 Polar Record 52 (265): 442–449 (2016). *doi:10.1017/S0032247416000085*

Characterisation of yeast and filamentous fungi from Brøggerbreen glaciers, Svalbard

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Received February 2015; first published online 7 March 2016

ABSTRACT. Cryoconite holes have ecological and biotechnological importance. This article presents results on culturable cryophilic yeasts and filamentous fungi isolated from cryoconite holes at Austre and Vestre Brøggerbreen glaciers, Svalbard. Based on DNA sequence data, these were identified as *Rhodotorula* sp., *Thelebolus* sp., and *Articulospora tetracladia*. Amongst these, *Articulospora tetracladia* (88.7–89.4% gene similarity with 5.8S rDNA) is a novel species, yet to be described. Filamentous fungus *Articulospora* sp. Cry-FB1 and Cry-FB2, expressed high amylase, cellulase, lipase and protease activities while yeast *Rhodotorula* sp. Cry-FB3 showed high amylase and cellulase activity. *Thelebolus* sp. Cry-YB 240 and Cry-YB 241 showed protease and urease activities. The effects of temperature, and salt on the growth of the cultures were studied. Optimum temperature of growth was on 10ºC at pH 7.0. Filamentous fungi and yeast in the cryoconite holes possibly drive the process of organic macromolecule degradation through cold-adapted enzyme secretion, thereby assisting in nutrient cycling in these supraglacial environments. Further, these cryophilic fungi, due to their enzyme producing ability, may provide an opportunity for biotechnological research in the Arctic.

Introduction

Cryoconite holes are variously shaped, water filled depressions that are distributed over the glaciers of polar, alpine and other mountainous areas of the world. These holes are the biologically active niches within glacial ecosystems (Säwström and others 2002). These contain soft, dark coloured granular material, mostly consisting of both organic and inorganic matter. The organic matter mainly includes algae, bacteria, (Takeuchi and others 2001; Säwström and others 2002; Anesio and others 2009; Kastovska and others 2005; Hodson and others 2008; Edwards and others 2011; Singh and others 2013a) and rotifers, while the inorganic matter is a mixture of minerals and trace elements (Singh and others 2012). Recently, Singh and Singh (2012) characterised yeast and filamentous fungi from cryoconite holes of Midre Lovénbreen glacier, Svalbard. Further, and subsequently, a novel species *Rhodotorula svalbardensis* was reported from cryconites (Singh and others 2014). Edwards and others (2013) analysed fungi from cryoconites of Austre Brøggerbreen (AB), Midre Lovénbreen (ML) and Vestre Brøggerbreen (VB) glaciers with terminal-restriction fragment length polymorphism (T-RFLP) profiles, and recorded the presence of *Articulospora* and *Varicosporium* through a cultured approach. However, studies on fungal community inhabiting cryoconite holes on glaciers in Svalbard are still scanty.

Cryoconite holes work as mico-ecosystems and have ecological and biotechnological importance. To date there has been no study on the enzyme producing ability of cryophilic fungi from cryoconites of Brøggerbreen glaciers of Svalbard, Arctic. This study aims to address this knowledge gap by characterising the fungi inhabiting these glaciers cryoconites.

Materials and methods

Study area and sample collection

Samples were collected from Brøggerbreen glaciers (Austre and Vestre) (Fig 1a). Cryoconite samples were collected from different locations of glaciers in ablation zone. Austre Brøggerbreen (11.7 km^2) and Vestre Brøggerbreen (5.3 km^2) glaciers are situated on the western part of Spitsbergen, Svalbard. These two glaciers are the main sources of water to the Bayelva river (also known as Red river) finally merging into Kongsfjorden. Cryoconites were collected into sampling bags following strict contamination-free procedures (using a sterile gloves, syringe and sterile HiMedia sample collector), transported to laboratory with dry ice and stored at -20°C until processed.

