier, Pakistan. The existence of psychrophilic fungi in this glacier has not been explored previously. In this study, 17 fungal strains were isolated at two temperatures 4°C (8 isolates) and 15°C (9 isolates) from glacial sediments (Damare, 2006), ice and water (Sati et al., 2014). After microscopic, morphological and molecular analysis (18S rRNA sequencing), it has been found that our fungal isolates belonged to 1 fungal genera, 1 family and 1 class. Major fungal isolates belonged to class *Leotiomycetes* followed by genus *Thelebolus* (Sonjak et al., 2006), *Pueraria* (Blanchette et al., 2010), *Penicillium*, *Cladosporium*, *Trichoderma*, *Periconia*, *Geomyces* and *Cryptococcus* (Maheswari, 2005).

The genus *Geomyces* (formerly known as *Chrysosporium pannorum*), frequently reported keratinophilic and psychrophilic fungus from Arctic, Alpine, temperate and Antarctic regions (Vishniac, 1996; Mercantini et al., 1989). In Antarctica, *G. pannorum* was isolated from thalli of seaweeds (Loque et al., 2010), as an endophyte (Rosa et al., 2010) and is associated with mosses (Tosi et al., 2002). According to Montemartini et al. (Montemartini et al., 1993), the genus *Thelebolus*, mainly *Thelebolus microsporus*, has been isolated as predominant genus from Arctic and Antarctic climate zones. The genus *Penicillium* has the ability to tolerate low temperature environments but in fact, many species demonstrated by their growth on food preserved in refrigerators (Pitt and Hocking, 1990) or are isolated from alpine, tundra (Domsch et al., 1980). *Penicillium* species have been identified from soils, lakes, historic woodlands and macroalgal thalli in Antarctical regions (Loque et al., 2010). In addition, *Cryptococcus* genus reported from soil from Southern Victoria Land and other locations in Antarctica (Adams et al., 2006; Thomas-Hall et al., 2002). The other genera (*Leotiomycetes*, *Cladosporium*, *Trichoderma*, *Periconia*) have been reported and isolated from various polar and non-polar cold habitats by other authors (Laura et al., 2013; Kostadinova et al., 2009).

In the present study, the fungal isolates showed great tolerance against different physiological parameters (temperature, pH and salt). The effects of pH on fungal growth were variable (from pH 1 to pH 13). Most of fungal isolates grow best over a pH range of 5-8. However, their growth was slow at pH extremes. Recca and Mrak (1952) and Battley and Bartlett (1966) found some of the fungal strains grown at pH 1.5 and pH 9. In addition, several fungi from cold habitats have been reported for their growth at both acidic and alkaline pH (Dhakar et al., 2014; Grzhimaylo et al., 2013). Most of the fungi were psychrotrophic in nature by growing at temperatures at 4-37°C. However, some of fungal isolates were able to grow outside this range i.e. at 45°C. Our results are supported by Zucconi et al. (1996), who isolated a thermotolerant-mesophilic fungal species from Victoria Land, Antarctica, having the ability to grow at 45°C. Azmi and Seppelt (1997) reported many fungal genera that show growth in between 4-35°C. The isolates in the present study showed growth up to 20% of NaCl thus showed halophilic nature. Kochkina et al. (2007) isolated a psychrophilic isolate of *Geomyces* from cryopegs. The isolate was capable of growth at up to 10% NaCl concentration. *Penicillium notatum* and *P. Chrysogenum* isolated from sandy soil of Al-Ain area, U.A.E, were reported to tolerate NaCl up to 20% (El-Mougith, 1993). Greiner et al. (2013) isolated different fungal strains from salt mine in Berchtesgaden, Bavaria, Germany. Among them, a new fungal species *Phialosimplex salinarum* was able to grow in the presence of 25% of salts.

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In this study, the fungal isolates were screened for their antibacterial and antifungal activities against clinically isolated bacterial and fungal human pathogens. Although, their bactericidal and fungicidal activities were not very effective but some of our isolates showed antimicrobial activity against Gram (+) bacterial and fungal strains. Fungi from cold habitats have not yet been reported against clinically isolated multi-drug resistant bacterial and fungal strains but fungi from other habitats have been extensively screened for this purpose and numerous antibiotics are being produced and commercially available. As the resistance against many antibiotics is increasing day by day, therefore new more effective antibiotics are the need of the day. Svahn et al. (2012) and Suay et al. (2000) have tested different filamentous fungi and yeasts against various human clinical pathogens (including MDR as well) and laboratory controls. Brunati et al. (2009) screened 160 filamentous fungi and 171 yeasts against bacterial and fungal human pathogens but none of them was MDR. It is evident from our results that MDR and resistant clinical isolates were inhibited. Metabolites from these fungal isolates can be characterized further.

Moreover, fungal isolates were checked for the production of extracellular enzymes. Generally, fungal isolates were good producers of lipase, protease and cellulase. Singh et al. (2012) has reported production of amylase, cellulase, phosphatase and pectinase enzymes at 4°C and 20°C from various filamentous fungi in Ny-Alesund, Spitsbergen. *Thelebolus microspore* has been found a good producer of amylase, lipase and chitinase enzymes from Larsemann Hills, Antarctica (Singh et al., 2014). Our results are also supported by Fenice et al. (1997) who screened 33 fungal strains for production of various extracellular enzymes, isolated from various sites of Victoria Land (continental Antarctica).

