COMMUNITY ANALYSIS AND CHARACTERIZATION OF FUNGI FROM BATURA GLACIER, KARAKORAM MOUNTAIN RANGE, PAKISTAN

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Abstract. Karakoram mountain range contains the tallest mountain peaks of the world with thousands of glaciers which are microbiologically untapped. This is a pioneer approach of isolation and characterization of fungi from Batura glacier, Karakoram, Pakistan. Total number (CFU/mL or g) was determined at 4 °C and 15 °C to isolate psychrophilic and psychrotrophic fungi, respectively. About 33 different fungi were isolated from sediment (29), ice (2) and water (2) and were identified morphologically and by sequencing of specific internal transcribed spacer region of the species through internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2) primers. Mostly, the fungal isolates belonged to genus *Penicillium*, followed by *Cladosporium*, *Geomyces*, *Cordyceps*, *Mrakia*, *Cadophora*, *Tetracladium*, *Eupenicillium*, *Trametes*, *Mortierella*, *Scopulariopsis*, *Beauveria*, *Candida* and *Pseudogymnoascus*. They showed growth at wide temperature range from 4–37 °C, few at 45 °C as well. Most of the isolates were able to grow at pH 1–13. Fungal isolates were able to grow in 2–26% NaCl, with the highest tolerance (26%) by *Mrakia robertii*. All study isolates showed inhibitory activity against *Staphylococcus* sp., and four industrially important enzymes (cellulase, DNase, lipase and phosphatase) were produced by *Mrakia robertii.* The fungal isolates of such cold habitats are potential sources of various industrial and environmental application.
Keywords: *non-polar glaciers, diversity, psychrotrophs, antimicrobial activity, industrial applied enzymes*

Introduction

Psychrophiles are known as cryophile or 'cold-loving' organisms (Margesin et al., 2007) having ability to grow and survive in cold environment. According to Morita (Morita, 1975), psychrophiles grow and survive below 20 °C providing 15 °C as optimum growth temperature. However, the psychrophiles in cold environment are also accompanied by psychrotrophs. The psychrotrophs possess ability to grow below and above 20 °C but their growth optimum has been observed beyond 20 °C (Baross and Morita, 1978; Cavicchioli et al., 2002; Gounot, 1986). Psychrotrophic community have been described as "the survivor's community" by Friedmann (1994) because of their survival strategies and their normal physiological state under low temperature (Morita, 2000).

Psychrophilic and psychrotrophic fungi have widely been studied for their presence in several cold environments as reviewed by Hassan et al. (2016) such as Arctic and Antarctic region (Azmi and Seppelt, 1997; Babjeva and Reshetova, 1998; Selbmann et al., 2005; Vishniac, 2006). Furthermore, different cold environments including permafrost (Broady et al., 1998), cold water (Botha and Wolfaardt, 2000; Dmitriev et al., 1997), ice

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in glacial habitats (Ma et al., 1999), snow and below snow-covered tundra (Schadt, 2000), polar water present in off shore area (Broady et al., 1998) and massive glacial sheets of ice and ice shelves freshwater (Bridge, 2010; Tojo and Newsham, 2012) have been investigated for cold adapted fungi. Limited research data is available on the fungal diversity and characterization in non-polar regions such as Hindukush, Karakoram, and Himalayas (HKKH). A proper investigation was needed for existence of cold adapted and psychrophilic life (microorganisms) in the HKKH glaciers.

The rate of discovery of new antibiotics is very slow as compared to emerging of antibiotic resistant pathogens in the current era. Treatment of several pathogens are now difficult due to dearth of operative antibiotics, e.g. Enterobacteriaceae bacteria producing extended spectrum beta-lactamase (ESBL), *Enterococci* sp. with vancomycin resistant abilities and *Staphylococcus aureus* with methicillin-resistant capabilities. It is important to explore extreme habitats for new antibiotics having diverse and unique characteristics. There is many diverse type of extreme environments however cold habitats could be among important sources for the discovery of novel antibiotics. Therefore, we need to investigate novel antibiotics from extreme and unexplored sites against both multi-drug resistant bacteria and fungi.

Pakistan has one of the world's largest glacier reserves in the Karakoram–Hindu Kush–Himalaya ranges. According to Rahman et al. (2008), the junction of the Karakoram Western Himalayan and Hindu Kush mountain ranges are accompanied by the Northern Areas of Pakistan. Batura glacier considered as the largest glaciers located exterior the polar region. It is present in the north of Passu 7,500 m above sea level. Batura glacier has not been explored yet for the presence of psychrophilic and psychrotrophic fungi. Isolation and characterization of the psychrophilic and psychrotrophic fungal community from samples of glacier (ice sediments and water) taken from Batura glacier was the ambition of the current research.

Materials and methods

Sampling

The length of Batura glacier is 57 km with a termination point of ~3000 m altitude in the valley of Hunza, Pakistan, given in *Figure 1*. Several highlighted peaks of Karakoram (>7000 m in south and >5500 m in the north) surrounded the Batura glacier. Ice, water and sediments samples were taken from Batura glacier (36°30.302N to 074°51.138E) following standard microbiological procedures. Approximately 200–300 g sediments were taken using sterilized gloves and scoops to avoid sample contamination. Nasco Whirl–Pak™ bags (Fisher Scientific) were used to place individual sample (for sediments) and sterilized polyethylene bottles (for ice and water samples). The pH of all samples were 7.0 whereas temperature of glacial sediments and water were 1 °C while glacial ice had –2 °C. All the collected samples were kept at 4 °C until transported to Department of Microbiology Quaid-i-Azam University Islamabad and stored at –20 °C.