Isolation of yeast and filamentous fungi

One gram of cryoconite sediment was processed following the serial dilution method (Waksman 1916) and plated on mycological media MEA (malt extract agar, pH 5.5), PDA (potato dextrose agar, pH 5.6 ± 0.2), SDA (Sabouraud dextrose agar, pH 5.6) and PCA (potato carrot agar, pH 6.8 ± 0.2) (HiMedia India), by pour plate as well as spread plate techniques. Plates were incubated in triplicates at 4, 15, 22 and 25ºC for 2–4 weeks. Culture plates were monitored regularly and on the basis of shape, colour, and different morphological features (hyphae, conidiophore, and conidial structure). The distinct colony

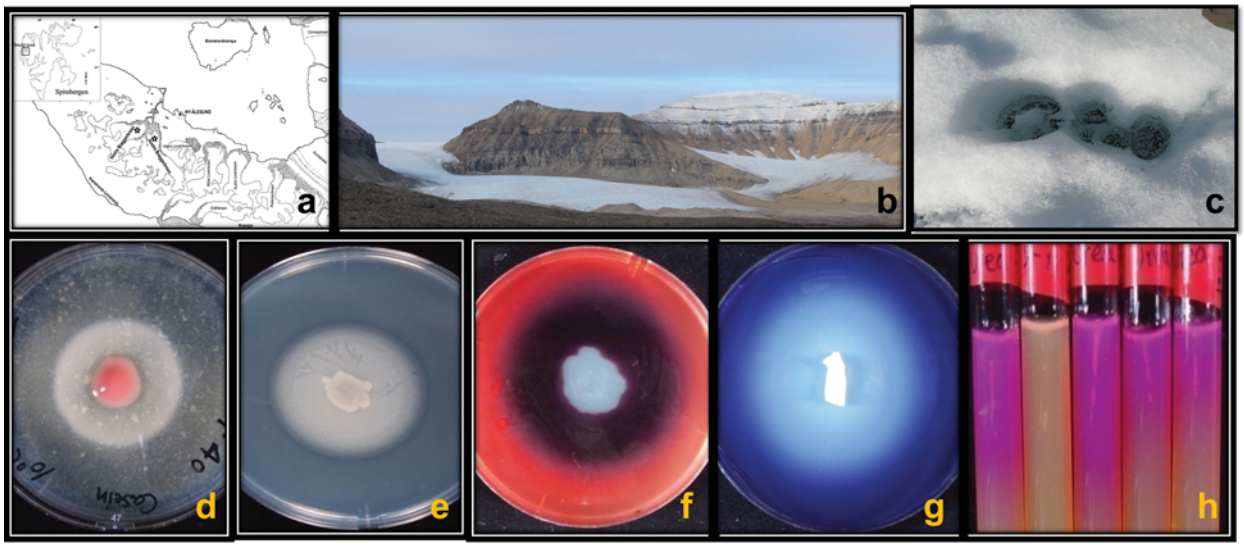

Fig. 1. a) Map showing the sampling areas, b) Landscape of Vestre Brøggerbreen glacier in Svalbard Arctic, c) Cryoconite holes d) protease activity e) lipase activity f) cellulase activity g) amylase activity h) urease activity.

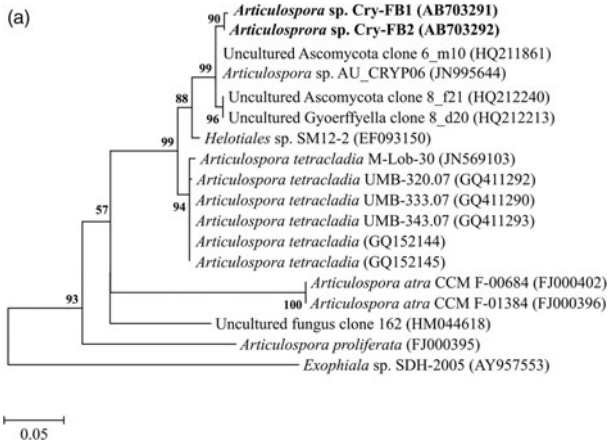

Fig. 2a. Phylogenetic analysis of *Articulospora* sp. using ITS region. The accession numbers of isolates are shown in parentheses. Tree was constructed with neighbor-joining method. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

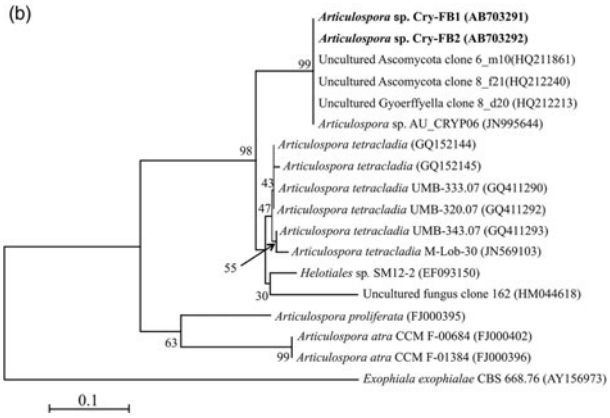

Fig. 2b. Phylogenetic analysis of *Articulospora* sp. using 5.8S rDNA region. The accession numbers of isolates are shown in parentheses. Tree was constructed with NJ method. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

