Fungal community associated with Arctic moss, *Tetraplodon mimoides* and its rhizosphere: bioprospecting for production of industrially useful enzymes

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Fungal community associated with terrestrial Arctic moss, *Tetraplodon mimoides* was studied by examining fresh thallus tissue and adhered soil. The study resulted in the isolation of 46 microfungi belonging to 20 species in 12 genera. These included seven non-sporulating morphotypes. To the best of our knowledge, species such as *Botrytis verrucosa*, *Mortierella simplex*, *M. schmuckeri*, *Penicillium frequentans*, *P. rugulosum* and *Cladosporium chlorocephalum* are new records to the study region. All isolates were tested for production of cold-adapted amylase, pectinase, cellulase, esterase, protease, phosphatase and urease. The cultures showed varying degrees of enzyme production, with two cultures producing all seven enzymes. The present study helps in understanding the fungal diversity associated with plants growing in extreme habitats.

Keywords: Bioprospecting, cold-adapted enzyme, fungal diversity, rhizosphere.

THE Arctic is low in its biological wealth owing to extreme environmental conditions. Plant life of the region is scarce and restricted only to grasses, mosses and lichens[1]. Mosses dominate these ecosystems and being major primary producers of the region, are essential for maintaining the thermal and hydrological regimes that influence important ecological processes[2,3].

Fungi thrive well in nutrient-rich environment. In the Arctic where conditions for survival of life are hostile, certain areas having organically rich soils support the growth of microbes[4]. Earlier mycological studies from the Arctic have focused on documenting mycorrhizal and herbaceous endophytes, lichenicolous fungi, and fungi from habitats such as soils, permafrost, deteriorated wood, ice and marine waters. Fungi associated with polar mosses have been studied, but from the Antarctic[5,6]. Studies on fungal diversity associated with *Sphagnum* moss and its substrate utilization were made from the boreal forests of Canada[7].

Psychrophilic enzymes have an edge over their mesophilic counterparts as they serve as energy savers during large-scale enzymatic conversions. Bioprospecting of Arctic microbes for the production of cold-active enzymes has been done for yeast and bacteria[8,9], but there are no reports of such work on filamentous fungi. The present study was therefore undertaken to document the mycoflora associated with an Arctic moss, *Tetraplodon mimoides* and the soil adhered with it, and to determine the significance of the isolated fungal strains with regard to production of cold-adapted industrially useful enzymes.

The moss samples along with the associated soil samples were collected from Ny-Ålesund (78°55′569″N, 11°54′033″E), Svalbard, Arctic, during the Indian Arctic Expedition 2009.

Isolation of fungi from the soil was done using soil dilution method followed by spread-plating[10] on to six different media, viz. malt extract agar (full strength) (MEA), 1/10 malt extract agar (1/10 MEA), potato dextrose agar (full strength) (PDA), 1/10 potato dextrose agar (1/10 PDA), Czapek-Dox agar (full strength) (CDA) and 1/10 Czapek-Dox agar (1/10 CDA).

Fungi from fresh moss tissue were isolated on PDA using the three-step sterilization technique[11]. All incubations were done at 5°C and 15°C. Identification of isolates was done morpho-taxonomically using IX-71 and BX51 microscopes (Olympus, Japan). Photomicrography was done using DP70 camera attached to the microscopes.

For enzyme assays substrate-specific media were used. The media were maintained at pH 5.5, except for urease which was maintained at 6.8. Incubation was done for 5 days at 15°C.

Amylase and pectinase activities were determined as described by Hankin and Anagnostakis[12]. For cellulase, 0.2% cellulose in mineral salt solution was used as a screening medium. The mineral salt solution was as proposed by Hankin and Anagnostakis[12]. Esterase production was determined using 1% polysorbate 20 (Tween 20)[13] in the medium constituting peptone (1%), $CaCl_2$ (0.01%) and agar (2%) as the medium base. Visible precipitation of calcium laurate crystals around the colony indicated positive activity. Phosphatase, protease and urease activities were assayed according to Pikovskaya[14], Damare *et al.*[15] and Ghasemi *et al.*[16] respectively.

Earlier studies have recorded the occurrence of a number of fungal species in Arctic habitats. Generally for soil fungi from colder regions, serial dilution using spread plate technique could be considered more advantageous than pour plate technique, as the latter may eliminate some heat-sensitive fungi from growing and also, some fungal spores do not germinate under submerged conditions[10]. The spread plate method was therefore used in the present study.

