



Identification and enzymatic activities of psychrophilic yeasts isolated from permafrost soil in Mongolia

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Abstract. Psychrophilic yeasts with unique properties have recently become a new source of biologically active products. However, psychrophilic microorganisms, including yeasts that inhabit Mongolian permafrost, are practically unknown. Therefore, this study was the first attempt to reveal living organisms hidden in Mongolian permafrost, in particular yeasts, by isolating and identifying yeast strains, investigating their physiological characteristics and the ability to produce extracellular enzymes. Fifteen strains were isolated at 0°C from soil samples collected from three different permafrost sites in Mongolia. The strains were identified by the D1 / D2 domain sequences of the LSU rRNA gene, and as a result, 6 strains belong to the genus *Cystofilobasidium*, 7 and 2 strains belong to the genera *Naganishia* and *Vishniacozyma*, respectively. Testing the effect of temperature on the growth of the 15 strains showed that all of them were able to grow at 0, 4 and 15°C, 6 strains were able to grow at 28°C, and none of them were able to grow at 37°C. In addition, the extracellular enzyme activity of all strains was determined, 10 strains exhibited lipolytic activity and 11 strains cellulolytic activity, respectively. These yeasts exhibited extracellular enzymatic activity at 0, 4, 15, and 28°C, indicating that they maintain active metabolism under permafrost conditions, which is of great importance both for studying cold-adapted enzymes and microbial activity in the degradation of the permafrost habitat.

Keywords: *Cystofilobasium* ssp., enzyme activity, *Naganishia* ssp., psychrophilic-psychrotolerant yeasts, *Vishniacozyma* ssp.

1 Introduction

Psychrophilic or cold-loving microorganisms, having an optimal growth temperature of ≤ 15 °C, effectively colonize all permanently cold environments, from the ocean depths to high mountains and polar regions [1]. Some microorganisms are capable of growth at low temperature but grow optimally above 15°C and are referred to as facultative psychrophiles or psychrotolerants [2].

Almost 71% of the Earth's surface is covered by oceans and 90% of this volume is below 5°C. The deep sea makes up the largest part of this low-temperature

environment, followed by snow (35%) and permafrost (24%) of the land surface, sea ice (13% of the Earth's surface), and glaciers (10% of the earth's surface). According to [3], permafrost is defined as lithosphere material (soil, sediment, or rock) that is permanently exposed to 0 °C temperature, is frozen for at least 2 years in a row, and can reach depths of more than 1000 m. Both high latitudes and high elevations have permafrost sections; mountains make up a sizable portion of the world's permafrost. Approximately 20 to 70% of the soil under permafrost is made up of ice and only 1% to 7% is not frozen. These salt solutions have low water activity ($A_w = 0.8-0.85$) [4].

Both prokaryotic and eukaryotic psychrophilic microorganisms thrive well in cold environments, and among these, yeasts can adapt to low temperature better than bacteria [2]. In the Siberian permafrost soil with an estimated age of 3 million years, viable basidiomycetous yeasts were discovered in significant amounts (up to 9,000 cfu / g dry mass) and belonged to the genera *Cryptococcus*, *Rhodotorula* and *Sporobolomyces* [5, 6].

By creating extracellular hydrolytic enzymes, psychrophiles and psychrotolerant organisms have the ability to break down a variety of polymeric substances. These "cold-adapted" or "cold-active" enzymes are appealing for industrial processes requiring high enzymatic activity at low temperatures because they have higher catalytic efficiencies than their mesophilic counterparts at temperatures below 20 °C and exhibit unusual substrate specificities [7].

This study aimed to isolate cold-adapted yeast species from permafrost in Mongolia, the southern boundary of Siberian permafrost, identify them at the molecular level by analysing the D1/D2 sequences, and search for yeast isolates capable of secreting cold-active extracellular enzymes.