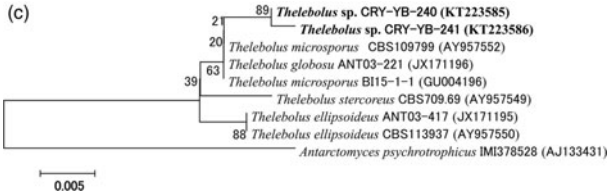

Fig. 2c. Phylogenetic tree of *Thelebolus* sp. using ITS region. The accession numbers of isolates are shown in parentheses. Tree was constructed with NJ method. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

was picked up, sub cultured, and observed for purity of cultures under a microscope. The purified fungal colonies were transferred onto PDA slants (in a test tube at about a 35º slant to provide more surface area for fungal growth), stored at 4ºC for detailed study.

For morpho-taxonomical studies, the fungal mounts were prepared on slides using lactophenol-cotton blue as a mounting medium, and observed under Olympus BX-51 and IX-71 model microscopes. Fungal cultures were initially identified on the basis of morphotaxonomy with the help of standard literatures (Ellis 1971, 1976; Barron 1977; Carmichael and others 1980; Kirk and others 2008; De Hoog and others 2005; Kurtzman and others 2011). The isolates with similar morphological characteristics were grouped together, and the representative isolates were subjected to DNA sequence analysis. All identified pure cultures were maintained on PDA slants and deposited at the National Fungal Culture Collection of India (NFCCI-WDCM 932) in Pune, India.

https://doi.org/10.1017/S0032247416000085 Published online by Cambridge University Press

Table 1a. Identification of *Articulospora* species (using ITS region), total sequence lengths after alignment, % sequence similarities, number of positions with base changes.

Sample detail	Sequence deposition no.	Total sequence length	No. of base changes	Bootstrap support %	ITS region gene sequences similarity (%)
***Articulospora* sp. Cry-FB1**	***AB703291***	553	9	99	Uncultured Ascomycota clone 6_m10 (HQ211861) by 98.4%.
		543	8	99	*Articulospora sp.* AU_CRYP06 (JN995644) by 98.5%.
		553	11	99	Uncultured Ascomycota clone 8_f21 (HQ212240) by 98.0%.
		553	11	99	Uncultured Gyoerffyella clone 8_d20 (HQ212213) by 98.0%.
		553	25	88	*Helotiales sp.* SM12-2 (EF093150) by 95.5%.
		514	30.	94	*Articulospora tetracladia* M-Lob-30 (JN569103) by 94.2%.
		514	29	94	*Articulospora tetracladia* UMB-320.07 (GQ411292) by 94.4%.
		514	28	94	*Articulospora tetracladia* UMB-333.07 (GQ411290) by 94.6%.
		514	28	94	*Articulospora tetracladia* UMB-343.07 (GQ411293) by 94.6%.
		554	32	94	*Articulospora tetracladia* (GQ152144) by 94.2%.
		554	33	94	*Articulospora tetracladia* (GQ152145) by 94.0%.
		561	111	100	*Articulospora atra* CCM F-00684 (FJ000402) by 80.2%.
		551	111	100	*Articulospora atra* CCM F-01384 (FJ000396) by 79.9%.
		516	68.	14	Uncultured fungus clone 162 (HM044618) by 86.8%.
		545	117	93	*Articulospora proliferata* (FJ000395) by 78.5%.
		420	119	-	*Exophiala* sp. SDH-2005 (AY957553) by 71.7%.
		553	6	100	*Articulospora* sp. Cry-FB2 (AB703292) by 98.9%.

Molecular characterisation (polymerase chain reaction (PCR), Sequencing and phylogenetic analysis)

Total DNA was extracted from cultures (grown on PDA for 3 weeks at 4ºC) using the ISOPLANT II kit (Wako pure chemical industries Ltd.,Japan). Extracted DNA was amplified by PCR method using KOD-plus DNA polymerase (Toyobo Co. Ltd. Japan). PCR was performed under following conditions: Initial denaturation at 95°C for 2 min followed by 35 cycles of each denaturation at 95°C for 1 min, annealing at 52°C for 30 sec, elongation at 72°C for 1 min and final elongation was carried out at 72°C for 7 min.