Isolation of fungi

For isolation of fungi Sabouraud Dextrose Agar (Sigma – Aldrich) (g/L; 10% mycological peptone 40% dextrose 15% Agar) Potato Dextrose Agar (Sigma – Aldrich) (g/L; 4% potato extract 20% dextrose 15% agar) and Malt Extract Agar (Sigma – Aldrich) (g/L; 30% malt extract 10% mycological peptone 15% Agar) were used as

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growth medium. Incubation temperatures were set at 4 °C and 15 °C. Total viable counts were determined in terms of fungal colony forming units (CFUs/mL or /g) and morphologically distinct colonies were further subcultured to get the pure cultures. Potato Dextrose Agar (PDA) slants were used for preservation of fungal isolates at 4°C and also under mineral oil till further use.

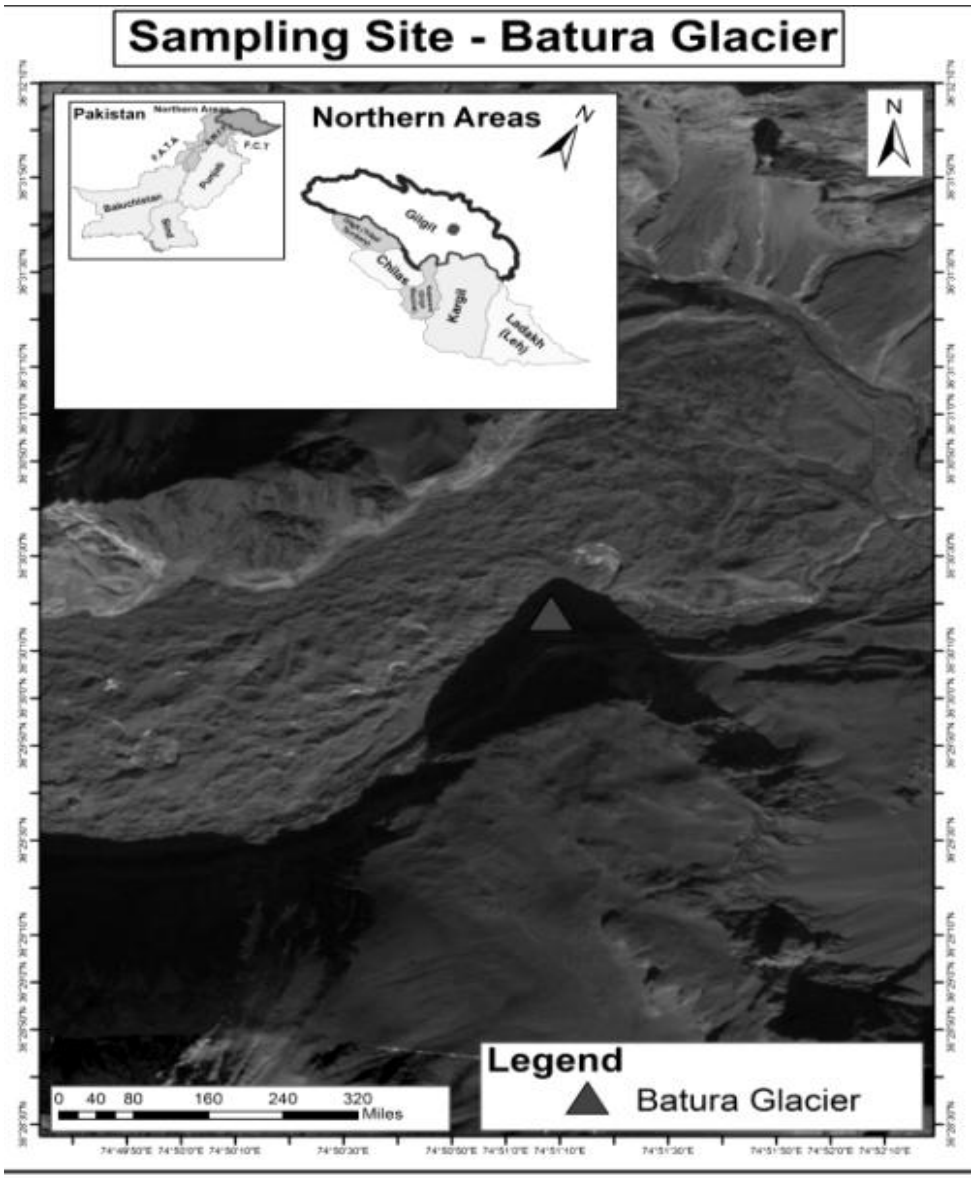

Figure 1. *Location of Batura glacier*

Morphological characterization

Different media such as PDA, Tryptic Soy Agar (Sigma – Aldrich) (g/L; 15% casein peptone 5% soya peptone 5% sodium chloride 15% agar) Sabouraud Dextrose Agar (SDA) and Malt Extract Agar (MEA) was used as growth media for fungal isolates. Color, texture and size (both front and reverse sides) were observed and recorded at 4 C and 15 C. Microscopic examination of fungal isolates was carried out following the protocol of Hassan et al. (2017).

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Physiological characterization

All the physiological parameters were measured using SDA, MEA and PDA. Cultures were grown for 10 days. Diverse temperatures from 4 to 50 C (4, 15, 37, 45, and 50 C) were used to determine temperature optima of the fungal isolates. pH tolerance of the fungal isolates was determined by inoculating them separately in a medium adjusted to varying pH values (1, 3, 5, 7, 9, 11 and 13). For determination of halophilic nature of the fungal isolates, SDA complemented with NaCl up to 26% was used. All physiological examinations were carried out at 4 °C and 15°C.