Conclusions

In our study, Siachen glacier was studied for the first time to look for the existence of fungi. Seventeen fungal isolates were isolated and identified through 18S rRNA sequencing. Majority of the fungal isolates belonged *Leotiomycetes,* followed by *Thelebolus*, *Penicillium*, *Cladosporium*, *Trichoderma*, *Periconia*, *Geomyces*, *Cryptococcus* and *Thelebolaceae* family. Some fungal isolates showed growth in the presence of 26% of salt, at pH 1 to 13 and at temperature 4°C to 45°C. Many isolates showed good antimicrobial activity and were good producers of industrially important enzymes.

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Authors' Contribution: Noor Hassan and Muhmmad Rafiq carried out all the research work, wrote the study design, drafted the manuscript and contributed equally to the research work, Shaukat Nadeem and Muhammad Hayat contributed in sampling, and experimental work, Aamer Ali Shah drafted the manuscript, Fariha Hasan wrote the study design, supervised research work and critically revised the manuscript. All authors read and approved the final manuscript.

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APPENDIX

Supplementary Table 1. *Colony morphology and microscopic characteristics of fungal isolates on SDA*

Isolate	Sample form	Temperature (°C)	Colony morphology		Microscopic characteristics
			Front	Reverse	
HS_1	Sediment	15	Cottony, initially yellow to green then turned to sea green with dim gray edges	Dark brown center with saddle brown edges	Hyphae hyaline to pale yellow and septate, scattered and erect conidiophores, and branched conidia
HS_2	Sediment	15	Cottony, initially dry mucoid, sandy brown then turned to pale goldenrod with light yellow margins	Saddle brown center and khaki edges	Spores are hyaline, *conidia* vary in shape and size, asci cylindrical shape.
HS_3	Ice	15	Mucoid, lemon chippon center with off-white margins	Off-white to yellow center and off-white margins	Round to ovoid shaped spores, branched or chain conidia and scattered
HS_4	Ice	15	Dry mucoid, deep burlywood center with off-white margins	Sandy brown to brown center and off-white margins	*Conidiophores* hyaline, septate hyphae, ovoid shaped conidia
HS_5	Ice	15	Velvety, initially dark olive green with light yellow edges then turned to black to dark green with white surface	Black center with Off-white edges	Branched, pale olivaceous brown hyphae, conidia ellipsoidal to limoni-form, smooth-walled or slightly verrucose, olivaceous brown

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HS_6	Water	15	Velvety, initially dark olive green with light yellow edges then turned to black to dark green	Black center with Off-white edges	Subglobose to broadly ellipsoid conidiophores, conidia are less branched and darker in nature
HS_7	Water	15	Cottony, initially white then surface turned to green	Off-white to yellow center with dark green margins	Phialides straight or sinuous and globose to subglobose chlamydospores
HS_8	Water	15	Mucoid, lemon chippon center with off-white margins	Off-white to yellow center and off-white margins	Spores are round to ovoid shaped, branched or chain hyphae and scattered
HS_9	Water	15	Cottony, initially dry mucoid, sandy brown then turned to pale goldenrod with light yellow margins	Saddle brown center and khaki edges	Spores are hyaline, *conidia* vary in shape and size, asci cylindrical shape.
LS_1	Sediment	4	Cottony, initially mucoid, brown then turned to goldenrod with light yellow margins	Brown center and khaki edges	Spores are hyaline, *conidia* vary in shape and size, asci cylindrical shape.
LS_2	Sediment	4	Mucoid, lemon chippon center with off-white margins	Off-white to yellow center and off-white margins	Round to ovoid shaped spores, branched or chain conidia and scattered

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LS_3	Sediment	4	Mucoid, light goldenrod yellow center with off-white margins	Off-white to yellow center and off-white margins	Ellipsoid to cylindrical ascospores, hyphae bundantly septate, branched, rich in oleaginous globules
LS_4	Ice	4	Cottony, deep brown center with white margin	Black to brown center and light brown margins	Conidia are spherical to globose shaped, septate hyphae and conidial heads are globose to ovoid
LS_5	Ice	4	Mucoid, pale goldenrod center with off-white edges	Off-white to yellow center and off-white margins	Ellipsoid shaped ascospores, hyphae septate, branched, rich in oleaginous globules
LS_6	Ice	4	Dry mucoid, deep burlywood center with off-white margins	Sandy brown to brown center and off-white margins	*Conidiophores* hyaline, septate hyphae, ovoid shaped conidia
LS_7	Water	4	Mucoid, salmon center and off-white margins	Off-white to yellow center and off-white margins	Spores are globose to ovoid shape, no true or hyphae pseudohyphae observed
LS_8	Water	4	Mucoid, dark salmon center and off-white edges	Burlywood center with off-white margins	Hyaline and septate hyphae, round to ovoid shaped spores and in scattered form

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