The ITS region was amplified using following primers: ITS1F (5'-GTA ACA AGG TTT CCG T) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC). The amplified DNA was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega KK, Tokyo, Japan). The purified DNA was sequenced on ABI DNA Sequencer following standard protocol.

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Table 1b. Identification of *Articulospora* species (using 5.8S rDNA region), total sequence lengths after alignment, % sequence similarities, number of positions with base changes

Sample detail	Sequence deposition no.	Total sequence length	No. of base changes	Bootstrap support %	5.8S rDNA region gene sequences similarity (%)
***Articulospora* sp. Cry-FB1**	***AB703291***	151	0	99	Uncultured Ascomycota clone 6_m10 (HQ211861) by 100%.
		151	0	99	*Articulospora sp.* AU_CRYP06 (JN995644) by 100%.
		151	0	99	Uncultured Ascomycota clone 8_f21 (HQ212240) by 100%.
		151	0	99	Uncultured Gyoerffyella clone 8_d20 (HQ212213) by 100%.
		151	15	30	*Helotiales sp.* SM12-2 (EF093150) by 90.1%.
		151	17	55	*Articulospora tetracladia* M-Lob-30 (JN569103) by 88.7%.
		151	16	43	*Articulospora tetracladia* UMB-320.07 (GQ411292) by 89.4%.
		151	16	43	*Articulospora tetracladia* UMB-333.07 (GQ411290) by 89.4%.
		151	15	55	*Articulospora tetracladia* UMB-343.07 (GQ411293) by 90.1%.
		151	16	43	*Articulospora tetracladia* (GQ152144) by 89.4%.
		151	17	43	*Articulospora tetracladia* (GQ152145) by 88.7%.
		134	32	99	*Articulospora atra* CCM F-00684 (FJ000402) by 76.1%.
		134	32	99	*Articulospora atra* CCM F-01384 (FJ000396) by 76.1%.
		146	26	30	Uncultured fungus clone 162 (HM044618) by 82.2%.
		146	44	63	*Articulospora proliferata* (FJ000395) by 69.9%.
		151	0	99	*Articulospora* sp. Cry-FB2 (AB703292) by 100%.

The sequences of isolates were analysed using the NCBI database and BLAST. Sequence alignment of ITS region isolates, together with the homologous sequences (retrieved from Genbank) of closely related species, was performed using Clustal W option of MEGA software version 6.0 (Tamura and others 2013). Subsequently the DNA sequences of the four isolates (AB703291, AB703292, KT223585 and KT223586) were deposited in the data bank.

To calculate the sequence divergence, the matrix was analysed using the neighbour joining method (Saitou and Nei 1987), the Tamura-Nei model (Tamura and Nei 1993) and the Maximum Parsimony method (Tamura and others 2011). To represent the evolutionary history of the taxa, the bootstrap consensus tree was inferred from 1000 replicates (Felsenstein 1985). The pairwise alignment was performed using EMBOSS Matcher - Pairwise Sequence Alignment tool (www.ebi.ac.uk/Tools/psa/emboss_matcher/nucleotide.html).

Screening for enzymatic activity

The enzyme activity (amylase, cellulase lipase and protease) was determined at 1, 4, 10, and 20˚C according to established procedures (Hankin and Anagnostakis

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Table 2. Identification of *Thelebolus* species (using ITS region), total sequence lengths after alignment, % sequence similarities, number of positions with base changes.

Sample detail	Sequence deposition no.	Total sequence length	No. of base changes	Bootstrap support %	ITS region gene sequences similarity (%)
Thelebolus sp. CRY-YB-240	KT223585	538	6	21	*Thelebolus microsporus* CBS109799 (AY957552) by 98.9%.
		504	6	63	*Thelebolus globosu* ANT03-221 (JX171196) by 98.8%.
		538	6	63	*Thelebolus microsporus* BI15-1-1 (GU004196) by 98.9%.
		482	12	39	*Thelebolus stercoreus* CBS709.69 (AY957549) by 97.5%.
		548	12	88	*Thelebolus ellipsoideus* ANT03-417 (JX171195) by 97.8%.
		480	12	88	*Thelebolus ellipsoideus* CBS113937 (AY957550) by 97.5%.
		1109	7	89	*Thelebolus* sp. CRY-YB-241 (KT223586) by 99.4%.