DNA extraction and amplification

Rosa et al. (2009) DNA extraction protocol was used to extract the DNA of all the fungal isolates. The extracted DNA was amplified using internal transcribed spacer 1 (ITS1) – 5.8S – internal transcribed spacer 2 ITS2 region prior to sequencing following the protocol described by Hassan et al. (2016). T100™ Thermal Cycler (Bio Rad) was used for PCR amplification. Agarose gel electrophoresis was used to confirm PCR products *using standard DNA ladder.*

Phylogenetic analysis

Sequencing was done commercially by Macrogen (Macrogen Inc. Seoul Korea) and the sequences obtained were analysed by Chromas Lite, while further evaluation was done using National Center for Biotechnology Information (NCBI) database by comparing with available nucleotide sequences (Thompson et al., 1997). The GenBank sequence database was used to compare the obtained FASTA sequences to data using BLASTn (Altschul et al., 1990; Zhang et al., 2000). Accession no. of all fungal isolates were obtained by depositing sequences to National Centre for Biotechnological Information (NCBI). Tamura–Nei model of Maximum Likelihood method used to infer evolutionary history of fungal isolates (Tamura and Nie, 1993). MEGA6 software following maximum likelihood method has been used to construct phylogenetic tree (Tamura et al., 2013) at the bootstrap value of 1,000.

Evaluation of antimicrobial activity

Antimicrobial activity of isolated fungal isolates was checked against *Staphylococcus aureus* (Multi-drug resistant), *E. coli* (MDR), *Staphylococcus* sp., *Klebsiella pneumoniae* (MDR), vancomycin resistant *Enterococcus* species and fungal isolates *Candida albicans* and *Aspergillus niger* following the protocol described by Hassan et al. (2017).

Screening for extracellular enzyme activity

Extracellular enzyme activity of all fungal isolates was determined on solid media. Ten days old fungal cultures were used as inoculum. Hankin and Anagnostakis (1975) protocol was used to determine amylase, deoxyribonuclease, lipase and protease activities. The phosphatase activity was determined on Pikovskaya's medium (Pikovskaya, 1948). Carboxymethylcellulose (CMC) was used as a substrate for screening cellulolytic activity of all fungal isolates. 0.5% Congo red solution and 1 M NaCl were used to visualize zones of inhibition around fungal colonies formed due to cellulolytic activity. 15 °C were used for all assays of qualitative extracellular enzyme activities.

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Results

33 fungal isolates were isolated in this study. The highest CFU/g/mL was observed in sediments ($1.11 \times 10^3$and 7.75×10^2 at 4 and 15 °C, respectively) and given in *Table 1*.

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

Temperature (°C)	Samples	No. of colonies/200 µL	CFU/mL/gm
4	Glacier ice	2	1.0×10^1
	Glacier water	3	1.5×10^1
	Glacier sediment	2.2×10^2	1.11×10^3
15	Glacier ice	7	3.5×10^1
	Glacier water	4	2.0×10^1
	Glacier sediment	1.55×10^1	7.75×10^2

Morphology

Colony morphology of all the fungal isolates were different, mostly they were of cottony to powdery textures irregular shapes and sizes and of blue to green in color. The macroscopic and microscopic features of the isolated fungal strains are specified in *Table A1* in the *Appendix*. The hyphae were septate colorless and spores were mostly cylindrical to ovoid in shape.

Molecular characterization

On the basis of DNA sequence of internal transcribed spacer region by (ITS1 – ITS2), all isolated fungal isolates have shown diverse groups of taxonomy. The evolutionary relationships, respective homology and resemblance directory of the isolated fungal strains with narrowly correlated species of fungi as well as their accession no. is summarized in *Table 2* and *Figure 2*.

Physiological characterization

Temperature tolerance of the fungal isolates showed variable results. All were capable to grow between 4 and 37 °C. Two isolates, HB_3 and LB_{13} were capable to grow above 37°C (*Table 3*). However, 4 and 15 °C temperatures were found to be optimum growth temperature values, except for HB_2, HB_7 and HB_{14} in the case of which the optimum growth temperature was 37 °C. The growth pattern of the fungal isolates at varying pH (1, 3, 5, 7, 9, 11 and 13) was very broad. The optimum pH for growth was found to be between 4 and 9. Most of the isolates (79%) grew in acidic range, 7 could not grow at pH 1 while all other isolates showed growth at the lowest pH. All fungal isolates have survived at extreme alkaline pH up to 13. The fungal isolates have tolerated salt concentration between 2 and 26% (33 between 2 and 14% 32 at 16% 24 at 18% 17 at 20% 11 at 22 and 24% and 1 showed growth at 26% salt concentration), given in *Table 3*. Based on such results, the fungal isolates could be categorized as polyextremophiles.