2 Materials and Methods

2.1 Sampling Sites and Sample Collection

Soil samples were collected from permafrost at depths of 5, 7, 9, and 10 metres in the permafrost regions of Mongolia, Uyanga sum of Uvurkhangai province, Otgon sum of Zavkhan province and Galuut sum of Bayankhongor province, in 2017 (Table 1). The sampling was carried out by permafrost researchers from the Institute of Geography and Geoecology (IGG) of the Mongolian Academy of Sciences (MAS) and the samples were stored frozen until use.

2.2 Isolation of yeast from soil

To isolate yeast, 1 g of soil sample was suspended in 9 ml of distilled water (1/10), stirred for 30 min and 0.1 ml of the resulting suspension was further serially diluted. Then 0.1 mL of 1/100 and 1/1000 dilutions were spread on Petri plates on the surface containing potato dextrose agar (PDA, Difco, Becton–Dickinson, Tokyo, Japan) and yeast malt extract agar (YM) (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, 20 g/L agar) supplemented with chloramphenicol. The plates were incubated at 0°C, 4°C, and 15°C for 3 weeks. The yeast colonies obtained from these

cultures were subsequently cultured onto YM agar plates for purification. The yeast isolates obtained from all dilutions were examined microscopically and preserved in 20% glycerol at -80°C.

2.3 Identification of Yeast Isolates

Yeast isolates were identified based on the sequences of the D1/D2 domain of the large subunit (LSU) ribosomal RNA (rRNA) gene.

Genomic DNA extraction was carried out using the PrepMan™ Ultra Sample Preparation Reagent (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region, 5.8S gene and the D1/D2 domain of the LSU rRNA gene was performed using the GoTaq® Green Master Mix (Promega Corp., Madison, WI, USA) and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'), primers described by [8]. The thermal profile was 94 °C 2 min, followed by 30 cycles of 98 °C 10 sec, 56 °C 30 sec, 68 °C 1:30 min, and a final extension step at 4 °C for 5 min. PCR products were verified on 1% agarose gels and purified using the AccuPrep® PCR/Gel Purification Kit (Bioneer, Korea), and sent to Macrogen, Korea for commercial sequencing with primers, NL1 (5' GCATATCAATAAGCGGAGGAAAAG 3') and NL4, for the D1/D2 domain of the LSU rRNA gene.

For phylogenetic analysis, the D1/D2 domain sequences were aligned with closest reference sequences, obtained from a BLAST homology search on the NCBI website, using the CLUSTAL_X software and phylogenetic trees were constructed using the neighbor-joining method [9].

2.4 Determination of the Effect of Temperature on the Growth of Yeasts

The yeasts were tested for the ability to grow at different temperatures (0, 4, 15, 28, and 37 °C) on YM agar plates. The plates were inoculated with yeast cells grown for 24-48 h, and incubated at different temperatures. Growth was monitored visually on a daily basis for a week, and growth of plates incubated at 0 °C and 4 °C was monitored for 2 weeks [10].

2.5 Production of Hydrolytic Enzymes

Sorbitan monooleate (Tween 80) was used to detect lipase activity. Tween 80 medium agar consisted of 10 g/L peptone, 5 g/L NaCl, 0.1 g/L CaCl₂.2H₂O, 20 g/L agar, 10 mL (v/v) tween 20 or tween 80 [11].

Screening for cellulose activity was done on YM agar supplemented with 0.5% sodium carboxymethylcellulase and the detection was done by Congo red solution (0.2%) and destained with NaCl 1M [12, 13].

The diameter of each clearance zone was measured, and the enzyme activity was calculated according to the following formula:

Extracellular enzyme secretion ability = (clear opaque zone diameter - colony diameter) / colony diameter [14].

3 Results

3.1 Isolation of Yeast from Permafrost Soil

Culturable yeasts in permafrost soils in Mongolia ranged from 2×10^2 to 2×10^3 colony forming unit per gram (cfu/g) (Table 1). After incubation at 0, 4, and 15 °C, number of yeast colonies grown from a sample taken at a depth of 5 m from permafrost in the Uyanga sum of Uvurkhangai aimag was 2×10^3 , 4×10^2 , and 3×10^2 , respectively.

Table 1. Permafrost sampling information and yeast abundance in each sampling sites.