1975; Buzzini and Martini 2002). Urease activity was also tested on YNBG (6.7 g/l Yeast Nitrogen Base, 20g/l glucose) containing 1 g/l urea solution (pH 5.5). The diameter of the clear zone was measured to apparently quantify the enzyme activity (Figs 1d-g). Change in color from orange to pink was considered positive (Kurtzman and others 2011).

Results

A total of 20 isolates was obtained which were representing two genera of yeasts and one filamentous fungi belonging to two classes: Ascomycota (*Articulospora* sp., *Thelebolus* sp.) and Basidiomycota (*Rhodotorula* sp.).

Sequencing of ITS region and subsequent BLAST search showed that isolates of *Articulospora* sp. Cry-FB1 and Cry-FB2 closely resembled *Articulospora tetracladia* M-Lob-30 (JN569103) by 94.2%., *A. tetracladia* UMB-320.07 (GQ411292) by 94.4%., *A. tetracladia* UMB-333.07 (GQ411290) by 94.6%, *A. tetracladia* UMB-343.07 (GQ411293) by 94.6%, *A. tetracladia* (GQvv152144) by 94.2%, *A. tetracladia* (GQ152145) by 94.0% and with *Articulospora* sp. AU_CRYP06 (JN995644) by 98.5% (Fig. 2a, Table 1a).

To confirm the novelty of *Articulospora* sp. Cry-FB1 and Cry-FB2 strains, the sequences of a more stable region (5.8S rRNA) which has slower evolutionary change rates were further analysed. 5.8S rRNA region showed closest sequences similarity (%) with *Articulospora tetracladia* M-Lob-30 (JN569103) by 88.7%, *A. tetracladia* UMB-320.07 (GQ411292) by 89.4%, *A. tetracladia* UMB-333.07 (GQ411290) by 89.4%, *A. tetracladia* UMB-343.07 (GQ411293) by 90.1%, *A. tetracladia* (GQ152144) by 89.4% and *A. tetracladia* (GQ152145) by 88.7% (Fig 2b, Table 1b). These analyses confirmed that *Articulospora* sp. Cry-FB1 (AB703291) and Cry-FB2 (AB703292) are novel strains. In phylogenetic tree the isolates *Articulospora* sp. Cry-FB1 and Cry-FB2 presented as novel species are yet to be established. The total sequence lengths after alignment, % sequence similarities, and number of positions with base changes are summarised in Tables 1a and 1b.

Sequence analyses of isolates *Thelebolus* sp. CRY-YB-240 (KT223585) and *Thelebolus* sp.CRY-YB-241 (KT223586) resembled *Thelebolus microsporus* CBS109799 (AY957552). *Rhodotorula* sp. Cry-FB3 indicated their closest relationship to the species of *Rhodotorula svalbardensis* Cry-YB-1 (AB734690). The phylogenetic tree of *Thelebolus* sp. is shown in Fig. 2c. The total sequence lengths after alignment, % sequence similarities, and number of positions with base changes of *Thelebolus* sp. CRY-YB-240, *Thelebolus* sp.CRY-YB-241 and closely related strains are shown in Table 2.

Most of the fungi isolated from the AB and VB glaciers cryoconites were capable of growing between 1 and 25˚C, indicating that fungi are cryophilic in nature. Isolates have shown salt tolerance ranging from 1–7% NaCl. It was worth investigating the potential of *Articulospora* sp, *Rhodotorula* sp. and *Thelebolus* sp. in terms enzyme production. The filamentous fungus like *Articulospora* sp., expressed high amylase, cellulase, lipase and protease activities whereas yeast *Rhodotorula* sp. showed high amylase and cellulase activity. *Thelebolus* sp. showed protease and urease activities (Table 3). The

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Table 3. Screening of fungal isolates for enzymes ability from Austre and Vestre Brøggerbreen glaciers of Svalbard.