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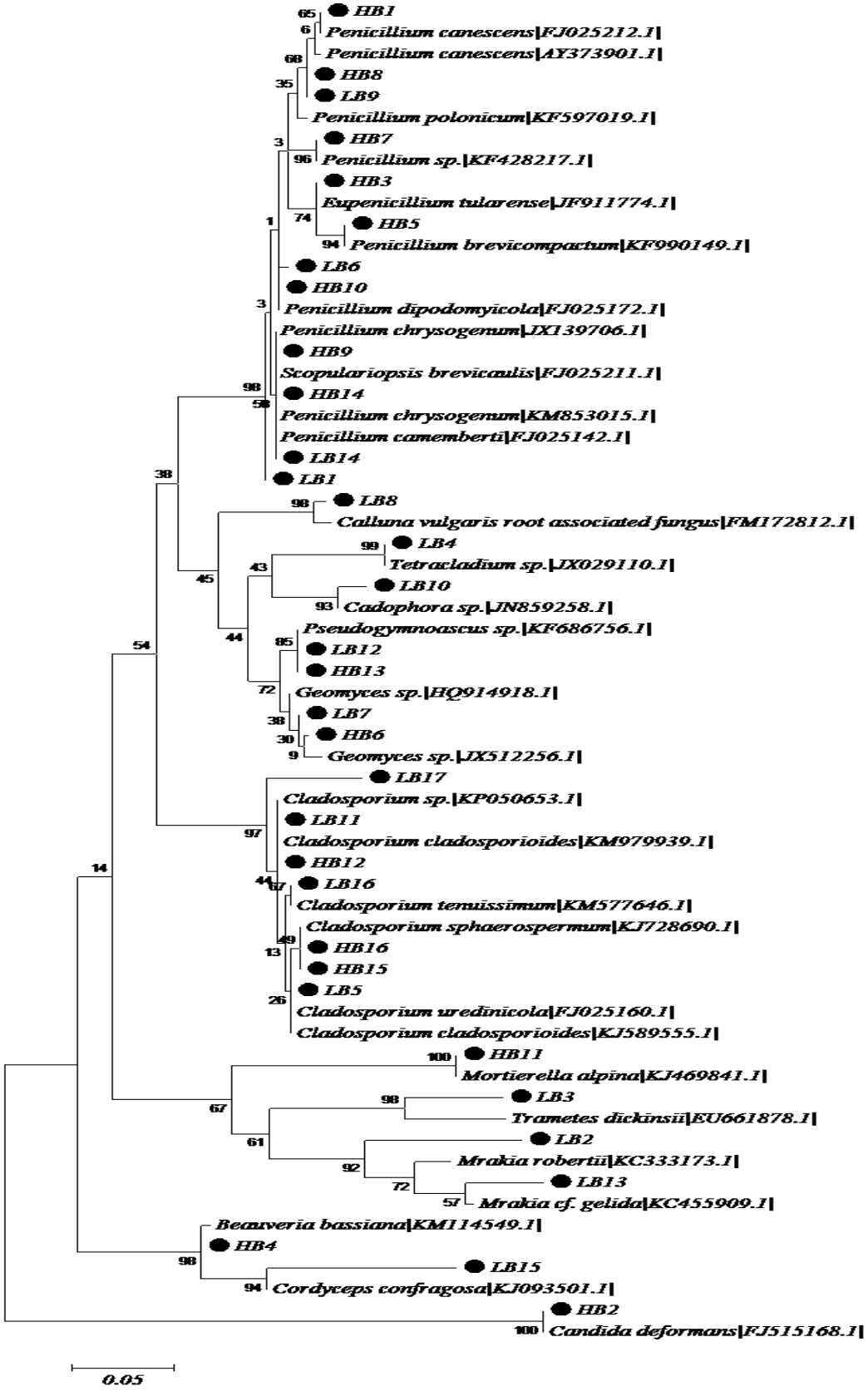

Figure 2. *Phylogenetic tree by Maximum likelihood method of the study isolates and homologous ITS data set from NCBI*

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Table 2. *The resemblance directory of the fungal isolates with respect to homologous strains*

Isolates	Accession no.	Homologous strains with accession no.	Identity (%)
LB_1	KR0(19737	*Penicillium camemberti* (FJ025142.1)	99
LB_2	KR0(19738	*Mrakia robertii* (KC333173.1)	84
LB_3	KR0(19739	*Trametes dickinsii* (EU661878.1)	87
LB_4	KR0(19740	*Tetracladium* sp. (JX029110.1)	100
LB_5	KR0(19741	*Cladosporium uredinicola* (FJ025160.1)	99
LB_6	KR0(19742	*Penicillium polonicum* (KF5970(19.1)	97
LB_7	KR0(19743	*Geomyces* sp. (HQ914918.1)	99
LB_8	KR0(19744	Calluna vulgaris root associated fungus (FM172812.1)	99
LB_9	KR0(19745	*Penicillium canescens* (AY373901.1)	100
LB_{10}	KR0(19746	*Cadophora* sp. (JN859258.1)	99
LB_{11}	KR0(19747	*Cladosporium cladosporioides* (KM979939.1)	100
LB_{12}	KR0(19748	*Geomyces* sp. (JX512256.1)	99
LB_{13}	KR0(19749	*Mrakia cf. gelida* (KC455909.1)	99
LB_{14}	KR0(19750	*Penicillium chrysogenum* (JX139706.1)	99
LB_{15}	KR0(19751	*Cordyceps confragosa* (KJ093501.1)	91
LB_{16}	KR0(19752	*Cladosporium tenuissimum* (KM577646.1)	100
LB_{17}	KR0(19753	*Cladosporium cladosporioides* (KJ589555.1)	95
HB_1	KR0(19754	*Penicillium canescens* (FJ025212.1)	99
HB_2	KR0(19755	*Candida deformans* (FJ515168.1)	100
HB_3	KR0(19756	*Eupenicillium tularense* (EU142874.1)	99
HB_4	KR0(19757	*Beauveria bassiana* (KM114549.1)	100
HB_5	KR0(19758	*Penicillium brevicompactum* (KF990149.1)	99
HB_6	KR0(19759	*Geomyces* sp. (HQ914918.1)	99
HB_7	KR0(19760	*Penicillium* sp. (KF428217.1)	100
HB_8	KR0(19761	*Penicillium canescens* (AY373901.1)	100
HB_9	KR0(19762	*Scopulariopsis brevicaulis* (FJ025211.1)	100
HB_{10}	KR0(19763	*Penicillium dipodomyicola* (FJ025211.1)	100
HB_{11}	KR0(19764	*Mortierella alpine* (KJ469841.1)	100
HB_{12}	KR0(19765	*Cladosporium* sp. (KP050653.1)	100
HB_{13}	KR0(19766	*Pseudogymnoascus* sp. (KF686756.1)	100
HB_{14}	KR0(19767	*Penicillium chrysogenum* (KM853015.1)	100
HB_{15}	KR0(19768	*Cladosporium sphaerospermum* (KJ728690.1)	99
HB_{16}	KR0(19769	*Cladosporium sphaerospermum* (KJ728690.1)	99

LB: Low (Temperature, 4 °C) Batura Isolates, HB: High (Temperature, 15 °C) Batura Isolates

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Table 3. *Temperature, pH and salt tolerance range of the fungal isolates. (Numbers between brackets are the optimal growth values)*