№	Sampling sites	Location	Soil depth (m)	Total yeast counts (cfu/g)		
				0 °C	4 °C	15 °C
1	Uvurkhangai province Uyanga sum	46°27'34.21"N 102°16'36.39"E	5	2×10^3	4×10^2	3×10^2
2	Zavkhan province Otgon sum	47°12'36.20"N 97°36'29.67"E	7	7×10^2	2×10^2	2×10^2
3	Zavkhan province Otgon sum	47°12'36.20"N 97°36'29.67"E	9	2×10^2	2×10^2	-
4	Bayankhongor province Galuut sum	46°42'1.43"N 100° 8'34.07"E	7	-	-	-
5	Bayankhongor province Galuut sum	46°42'1.43"N 100° 8'34.07"E	10	-	-	-

A sample taken at a depth of 7 m from permafrost in the Otgon sum of Zavkhan aimag yielded 7×10^2 , 2×10^2 , and 2×10^2 colonies at these temperatures, while a sample from a depth of 9 m yielded 2×10^2 colonies at 0 and 4 °C, and no growth was observed at 15 °C. Also, no yeast growth was observed in the samples collected in the Galuut sum of Bayankhongor aimag.

Fifteen were selected from yeast colonies grown at 0 °C on two different culture media based on colony characteristics such as texture, elevation, size and pigmentation for further study.

3.2 Identification of Yeast

A total of 15 yeast strains isolated at 0 °C were identified based on the D1/D2 domain of the 26S rRNA gene. The strains were basidiomycetous yeasts belonging to 3 genera and 6 species (Table 2). Using the CLUSTAL_X program, the D1/D2 domain sequences of the strains were compared with the most similar sequences obtained as a result of the BLAST search, as well as with the sequences of type species, and phylogenetic trees were constructed using the neighbor-joining method.

The sequences of six strains U5-23, U5-32, U5-36, O7-34, O7-31, and O9-28 were 99.67-100% similar to the sequences of representatives of *Cystofilobasidium macerans* (Table 2) and grouped into clusters on the phylogenetic tree (Fig. 1A), which indicates that these strains belong to *Cystofilobasidium macerans*.

Table 2. Yeast strains isolated from permafrost soils in Mongolia identified by LSU D1/D2 domain sequence comparison with the BLAST match with the NCBI GenBank database.

№	Strain	Obtained GenBank accession No.	Top BLAST search results	Similarity %	Reference accession No.
1	U5-23	LC772161	<i>Cystofilobasidium macerans</i>	99.83	NG_059011
2	U5-24	LC772162	<i>Naganishia adeliensis</i>	100	KF891469
3	U5-25	LC772163	<i>Vishniacozyma</i> sp.	99.66	OP941491
4	U5-30	LC772164	<i>Naganishia albidus</i>	100	MW990007
5	U5-32	LC772165	<i>Cystofilobasidium macerans</i>	100	MF448255
6	U5-33	LC772166	<i>Naganishia albidus</i>	100	MW990007
7	U5-36	LC772167	<i>Cystofilobasidium macerans</i>	99.67	NG_059011
8	O7-26	LC772168	<i>Naganishia adeliensis</i>	100	MF462742
9	O7-27	LC772169	<i>Naganishia albidosimilis</i>	99.83	MF448297
10	O7-29	LC772170	<i>Naganishia adeliensis</i>	99.83	KF891469
11	O7-31	LC772171	<i>Cystofilobasidium macerans</i>	100	MF448255
12	O7-34	LC772172	<i>Cystofilobasidium macerans</i>	100	MF448281
13	O7-35	LC772173	<i>Vishniacozyma victoriae</i>	100	MN848499
14	O9-28	LC772174	<i>Cystofilobasidium macerans</i>	100	MF448255
15	O9-37	LC772175	<i>Naganishia adeliensis</i>	100	KF891469

The sequences of strains U5-24, O7-26, O7-29 and O9-37 were 99.83-100% similar to those of members of *Naganishia adeliensis*, and the sequences of strains U5-33 and U5-30 were 100% similar to those of members of *Naganishia albidus*, the sequence of strain O7-27 was 99.83% similar to that of *Naganishia albidosimilis* isolate lhWW59 (Table 2), and they were clustered with members of the corresponding species (Fig. 1B).