Culture Code	Identification of strains	Protease (casein)				Lipase (Esterase)				Cellulase				Amylase				Urease			
		1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C
Cry-FB1	*Articulospora* sp.	+	+	++	++	-	+	w	+	+	+	++	-	+	+	+	++	-	+	+	-
Cry-FB2	*Articulospora* sp.	+	+	++	++	+	++	+	+	+	+	++	+	+	+	-	++	-	+	+	-
Cry-FB3	*Rhodotorula* sp.	+	+	+	w	+	+	+	+	+	+	++*	-	+	+	+	++	-	w	+	-
Cry-YB 240	*Thelobolus* sp.	+	-	++	w	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
Cry- YB 241	*Thelobolus* sp.	-	-	-	w	-	-	-	-	-	-	+	-	+	+	-	+	-	++*	++	-

++, Strong positive; +, Positive; W, weak; -, Negative.
[Halo zone size (1mm-15 mm) = +, (16-20 mm & above) = ++]

change in colour from orange to pink (Kurtzman and others 2011) was considered positive (Fig. 1h).

Discussion

Literature on culturable fungal diversity and biotechnological potential of isolates from glacier cryconites is scarce. Recently, Singh and Singh (2012) reported five genera (*Cryptococcus*, *Mrakia*, *Rhodotorula*., *Phialophora* and *Articulospora*) from ML glacier, while Edward and others (2013) reported two genera (*Articulospora* and *Varicosporium*) from AB, ML and VB glaciers. In this study, fungi from three genera (*Articulospora*, *Thelebolus*, *Rhodotorula*) from AB and VB glaciers were isolated and their enzymatic potential analysed. The ecological role of these fungi in the cryoconite holes possibly drive the process of organic macromolecule degradation through enzyme secretion, thereby assisting in nutrient cycling in these supraglacial environments. Edward and others (2013) mentioned the role of *Articulospora* and *Varicosporium* as decomposers in the carbon dynamics of cryoconite holes.

The capability of subsisting at the midst of cryoconite holes at low temperatures (0.1-1.5°C), and maintaining physiological processes depend on the biomacromolecules like enzymes that are cold-active. Most of the catabolic and anabolic reactions are energy consuming process and possibly the enzymes produced by the fungal isolates (*Articulospora*, *Thelebolus*, *Rhodotorula*) help them in cold adaptation mechanism and survival in oligotrophic glacier environment. The role of psychrophilic enzymes in cold adaptation has been reported earlier (Feller 2003; Lonhienne and others 2000). There are various interactions at the subcellular level, in which certain enzymes act as inducers for chains of further processes which are related to precursors of other reaction chains (Booth 1999; Orange 1994). During extreme environmental conditions, enzymes regulate the osmotic status of the cell by producing sugars, sugar alcohols, and polyols. Thus, enzyme producing ability of fungal isolates of cryoconites has an ecological significance.

The yeast colonies isolated from cryoconites showed orange and brown colour due to the presence of pigments which probably play some roles in maintaining the membrane fluidity. It is known that the modulation of membrane fluidity is brought about by changing the levels of polar and non-polar carotenoids (Jagannadham and others 2000). Thus, it seems that the pigments interacting with cell membranes increase its rigidity and enable cold-adaptation in cryoconite environment.

The screening results for various enzymes such as amylase, cellulase, lipase, protease, and urease of the isolates exhibited strong enzyme activities. These enzymes from AB and VB glaciers fungi promise biotechnological potentials. Studies on the cold-active enzymes from the polar regions are still fragmentary (Bej and Mojib 2009; Männistö and Häggblom 2006; Medigue and others 2005, Singh and others 2013b) although they find applications in health, agriculture and industry (Feller and Gerday 2003). Further studies on purification and characterisation of enzymes of these fungal isolates (*Articulospora*, *Thelebolus*, *Rhodotorula*) would provide better understanding about the biotechnological potentials, and its application in health, agriculture and industry.

Based on DNA sequence analyses, *Articulospora* sp. Cry-FB1 (AB703291) and *Articulospora* sp. Cry-FB2 (AB703292) are potentially novel strains with little 5.8S rDNA sequence homology to known species. Characterisation of these putative novel species of *Articulospora svalbardensis* sp. nov. will be addressed in a separate study.

Acknowledgments

We would like to thank the Directors, BITS, NIPR and NCAOR for facilities. Authors are thankful to Department of Science and Technology (DST) India for providing the financial support [SR/WOSA/LS-419/2013(G)]. The authors also acknowledge Dr. S.M. Singh of Polar Biology Laboratory, National Centre for Antarctic Ocean & Research (NCAOR) Goa for his valuable suggestions.

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