Isolates	Temperature (°C) range	pH range	Salt range (%)
LB_1	4−37, opt. 4	1−13, opt. 5–8	2−24, opt. 2–8
LB_2	4−37, opt. 4	1−13, opt. 5–7	2−26, opt. 2–10
LB_3	4−37, opt. 4	1−13, opt. 5–7	2−16, opt. 2–6
LB_4	4−37, opt. 4	1−13, opt. 5–8	2−18, opt. 2–6
LB_5	4−37, opt. 4	1−13, opt. 5–8	2−16, opt. 2–6
LB_6	4−37, opt. 4	1−13, opt. 5–7	2−24, opt. 2–8
LB_7	4−37, opt. 4	2−13, opt. 5–7	2−16, opt. 2–6
LB_8	4−37, opt. 4	1−13, opt. 5–8	2−16, opt. 2–6
LB_9	4−37, opt. 4	1−13, opt. 5–8	2−18, opt. 2–6
LB_{10}	4−37, opt. 4	1−13, opt. 5–8	2−16, opt. 2–6
LB_{11}	4−37, opt. 4	1−13, opt. 5–7	2−18, opt. 2–6
LB_{12}	4−37, opt. 4	1−13, opt. 5–7	2−16, opt. 2–6
LB_{13}	4−45, opt. 4	1−13, opt. 5–7	2−18, opt. 2–6
LB_{14}	4−37, opt. 4	1−13, opt. 5–7	2−18, opt. 2–6
LB_{15}	4−37, opt. 4	1−13, opt. 5–8	2−16, opt. 2–6
LB_{16}	4−37, opt. 4	1−13, opt. 5–8	2−16, opt. 2–6
LB_{17}	4−37, opt. 4	1−13, opt. 5–8	2−20, opt. 2–6
HB_1	4−37, opt. 15	2−13, opt. 5–7	2−24, opt. 2–10
HB_2	4−37, opt. 15	1−13, opt. 5–8	2−20, opt. 2–8
HB_3	4−37, opt. 15	2−13, opt. 5–7	2−24, opt. 2–10
HB_4	4−37, opt. 15	1−13, opt. 5–8	2−20, opt. 2–8
HB_5	4−37, opt. 15	2−13, opt. 5–7	2−24, opt. 2–10
HB_6	4−37, opt. 15	1−13, opt. 5–8	2−14, opt. 2–6
HB_7	4−37, opt. 15	1−13, opt. 5–8	2−24, opt. 2–10
HB_8	4−37, opt. 15	2−13, opt. 5–7	2−18, opt. 2–8
HB_9	4−37, opt. 15	1−13, opt. 5–8	2−20, opt. 2–8
HB_{10}	4−37, opt. 15	1−13, opt. 5–8	2−24, opt. 2–8
HB_{11}	4−37, opt. 15	2−13, opt. 5–7	2−18, opt. 2–8
HB_{12}	4−37, opt. 15	2−13, opt. 5–7	2−24, opt. 2–8
HB_{13}	4−37, opt. 15	2−13, opt. 5–7	2−24, opt. 2–10
HB_{14}	4−37, opt. 15	2−13, opt. 5–7	2−20, opt. 2–8
HB_{15}	4−37, opt. 15	1−13, opt. 5–7	2−20, opt. 2–8
HB_{16}	4−37, opt. 15	1−13, opt. 5–8	2−20, opt. 2–8

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Evaluation of antimicrobial activity

Screening of the fungal isolates for antimicrobial activity showed greater antibacterial than antifungal activities and summarised in *Table 4*. Five fungal isolates exhibited inhibitory activity against *Staphylococcus aureus*, 21 showed such activity against *Staphylococcus* sp., 3 against *Enterococcus* sp., 2 against *E. coli* but none showed antimicrobial activity against *Klebsiella pneumoniae*. The number of fungal isolates that displayed inhibitory activity against *Candida albicans* and *Aspergillus niger* were 10 and 4, respectively.

Table 4. *Production of various extracellular enzymes by the fungal isolates*

Isolates	Enzymes					
	Amylase	**Cellulase**	**DNase**	**Lipase**	**Phosphatase**	**Protease**
LB_1	−	−	−	−	+	−
LB_2	−	+	+	+	+	−
LB_3	−	−	−	−	−	−
LB_4	−	−	−	+	−	−
LB_5	−	−	−	−	−	−
LB_6	++	+	−	−	+	−
LB_7	++	++	−	++	−	−
LB_8	−	−	−	−	−	−
LB_9	−	−	−	−	+	−
LB_{10}	++	−	−	−	−	−
LB_{11}	−	−	−	−	−	−
LB_{12}	−	−	−	++	−	−
LB_{13}	+	−	−	−	−	−
LB_{14}	−	+	−	−	+	−
LB_{15}	−	−	−	−	+	−
LB_{16}	++	−	−	−	−	++
LB_{17}	−	−	−	−	−	−
HB_1	−	−	−	−	+	−
HB_2	−	−	−	−	−	−
HB_3	−	−	−	++	+	−
HB_4	−	−	−	−	++	−
HB_5	+	−	−	−	+	−
HB_6	−	−	−	++	−	−
HB_7	−	−	−	++	+	−
HB_8	−	++	−	+++	−	−
HB_9	−	+++	−	−	+	−
HB_{10}	−	++	−	−	+	−
HB_{11}	−	−	−	−	−	−
HB_{12}	−	−	−	−	−	−
HB_{13}	−	−	−	−	−	−
HB_{14}	−	−	−	−	−	−
HB_{15}	−	−	−	−	−	−
HB_{16}	−	−	−	−	−	−

(–) No Zone, (+) Zone up to 6 mm, (++) Zone up to 12 mm, (+++) Zone above 12 mm

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Screening for extracellular enzyme activity

In this study, maximum number of fungal isolates were found to produce phosphate (13 fungal isolates) followed by lipases (10), cellulases (7), amylases (6), DNases (1) and proteases (1) and summarized in *Table 5*. They were good in lipase production but were very poor in the production of DNase and protease. This research openly revealed that fungal isolates could be a good source of industrially important biocatalysts.