The resting 2 strains belonged to the genus *Vishniacozyma*, strain U5-25 belonged to *Vishniacozyma carnescens* and strain O7-35 belonged to *Vishniacozyma victoriae*, respectively (Table 2, Fig 2). The sequence of strain U5-25 was 99.66% and 99.32% similar to that of *Vishniacozyma* sp. isolate KBP_Y-6982 and *Vishniacozyma carnescens* CBS 973^T fell into one cluster with them (Fig. 1B).

3.3 Growth of Yeast Strains at Different Temperatures

The ability of yeast strains to grow at temperatures from 0 to 37 °C was tested on YM plates to assess their growth characteristics. After incubation for 7 days, all strains were able to grow at 4 and 15 °C. Then after 14 days, strains belonging to the genera *Naganishia* and *Vishniacozyma* showed from visible to strong growth at 0 °C, strong growth at 4, 15, and 20 °C, except *V. carnescens* U5-25, which showed visible growth at 20 °C. Strains belonging to the genus *Cystofilobasidium* showed from visible to strong growth at 4 and 15 °C, weak to strong growth at 0 °C, and less growth at 20 °C.

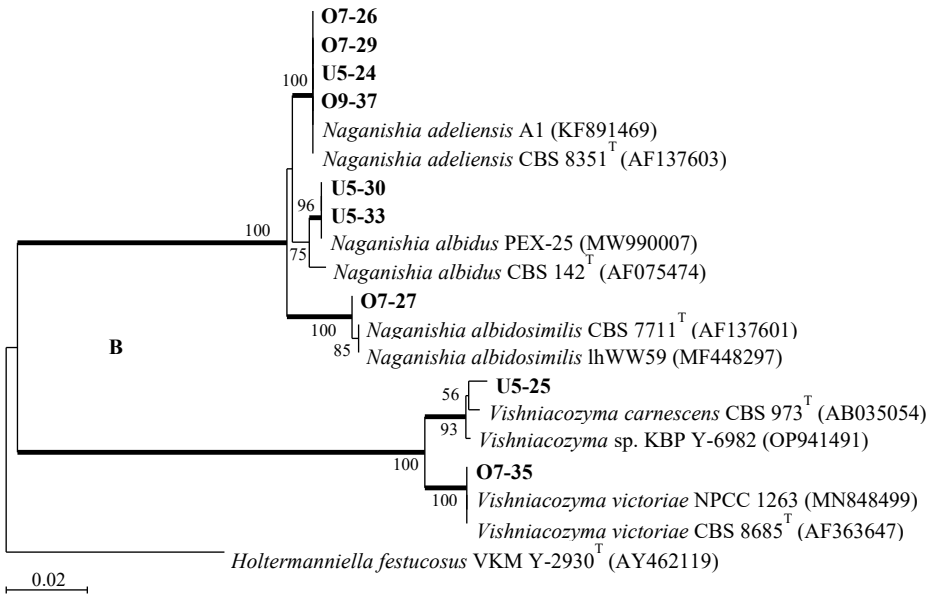
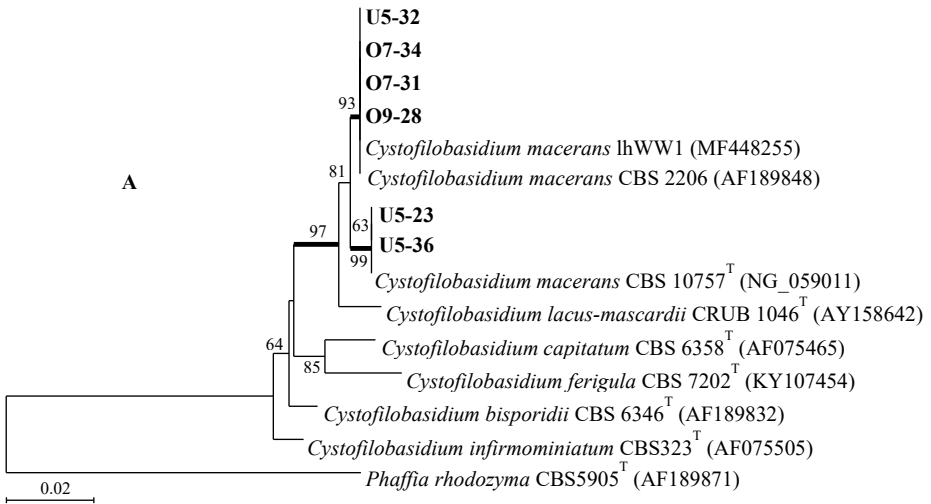