The study resulted in the documentation of 46 isolates belonging to 20 species in 12 genera. Most of the species isolated were Hyphomycetes (15) followed by Zygomycetes (5). Besides, seven non-sporulating morphotypes were also isolated, three of which were identified to belong to Zygomycetes based on mycelial characters. Taxonomic details are given in Table 1.

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Table 1. Characteristics of fungal strains isolated from *Tetraplodon mimoides* and their enzymatic activities

Fungus	Substrate	Isolation temperature (°C)	Media	Amylase	Pectinase	Cellulase	Esterase	Protease	Phosphatase	Urease
Aureobasidium pullulans strain 1	Soil	15	1/10 MEA	–	+	+++	–	–	–	+
Aureobasidium pullulans strain 2	Soil	5	MEA	–	++	+++	–	++	–	+
Aureobasidium sp.	Soil	5	MEA	–	–	+	–	+	–	+
Botrytis verrucosa	Soil	15	PDA	–	+	–	+	+	+++	+++
Cladosporium chlorocephalum	Soil	15	CDA	–	–	–	–	–	–	–
Cladosporium cladosporioides	Soil	5	MEA	–	++	–	+	–	–	–
Fusarium oxysporum	Soil	15	MEA	–	+	+	++	–	++	–
Geomyces pannorum strain 1	Soil	15	MEA	–	–	–	++	+	+++	+++
Geomyces pannorum strain 2	Soil	5	MEA	–	–	–	+	+	++	+++
Microdochium sp. strain 1	Soil	15	1/10 PDA	–	+	+++	–	–	++	++
Microdochium sp. strain 2	Soil	15	1/10 PDA	–	–	++	+	–	++	++
Mortierella alpina strain 1	Soil	15	PDA	–	–	–	–	+	+++	–
Mortierella alpina strain 2	Soil	15	PDA	–	–	–	–	–	–	–
Mortierella alpina strain 3	Soil	15	1/10 PDA	–	–	–	–	–	–	+
Mortierella alpina strain 4	Soil	15	1/10 CDA	–	–	–	–	+	–	–
Mortierella schmuckeri	Soil	15	MEA	–	–	–	–	–	+	–
Mortierella simplex	Soil	15	PDA	–	+	–	–	–	+	++
Mortierella sp.	Soil	15	1/10 CDA	–	–	+++	–	–	–	–
Mucor hiemalis strain 1	Soil	15	PDA	–	–	–	–	–	++	++
Mucor hiemalis strain 2	Soil	5	PDA	–	–	–	–	–	++	++
Mucor hiemalis strain 3	Soil	5	PDA	–	–	–	–	–	+	++
Penicillium citrinum strain 1	Soil	15	1/10 MEA	++	++	++	–	–	++	+
Penicillium citrinum strain 2	Soil	15	MEA	+	+	+	++	+	+++	+++
Penicillium citrinum strain 3	Soil	5	MEA	–	++	+	–	–	+++	–
Penicillium citrinum strain 4	Soil	5	MEA	–	+++	+	–	+	–	–
Penicillium citrinum strain 5	Soil	5	MEA	+++	+	+++	+	+	+++	–
Penicillium citrinum strain 6	Soil	15	1/10 PDA	–	–	–	–	–	–	–
Penicillium citrinum strain 7	Soil	15	1/10 PDA	–	+	+++	–	+	+++	–
Penicillium citrinum strain 8	Soil	15	CDA	–	–	+++	–	–	–	–
Penicillium frequentans	Soil	15	CDA	–	+++	+++	+	–	+++	–
Penicillium rugulosum	Soil	15	MEA	–	+++	+	–	+	+++	+
Penicillium sp.	Soil	15	CDA	–	–	++	–	–	–	–
Phialophora sp. strain 1	Soil	15	MEA	–	+	+++	–	–	–	+
Phialophora sp. strain 2	Soil	15	MEA	–	++	+++	–	–	+	++
Phialophora sp. strain 3	Soil	15	MEA	–	++	–	–	–	+	–
Phialophora sp. strain 4	Soil	15	MEA	–	++	+	+	–	+	–
Phialophora sp. strain 5	Soil	15	PDA	–	+	++	–	–	–	+++
Pithomyces chartarum	Soil	15	MEA	+++	+	++	–	–	++	+++
Trichosporiella cerebriformis	Soil	15	MEA	–	++	–	–	–	+	++
NSM 1	Soil	5	MEA	+	+	+	+	+	++	+
NSM 2	Fresh plant	15	PDA	–	–	++	–	+	–	–
NSM 3 (Zygomycetes)	Fresh plant	5	PDA	–	+++	++	+++	–	–	–
NSM 4	Soil	5	MEA	–	–	–	+	–	–	+
NSM 5	Soil	15	CDA	–	–	++	–	–	–	+
NSM 6 (Zygomycetes)	Soil	15	1/10 CDA	–	–	+	–	++	–	–
NSM 7 (Zygomycetes)	Soil	15	1/10 PDA	–	–	++	–	+	+	+