Table 5. *Screening for antimicrobial compounds against different bacteria and fungi*

Isolates	Bacteria					Fungi	
	E. coli	***Klebsiella pneumonie***	***Staph. Aureus***	***Staph.* sp.**	***Enterococcus* sp.**	***Aspergillus niger***	***Canda albicans***
LB_1	–	–	–	++	–	–	+
LB_2	–	–	–	–	++	–	–
LB_3	–	–	–	+++	–	–	++
LB_4	+	–	–	+++	–	–	+
LB_5	–	–	–	+++	–	–	–
LB_6	–	–	–	–	–	–	–
LB_7	–	–	–	+++	–	–	–
LB_8	–	–	–	+++	++	–	+
LB_9	–	–	+	+	–	++	++
LB_{10}	–	–	–	+++	–	–	–
LB_{11}	–	–	–	+++	–	–	+
LB_{12}	–	–	–	++	–	–	–
LB_{13}	–	–	–	+++	–	–	–
LB_{14}	–	–	–	+++	–	–	++
LB_{15}	–	–	++	++	–	–	+
LB_{16}	–	–	–	++	–	–	–
LB_{17}	–	–	–	–	–	–	–
HB_1	–	–	–	–	–	++	–
HB_2	–	–	–	+++	–	–	–
HB_3	–	–	–	–	–	–	–
HB_4	–	–	++	+	–	–	–
HB_5	–	–	+++	–	++	+	–
HB_6	–	–	–	–	++	–	–
HB_7	–	–	–	+	–	–	–
HB_8	–	–	++	–	–	++	+
HB_9	–	–	–	–	–	–	–
HB_{10}	–	–	–	++	–	–	–
HB_{11}	–	–	–	+++	–	–	–
HB_{12}	++	–	–	+++	–	–	–
HB_{13}	–	–	–	+++	–	–	–
HB_{14}	–	–	–	–	–	–	–
HB_{15}	–	–	–	–	–	–	–
HB_{16}	–	–	–	–	–	–	–

(–) No Zone, (+) Zone up to 8 mm, (++) Zone up to 16 mm, (+++) Zone above 16 mm

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Discussion

The main objective of this research was to isolate and characterize the psychrotrophs and in the current study, a combination of morphological analysis and molecular methods were carried out for the identification of the fungal isolates. Out of 33 fungal isolates, four isolates LB_2 LB_3 LB_{15} and LB_{17} showed a similarity index up to 84, 87, 91 and 95% respectively and their sequences are deposited in database and to determine their taxonomic affiliation further investigations are needed. The fungal isolates were found to belong to mostly genus *Penicillium* (10) followed by *Cladosporium* (7) *Geomyces* (3) *Cordyceps* (1) *Mrakia* (2) *Cadophora* (1) *Tetracladium* (1) *Trametes* (1) *Mortierella* (1) *Scopulariopsis* (1) *Beauveria* (1) *Candida* (1) *Eupenicillium* (1) and *Pseudogymnoascus* (1).

The members of the genus *Geomyces,* are keratinophilic, psychrophilic and psychrotolerant in nature as have been reported by Blehert et al. (2009) and Arenz et al. (2011) from Antarctica and Arctic habitats as well as characterized as halotolerant and moderately cellulolytic. Fell and Stalzell-Tallman (1998) and Margesin et al. (2005) have earlier been documented *Mrakia* species with obligate psychrophilic nature from Antarctica, Alpine and Arctic habitats. Fungal species isolated in the present study that related to *Penicillium*, Candida, *Cladosporium*, *Pseudogymnoascus* and *Cadophora* have previously been reported in Antarctica and Alpine habitats (Burgaud et al., 2010; Dhakar et al., 2014; Duncan et al., 2006; Goncalves et al., 2012; Wang et al., 2015).

Tetracladium species have been found in alpine glaciers and snow-covered soil therefore considered to be cold-adapted (Kuhnert et al., 2012; Robinson et al., 2000). *Mortierella* species have been reported from Antarctic soil and mosses (Bridge and Newsham, 2009). The fungal species *Eupenicillium tularense Beauveria bassiana* and *Scopulariopsis brevicaulis* have not been reported from the low temperature habitats previously these species have been isolated for the first time from non-polar glacier in this study. Although *Trametes* sp. and *Cordyceps* sp. have been found for the first time in non-polar glaciers in this study but as the isolates showed low similarity (87% and 91% respectively) which most probably belong to genera other than *Trametes* and *Cordyceps* because they have homology less than 97% (Goncalves et al., 2012).

Batura glacier is one of the coldest non-polar glacier having very low temperature often below freezing point (–2 °C). In this study fungal isolation from such low temperature habitat indicate the possible role of the spores in their survival. Spores facilitate the existence of both bacteria and fungi in extreme environments as they protect them from UV-radiation and DNA damages (Ma et al., 2000). According to Robinson (2001), fungal persistence may possible due to cold circumvention rather than cold tolerance in Arctic and Antarctic habitats. Vishniac (1996) believed that spores helped more fungi to endure freezing than vulnerable hyphomycete hyphae. Catranis and Starmer (1991) and Ma et al. (2000) have isolated several of fungal isolates up to 140,000 years old from ice cores of Greenland. These studies show that fungal spores are central to survival of fungi in cold environments.