Fig. 1. Phylogenetic trees based on the sequences of the D1/D2 domain of the LSU rRNA gene showing the position of representative yeast strains in relation to closely related species. The trees were constructed by the neighbor-joining method, and bootstrap percentages based on 1000 replications are displayed for each node; bootstrap values higher than 50% are shown. Strains isolated in the course of this work and branches supported by 90% or more are highlighted in bold. The trees were constructed for genera: *Cystofilobasidium* (A), *Naganishia* and *Vishniacozyma* (B).

Six strains (40% of the total strains) could grow at 28 °C, and all of them were belonged to the genus *Naganishia*. On the other hand, none of the yeast strains could grow at 37°C (Table 3).

Table 3. Growth ability of yeast strains isolated from permafrost soils in Mongolia at different temperatures.

№	Strain	7 days					14 days						
		0 °C	4 °C	15 °C	20 °C	28 °C	37 °C	0 °C	4 °C	15 °C	20 °C	28 °C	37 °C
1	<i>C. macerans</i> U5-23	-	+	++	-	-	-	+	++	++	+	-	-
2	<i>N. adeliensis</i> U5-24	-	+	++	++	-	-	++	++	++	++	-	-
3	<i>V. carnescens</i> U5-25	-	++	++	w	-	-	++	++	++	+	-	-
4	<i>N. albidus</i> U5-30	-	++	+	++	w	-	++	++	++	++	++	-
5	<i>C. macerans</i> U5-32	-	w	w	-	-	-	w	+	+	+	-	-
6	<i>N. albidus</i> U5-33	-	w	++	+	w	-	+	++	++	++	++	-
7	<i>C. macerans</i> U5-36	-	+	+	-	-	-	+	++	+	w	-	-
8	<i>N. adeliensis</i> O7-26	-	+	++	++	w	-	+	++	++	++	++	-
9	<i>N. albidosimilis</i> O7-27	-	+	++	++	w	-	+	++	++	++	+	-
10	<i>N. adeliensis</i> O7-29	-	+	++	++	w	-	++	++	++	++	+	-
11	<i>C. macerans</i> O7-31	-	+	++	+	-	-	++	++	++	+	-	-
12	<i>C. macerans</i> O7-34	-	+	+	-	-	-	+	++	++	+	-	-
13	<i>V. victoriae</i> O7-35	-	+	++	w	-	-	++	++	++	++	-	-
14	<i>C. macerans</i> O9-28	-	w	+	+	-	-	w	+	+	+	-	-
15	<i>N. adeliensis</i> O9-37	-	++	++	+	w	-	++	++	++	++	++	-

Growth of yeast strains: ++ strong growth, + visible growth, w weak growth, - absence of growth

3.4 Screening of enzymes activities

The ability of yeast strains to produce hydrolytic enzymes was evaluated at 0, 4, 15, and 28°C. All strains except *N. adeliensis* O7-26 exhibited at least one extracellular enzymatic activity at 0, 4, 15, and 28°C.