MEA, Malt extract agar; PDA, Potato dextrose agar; CDA, Czapek-Dox agar; NSM, Non-sporulating morphotype; Enzyme activity: +++, Good activity; ++, Moderate activity; +, Low activity and –, No activity.

Most of the cultures (44) were isolated from associated soil and only two from fresh tissue. Those from fresh tissue, isolated at two different temperatures, were non-sporulating. Of the 44 soil isolates, 11 were isolated at 5°C and 33 at 15°C. Species such as *Aureobasidium pullulans*, *Aureobasidium* sp., *Botrytis verrucosa*, *Cladosporium chlorocephalum*, *C. cladosporioides*, *Fusarium oxysporum*, *Geomyces pannorum*, *Microdochium* sp., *Mortierella alpina*, *M. schmuckeri*, *M. simplex*, *Mortierella* sp., *Mucor hiemalis*, *Penicillium citrinum*, *P. frequentans*, *P. rugulosum*, *Penicillium* sp., *Phialophora* sp., *Pithomyces chartarum* and *Trichosporiella cerebriformis* were isolated from the associated soil. Photomicrographs of most common species are given in Figure 1.

Fungi belonging to genera such as *Aureobasidium*, *Chrysoporium*, *Fusarium*, *Mortierella*, *Mucor*, *Cladosporium* and *Penicillium* are ubiquitous soil inhabitants known from all over the world, including Svalbard and its