In the current study, the fungal isolates survived and grown at various extreme physiological parameters including temperature, NaCl and pH. Based on such results, fungal isolates grouped as psychrotolerants, halotolerants and thermotolerants as they tolerated broad range of temperature i.e. low and high temperature (from 4 to 45 °C) and the highest NaCl concentration (up to 26%). The isolates are highly diverse in terms of temperature requirement. They showed growth at lower temperature range (4–15 °C) which is a key property of psychrophilic organisms but they also showed growth at

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higher temperatures. The ability of fungal isolates to grow at low and high temperature and high concentrations of salt possibly indicated mechanisms involved in the protection against such stressful conditions. Production of polyols, lipid/fatty acids and antifreeze proteins, etc. by cold adapted microbes are central mechanisms to overcome stressful conditions (Robinson et al., 2001). In this study the isolate *Mrakia cf. gelida* was capable of surviving at 45 °C and this was the first report of *Mrakia cf. gelida* from the glacier habitat that shown such a thermophilic nature because members of genus *Mrakia* have been reported as of psychrophilic nature (Xin and Zhou, 2007). Maheshwari[46] defined thermophilic fungi as they are able to grow between 20 and 55 °C. Our results are supported by Zucconi et al. (1996), who isolated thermotolerant-mesophilic fungi from Victoria Land Antarctica that was able to grow at 45 °C.

Fungal isolates showed growth on wide range of pH (1–13) which is a remarkable discovery of current research. Generally, acidic environment favors growth of fungi. On the other hand, the isolated fungal strains tolerated diverse salt concentrations (2–26%). Several fungal isolates showed growth at 24% (NaCl), whereas 1 of the fungal isolates could grew at 26% salt concentration as well. According to existing literature, many of the fungal isolates representing different genera isolated from the low temperature environments (*Cladosporium Cordyceps Mrakia Cadophora Tetracladium Trametes, Mortierella, Scopulariopsis, Beauveria, Candida, Eupenicillium* and *Pseudogymnoascus*) were characterized for the first time at such an extreme pH and salt conditions in the present study. However, species of the genera *Penicillium, Geomyces* and a few others (isolated from cold and other habitats) have been observed to grow at both high and low pH as well as the highest salts concentrations (Dhakar et al., 2014; Eliades et al., 2006; Grum-Grzhimaylo et al., 2013; Kochkina et al., 2007).

Secondary metabolites production by fungal isolates most importantly represented by antimicrobial activity have also been investigated in this study. Previously many fungal isolates have been found to produce secondary metabolites with bactericidal and fungicidal activity. In our study, fungal isolates exhibited decent antimicrobial activities against genus *Staphylococcus* as compared to other bacterial fungal genera. Screening of fungi from cold habitats against clinical bacterial isolates and fungal strains has not yet been reported. However, Brunati et al. (2009) screened more than 150 fungi and 165 yeasts (benthic mats of Antarctic lakes) against bacterial and fungal human pathogens from Merck Culture Collection (MB MY) and American Type Culture Collection (ATCC) including *Staphylococcus aureus, Enterococcus faecium, Escherichia coli, Moraxella catarrhalis, Pseudomonas aeruginosa, Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans* but they were not found to be multi-drug resistant. A total of 47 (29%) filamentous fungi repressed growth of *Escherichia coli* (10%), *Staphylococcus aureus* (14%) and *Cryptococcus neoformans* (8%) *Candida albicans* (11%).

Based on the previous studies Arctic and Antarctic fungal isolates have been very poorly studied for the enzyme production. Currently, applications of cold adaptive fungi in biotechnological industries are becoming of great interest. Due to the cold adapted nature of various enzymes produced by psychrophilic fungi such as amylases, lipases, cellulases and proteases, their applications are increasing in various industrial fields (Feller and Gerday, 2003; Leary, 2008). In our study fungal isolates showed a broad range of extracellular enzyme production. Generally fungal isolates were reported as good producers of lipases phosphatases and cellulases. In various research studies many of the fungi from cold regions have been found good producers of different extracellular

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enzymes cluding amylase, lipase, cellulose, chitinase, polygalacturonase and phosphatase (Fenice et al., 1997; Inglis et al., 2000; Singh et al., 2012, 2014).

Conclusions

Diversity evaluation of psychrotrophic and psychrophilic fungi in Batura glacier was conducted for the first time. Most of the fungal isolates belonging to Ascomycetes followed by Basidiomycetes and Zygomycetes were identified in this study. They were very versatile, capable to survive and grow at different extreme salt concentrations, temperature and pH and were also capable of producing antibiotics and enzymes of industrial importance at low temperatures. In the future, the isolated fungi of this study would be a great source of novel antibiotics and cold active enzymes that need to be properly characterized via both genetic and analytical tools. But utilizing cloning and other genetic manipulation approaches, the polyextremophilic nature of fungal isolates could be a good source of acid, salinity and alkalinity stable enzymes that would contribute greatly to the food, feed, paper and pulp industries, known for their extreme and environmental friendly processes.

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Conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions. All authors have made contributions to the work and writing of this manuscript.

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APPENDIX

Table A1. *Colony morphology and microscopic characteristics of fungal isolates on SDA*

Isolate	Sample form	Temperature (°C)	Colony morphology		Microscopic characteristics
			Front	Reverse	
LB_1	Sediment	4	Powdery, initially cottony, white with white margins then turned to dim gray	Golden to brown center with dim gray margins	Branched and septate hyphae, smooth-walled to broadly ellipsoidal and scattered conidia, cylindrical phialides and metulae
LB_2	Sediment	4	Mucoid, light goldenrod center with off-white edges	Lemmon chiffon center with off-white margins	Cylindrical to ovoid shaped and scattered spores, no pseudo- hyphae observed
LB_3	Sediment	4	Cottony, initially salmon to white with salmon edges then turned to slate gray to white	Saddle brown center with golden edges	Hyaline, thin-walled and branched hyphae with frequent clamp connections, chlamydospores hyaline, pear to oval shaped.
LB_4	Sediment	4	Velvety, initially dry mucoid to yellow then turned to dark orange with goldenrod margins	Saddle brown center with golden edges	Hyphae septate, branched, Conidiophores simple or sparsely branched and scattered conidiophores