Ten strains were positive for lipase, depending on the temperature at which it was tested. Two strains belonging to the genus *Vishniacozyma*, *V. carnescens* U5-25 and *V. victoriae* O7-35, showed lipolytic activity regardless of the temperature at which it was tested, but the highest at 15 °C. *N. albidosimilis* O7-27 also demonstrated lipolytic activity at all temperatures, but the highest at 28 °C. Out of 6 strains belonging to *C. macerans*, 4 had lipolytic activity depending on the temperature (Fig. 2A). Interestingly, the representative strains of this species formed 2 clusters on the phylogenetic tree (Fig. 1A), and 2 lipase-negative strains fell into a cluster with the type strain of the species, *C. macerans* CBS 10757^T, while 4 lipase-positive strains clustered with other strains. Moreover, *C. macerans* U5-32 and *C. macerans* O7-31 had highest activity at 4 °C.

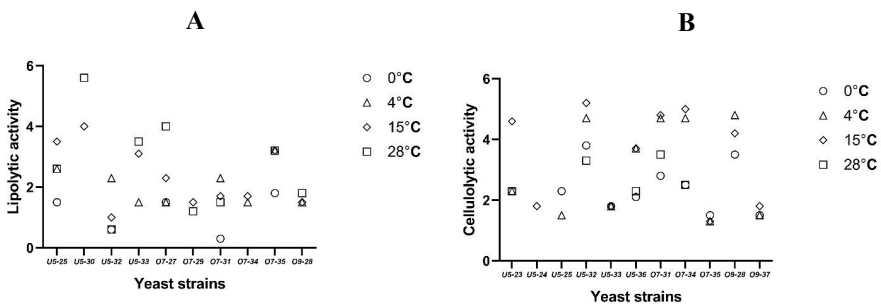


Fig. 2. Extracellular enzymatic activity of yeast strains at different temperatures. (A) Lipolytic activity, (B) Cellulolytic activity.

Eleven strains were positive for cellulase, depending on the temperature at which it was tested. Two strains belonging to the genus *Vishniacozyma* and 3 strains belonging to *Naganishia* showed cellulolytic activity at low temperatures, at 15°C and below. All 6 strains belonging to *C. macerans* had a pronounced cellulolytic activity in a wide temperature range with maximum activity at 15°C, with the exception of *C. macerans* O9-28, which did not show activity at 28°C (Fig. 2B).

4 Discussion

In this study, the abundance of yeast in the permafrost soils in Mongolia ranged from 2×10^2 to 2×10^3 cfu/g. Studies of the distribution and diversity of yeasts in glacial habitats of different geographic areas revealed varying numbers of yeasts; from 5 to over 10^5 cfu/g in soil of Ross Dependency of Antarctica [15], 9×10^3 cfu/g in Siberian permafrost soil [5], over 10^4 cfu/mL in snow and ice cores of the high-altitude Belukha

glacier of Altay Mountains [16], up to 9.6×10^3 cfu/g in subglacial sediment in glaciers of the Italian Alps [17], and 10^3 cfu/g in sediments of Calderone Glacier [18].

Forty percent of all yeast strains isolated in the present study belonged to *C. macerans*. This species is the sexual stage of *Cryptococcus macerans*, and recognized as *Cystofilobasidium macerans* sp. nov. with the self-fertile type strain CBS 10757^T after observing the entire sexual cycle [19]. Indeed, *C. macerans* has been extensively isolated from glacial habitat samples; soil of Antarctica (original taxonomic designation *Rhodotorula macerans*) [2, 15], frozen environmental samples of Iceland [20], high Arctic glacier ice of Norway [21], glacial meltwater of Argentina [10], and sediments of glacier of Italy [18], suggesting that it is a cold-adapted species. Sixty percent of all yeast strains isolated in this study belonged to 3 species of the genus *Naganishia*, namely *N. adeliensis*, *N. albidus* and *N. albidosimilis*, and 2 species of the genus *Vishniacozyma*, namely *V. carnescens* and *V. victoriae*. These species previously belonged to the genus *Cryptococcus* [22], the main genus representing glacial habitats; about 25-40% of the total species isolated from Antarctica, European and South American glaciers belong to this genus [2]. All 5 species were found in Antarctica and the Arctic, while *C. adeliensis*, *C. albidosimilis*, and *C. victoriae* were also found in European glaciers, and *C. adeliensis* and *C. victoriae* in South American glacier-associated habitats [2]. Further, *C. victoriae* and *C. carnescens* were isolated from glacial biomes in Argentina and Norway, where the latter was the dominant species (de García et al., 2012).