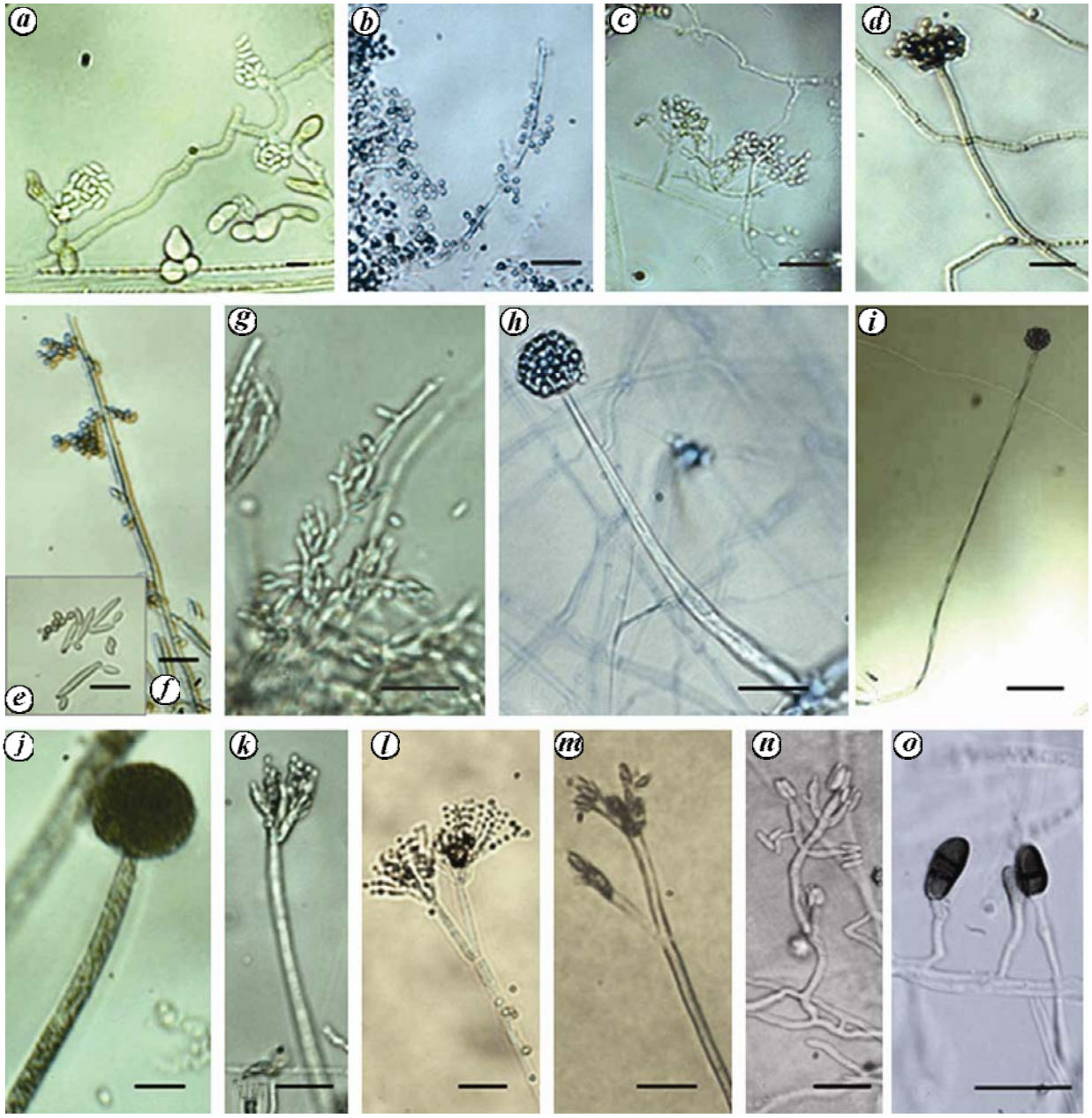

Figure 1. Photomicrographs of fungi reported in the study. ***a***, *Aureobasidium pullulans*; ***b***, *Botrytis verrucosa*; ***c***, *Geomyces pannorum*; ***d***, *Cladosporium chlorocephalum*; ***e***, ***f***, *Cladosporium cladosporioides*; ***g***, *Microdochium* sp.; ***h***, *Mortierella alpina*; ***i***, *Mortierella simplex*; ***j***, *Mucor hiemalis*; ***k***, *Penicillium citrinum*; ***l***, *Penicillium frequentans*; ***m***, *Penicillium rugulosum*; ***n***, *Phialophora* sp. and ***o***, *Pithomyces chartarum* (bar = 20 μm).

surroundings[17–19]. To the best of our knowledge however, *B. verrucosa*, *C. chlorocephalum*, *M. schmuckeri*, *M. simplex*, *P. frequentans* and *P. rugulosum* appear to be new records for the study region. Enzymatic analysis showed *C. chlorocephalum* to produce none of the screened enzymes, whereas *C. cladosporioides* produced only moderate amounts of cellulase and low levels of esterase. *F. oxysporum* produced moderate amount of esterase and phosphatase and low amount of pectinase and cellulase. Of the four *M. alpina* strains, only one showed good phosphatase activity whereas two other strains showed mild activity for protease and urease. *M. schmuckeri* and *M. simplex* showed mild activity for phosphatase. *M. simplex* also showed moderate activity for urease and low activity for pectinase. Another species of *Mortierella* exhibited good cellulase activity. Previous studies have reported production of pectinase and esterase by *P. frequentans*[6,20], whereas *P. citrinum* is known to decompose pectin, cellulose and protein[21–23]. In the present study it was found that eight strains of *P. citrinum* produced various enzymes amongst those tested, and *P. frequentans* produced good levels of pectinase, cellulase and