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LB_5	Sediment	4	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	
LB_6	Sediment	4	Cottony, initially white velvety with off-white margins then turned to dim gray	Golden to brown center with dim gray margins.	Terverticillate to quaterverticillate conidiophores, smooth-walled, globose to suglobose conidia and cylindrical metulae and phialides	
LB_7	Sediment	4	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	
LB_8	Sediment	4	Velvety, initially cottony white then turned to gray with slate gray margins	Dark brown center with light goldenrod margins	Septate and branched hyphae, globose to subglobose shaped conidia, scattered conidiophores	
LB_9	Sediment	4	Cottony, initially velvety with blue to green center, white margin then turned to light gray to dark sea green	Dark orange to brown center with off-white margins	Branched and septate hyphae, conidiophores biverticillate, grey to green and echinulate to globose shaped conidia	
LB_{10}	Sediment	4	Velvety, initially gray to black center with off-white edges then turned to light slate gray	Gray to black center and light yellow edges	Hyaline, smooth and thin-walled conidia, ovoidal to ellipsoidal shaped spores	
LB_{11}	Sediment	4	Cottony, initially slate gray center with white margins then turned to dim gray	Brown center and yellow to off-white margins	Conspicuously roughened and branched conidiophores, septate hyphae, scattered conidia	
LB_{12}	Sediment	4	Velvety, initially white center with off-white margins then turned into light gray	Black to brown center with brown margins	Scattered and branched hyphae, curved, branched and pale greenish conidia	
LB_{13}	Sediment	4	Dry mucoid, initially white center with off-white margins then turned to light goldenrod	Golden center and light goldenrod margins	Spores are globose to ovoid shape, no pseudohyphae was observed	
LB_{14}	Sediment	4	Powdery, initially cottony, white with white margins then turned to dim gray	Golden to brown center with dim gray margins	Cylindrical metulae and phialides, scattered and branched hyphae, scattered conidiophores	

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LB_{15}	Sediment	4	Velvety, initially white with white edges then turned to light cyan	Saddle brown center with light goldenrod yellow edges	Septate to multiseptate and branched hyphae, colorless and cylindrical to ovoid shape spores	
LB_{16}	Ice	4	Velvety, initially dark olive green gray center with off-white edges then turned to dark green to black	Black center with off-white edges	Septate and branched hyphae, elliptical to cylindrical in shape, pale to dark brown in color	
LB_{17}	Water	4	Velvety, dark olive green with light yellow edges	Black center with light goldenrod yellow edges	Branched hyphae, conidia ellipsoidal to limoni-form, smooth-walled or slightly verrucose, olivaceous brown	
HB_1	Sediment	15	Cottony, initially green to blue then turned to dim gray with white margins	Brown center with golden-rod margins	Septate, branched hyphae, longer and visibly roughened conidiophores producing smooth to finely roughened conidia	
HB_2	Sediment	15	Cottony, initially white to light yellow then converted to gray with whit to off-white edges	Khaki center with pale goldenrod edges	Pseudomycelium and septate hyphae, cylindrical to ovoid shaped and scattered conidia	
HB_3	Sediment	15	Powdery, initially dark sea green then turned to black with pale green edges	Black center with dark orange to golden edges	Branched, septate hyphae, numerous, large and densely packed phialides conidiophore	
HB_4	Sediment	15	Cottony, light yellow to light golden-rod yellow center with white margins	Golden center with light goldenrod margins	Septate and hyaline hyphae, short and globose to ovoid conidiophore, scattered conidia	
HB_5	Sediment	15	Cottony, initially white to gray then turned to brown-black with off-white edges	Off-white to yellow center and light yellow edges	Round to ovoid in shape conidia, septate and branched hyphae, and scattered conidiophores	
HB_6	Sediment	15	Cottony, white to light goldenrod yellow center with white margins	Dark orange to brown center with goldenrod margins	Short distinct branched conidiophores, conidia are 1-celled and either white or yellow	
HB_7	Sediment	15	Cottony, initially green center with white margins then turned to light gray to white	Off-white to yellow center and light yellow edges	Conspicuously roughened and branched conidiophores, septate hyphae, scattered conidia	
HB_8	Sediment	15	Cottony, initially velvety with blue to green center, white margin then turned to light gray to dark sea green	Dark orange to brown center with off-white margins	Conidiophores biverticillate, grey to green and echinulate to globose shaped conidia	

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HB_9	Sediment	15	Powdery, initially cottony with dark green center and white edges then turned to dim gray	Off-white to yellow center and light yellow edges	Hyphae are septate and hyaline, kidney-shaped to brush-shaped conidiophores on conidia
HB_{10}	Sediment	15	Cottony, initially gray center with white edges then turned to light gray	Off-white to yellow center and light yellow edges	Divergent biverticillate to terverticillate conidiophores, smooth-walled and scattered conidia
HB_{11}	Sediment	15	Cottony, white center with white margins	Golden to yellow center and off-white margins	Sporangiophores subulate, hyaline, smooth, unbranched. Sporangia mainly globose
HB_{12}	Sediment	15	Powdery, initially white center with dark sea green margins then turned into black to brown	Black center with dark olive green margins	Septate and brown hyphae erected and pigmented conidiophores with conidia
HB_{13}	Sediment	15	Cottony, white center with dark salmon margins	Golden to brown center and yellow margins	Short chains or branched conidiophores, obovoidal and scattered conidia
HB_{14}	Sediment	15	Powdery, initially velvety with blue to green center, white margin then turned to gray to green	Light golden-rod center with light golden-rod yellow margin	Septate and branched hyphae, chains of spores (or conidia) from brush-shaped conidiophores
HB_{15}	Ice	15	Velvety, initially dark olive green with light yellow edges then turned to black to green	Black center with light goldenrod yellow edges	Septate, light colored hyphae, erect, pigmented, scattered conidiophores, and conidia
HB_{16}	Water	15	Velvety, dark olive green with light yellow edges	Black center with light golden red yellow edges	Septate and branched hyphae, elliptical to cylindrical in shape conidia, scattered conidiophores

LB: Low (Temperature, 4 °C) Batura Isolates, HB: High (Temperature, 15 °C) Batura Isolates

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