Among them, *C. carnescens* and *C. victoriae* are known to be psychrophilic or psychrotolerant, and *C. adeliensis*, *C. albidus* and *C. albidosimilis* are mesophilic as their maximum temperatures for growth are ≥ 30 °C [23]. However, in the present study, strains belonging to *N. adeliensis*, *N. albidus* and *N. albidosimilis* (synonyms of *C. adeliensis*, *C. albidus* and *C. albidosimilis*) showed strong growth at ≤ 20 °C, even some could not grow at 28 °C. Four of them showed strong growth at 28 °C, but the same growth was observed at lower temperatures. According to the results obtained, all strains were classified as facultative psychrophiles or psychrotolerants.

Lipolytic activities (hydrolysis of Tween-80 and/or tributirin) was the most predominantly expressed extracellular enzyme activity and was higher at 4 °C than at 20 °C in most studies [10, 17, 24, 25, 26, 27]. Among the isolates reported in the above studies, one isolate of *C. macerans* showed the highest lipolytic activity at 4 °C on tributirin agar [10], and *C. adeliensis*, *C. carnescens* and *C. victoriae* expressed lipolytic activity at 5 °C and 20 °C [25]. In the present study, 2 strains of *C. macerans* exhibited the highest lipolytic activity at 4 °C, which is consistent with the results of the above studies. Representative strains of *V. carnescens*, *V. victoriae* and *N. albidosimilis* had lipolytic activity at wide range of temperatures, from 0 °C to 28 °C, and the similar results were reported on yeasts isolated from East Ongul Island of Antarctica [14]. Also, [26] reported that most *C. victoriae* isolates hydrolyzed Tween 80 and cellulose at 4 and 20°C.

In some studies, yeast isolates were not able to hydrolyze cellulose at the assessed temperatures [10, 17, 24]. However, [25] found cellulase activity in 53.0% of 148 strains tested, including *C. adeliensis*, *C. carnescens*, and *C. victoriae*, while [27] detected hydrolysis of carboxymethyl-cellulose in 96 out of 212 strains. Interestingly,

yeasts from permafrost in Mongolia showed high cellulolytic activity in terms of both percentage of positive strains (73.3%) and enzyme secretion ability (highest 5.1 or halo zone of 37mm) over a wide temperature range from 0 °C to 28 °C. All *C. macerans* strains had a pronounced cellulolytic activity. Moreover, strains belonging to *V. carnescens*, *V. victoriae*, *N. adeliensis*, and *N. albidus* showed cellulolytic activity at $\leq 15^{\circ}\text{C}$, in contrast to results of [14] where no activity was detected for *N. adeliensis* and higher activity at $\geq 15^{\circ}\text{C}$ for *V. victoriae*. The high cellulase activity of yeasts from Mongolian permafrost compared to the yeasts isolated from aquatic environments such as glacial and subglacial sediments, water and ice [10, 24] can be associated with the vegetation cover of the active soil layer above the permafrost; they may involve in the decomposition cellulose, which constitutes up to 20% of the biomass of land plants.

5 Conclusion

Psychrophilic basidiomycetous yeasts, belonging to the taxa that extensively inhabit the global glacial environments, were isolated for the first time from permafrost in Mongolia. The yeast strains exhibited pronounced extracellular enzymatic activity at 0, 4, 15, and 28 °C, which indicates that they maintain an active metabolism under permafrost conditions. These results are of great importance both for the study of cold-adapted enzymes, which have the potential for industrial application, and for the study of microbial activity in the Mongolian permafrost degrading due to climate warming. Furthermore, bacteria and filamentous fungi were also isolated (data not shown), confirming that Mongolian permafrost is a reservoir of both prokaryotic and eukaryotic microorganisms.

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