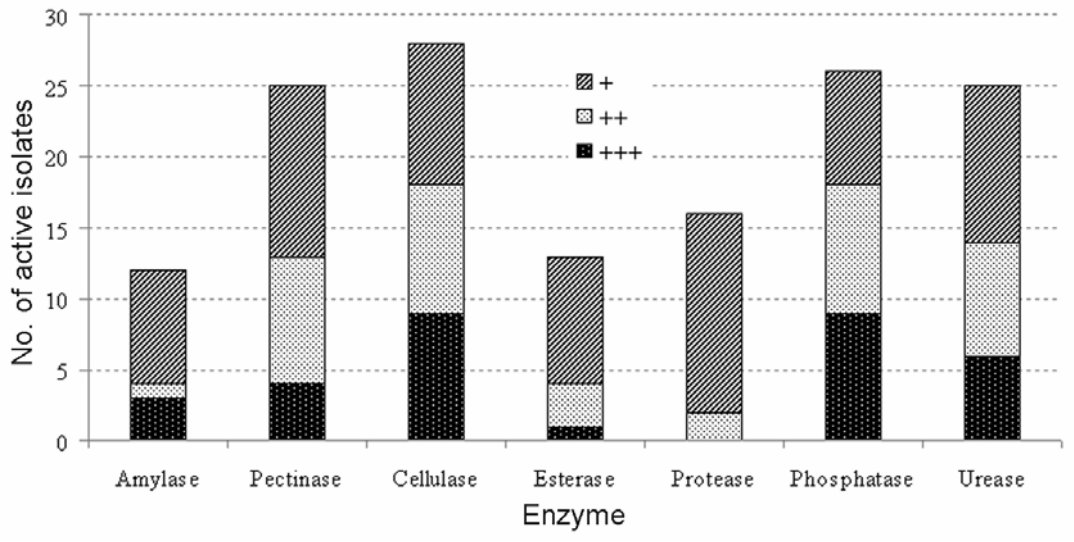

Figure 2. Enzyme activity of the isolates. +++, Good; ++, Moderate, and + Low activity.

phosphatase and low levels of esterase. *P. rugulosum* produced high levels of pectinase and phosphatase and low levels of cellulase, protease and urease.

A. pullulans because of its adaptation to low temperature, is predominant in the colder regions of the world, especially the temperate regions[24]. It has been reported from surface soils of Canada, Alaska, Poland and Antarctica. Its presence in recently deglaciated and tundra soils suggests that it does not necessarily depend on rich organic soils. It has also been reported from the rhizosphere of grasses[18]. Previous studies have shown its presence in ice from glacial and sub-glacial environments of Svalbard[25,26]. The fungus is known to produce extracellular amylase[27,28], pectinase[29], cellulase[30] and protease[31]. In the present study the two strains were observed to produce only pectinase and cellulase. Protease production was seen in only one strain, and amylase production was absent in both the strains tested. The fungus was also seen to produce urease, but in low amounts.

In Svalbard, the occurrence of several species of *Botrytis* has been reported[17], but *B. verrucosa* has not been reported earlier. The fungus was observed to be a good producer of phosphatase and urease, while it also produced pectinase, esterase and protease in low quantities. *G. pannorum* occurs frequently in the cold regions of the world such as tundra, Canada, Alaska, Svalbard and high altitudes[17,18]. From the literature it was observed that the fungus degrades starch, pectin and cellulose[18]. In the present study, however, the cold-adapted activity of any of the above-mentioned enzymes was not seen in both the isolated strains. Instead, the fungus produced good amounts of phosphatase and urease, and moderate to low amounts of esterase and protease.

M. hiemalis has been previously reported from the Arctic and Alpine regions[17,18]. From the literature it can be seen that the fungus utilizes cellulose, starch and pectin[7]. In the present study all the three strains of the fungus produced phosphatase and urease in moderate amounts. Among others, genera such as *Microdochium* and *Phialophora* have also been recorded from Svalbard[17]. Enzyme profile of the two *Microdochium* strains showed moderate to high level production of cellulase, moderate amounts of phosphatase and urease, and low levels of pectinase and esterase, whereas *Phialophora* sp. produced good amounts of pectinase and cellulase and low levels of phosphatase and urease.

P. chartarum, a saprotrophic tropical species, also known from Svalbard, was reported in the present study as well. The culture produced good amounts of amylase and urease, moderate amounts of cellulase and phosphatase, and low amounts of pectinases. Another species of soil fungus, *T. cerebriformis*, reported previously from the poles[19,32], was also found during the present study. The fungus was reported to produce moderate levels of pectinase and urease, and low levels of phosphatase. Besides the above-mentioned fungi, non-sporulating fungal cultures were also isolated during the present study and these cultures were seen to produce a range of enzymes.

In total, 7 isolates were positive for amylase, 25 for pectinase, 28 for cellulase, 13 for esterase, 16 for protease, 26 for phosphatase and 25 for urease (Table 1 and Figure 2). Based on the extent of substrate degradation, the activity was categorized into four groups: good, moderate, low and no activity. Amongst those showing activity two were good producers of amylase, four of pectinase, ten of cellulase, one of esterase, two of protease, nine of phosphatase and six of urease. Two isolates (*P. citrinum* strain 2 and NSM 1) were active for all the enzymes screened, whereas *P. citrinum* strain 5 also produced all enzymes, except urease. Three isolates (*M. alpina* strain 2,

C. chlorocephalum and *P. citrinum* strain 6) did not produce any of the screened enzymes. Rest of the cultures varied in the type of enzyme produced.

Although fungi reported here were known previously to produce enzymes of mesophilic nature, the present study reports the fungi that produce cold-adapted enzymes. Observations related to substrate utilization by Arctic fungi indicate their potential to produce industrially important enzymes. Optimization and characterization of culture conditions for increased production of these cold-active enzymes is therefore worth pursuing.

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