Study on the cold adaptation strategies and biotechnological potential of cryophilic basidiomycetous yeast *Mrakia blollopis*

A Dissertation Submitted to Division of Biocontrol and Bioenvironmental Sciences, United Graduate School of Agricultural Science, Iwate University, in Fulfillment of the Degree of Doctor of Agriculture

by

Masaharu Tsuji

辻　雅晴

Contents

Abbreviation

AFP Anti-freeze protein

AS Activated sludge

ASM Activated sludge containing *M. blollopis* SK-4

BOD Biological oxygen demand

BSA Basidiomycetes selective agar

CFU Colony forming unit

DEF Direct ethanol fermentation

EPS Extracellular polysaccharide

FAME Fatty acid methyl ester

FPU Filter paper unit

GPS Global positioning system

HPLC High performance liquid chromatography

ITS Internal transcribed spacers

JARE Japan Antarctica research expedition

OD600 Optical density at 600 nm

PDA Potato dextrose agar

PDB Potato dextrose broth

PUFA Polyunsaturated fatty acid

YPD Yeast peptone dextrose broth

Chapter 1

General information

1-1. Biodiversity of fungi in the Antarctica

The first fungus recorded from Antarctica was *Scleotium antarcticum* collected at Danco Land, Antarctic Peninsula on an SY Belgica expedition (Bommer and Rouissean, 1900). Pegler et al. (1980) described over 90 species of macro-fungi. A further 90 species of filamentous micro-fungi isolated from terrestrial environments were listed by Vishniac (1996). In general, fungi live with large organisms; however, Antarctic fungi exist in the absence of large organisms and are restricted to higher plants. Most species of large Basidiomycota live on either decaying or old wood, which is very rare in Antarctica. These fungi have been collected from various sites on imported materials.

Over 1,000 fungal species from 421 genera have been isolated and recorded from Antarctica (Bridge and Spooner, 2012); the list of known species from culturing and collection consists of 68% ascomycetes, 23% basidiomycetes, 5% zygomycetes, and the final 4% is made up of various other lineages. Fell et al. (2006) reported that approximately 40% of fungi isolated in Dry Valley, a low-temperature and -moisture region, are occupied by basidiomycetous yeasts.

Di Menna (1966b) reported that approximately 24% of culturable yeasts from Antarctic soil were *Mrakia* spp. Furthermore, *Mrakia* spp. have been identified in various polar regions, such as the Arctic, Siberia, the Alps, and Antarctica (Pathan et al., 2010; Margesin et al., 2005; Thomass-Hall et al., 2010; Singh and Singh, 2012). *Mrakia* spp. make up the majority of the mycobiota and are one of the most adaptive fungi in Antarctica.

1-2. A history of the genus *Mrakia*

The genus *Mrakia* currently comprises eight known species: *M. gelida*, *M. frigida*, *M. nivalis*, *M. stokesii*, *M. psychrophila* (Xin and Zhou, 2007), *M. curviuscula* (Bab'eva et al., 2002), *M. blollopis*, and *M. robertii* (Thomass-Hall et al., 2010).

Sinclair and Stokes (1965) reported a new species, *Candida stokesii*, from Antarctic snow and di Menna (1966a) collected three new *Candida* spp. isolates from Scott Base, Ross Island, Antarctica. These three isolates have several characteristics in common e.g., NO_3^- reducibility and starch (e.g., extracellular polysaccharide compounds) production. However, their modes of carbon source assimilation and sugar fermentation differ; the three isolates

were described as *Candida nivalis*, *C. gelida*, and *C. frigida* based on these characteristics. Fell et al. (1969) reported a heterobasidiomycetous life cycle in *Candida* spp. isolated from Antarctic soil. *Candida stokesii*, *C. nivalis*, *C. gelida*, and *C. frigida* were subsequently transferred to *Leucosporidium* as *Leucosporidium stokesii*, *L. nivalis*, *L. gelidum*, and *L. frigidum*, respectively. Approximately two decades after Fell's article, the genus *Leucosporidium* was taxonomically revised on the basis of the CoQ system. *Leucosporidium stokesii*, *L. nivalis*, *L. gelidum*, and *L. frigidum* are equipped with CoQ8 and these species were reassigned to the newly established genus *Mrakia*, as *M. stokesii*, *M. nivalis*, *M. frigida*, and *M. gelida*, respectively. Other *Leucosporidium* spp. are equipped with either CoQ9 or CoQ10 (Yamada and Komagata, 1987).

Fell and Kurtzman (1990) identified closely related homothallic species, such as *Rhodotorula*, *Candida*, and *Mrakia*, using 230 bp of the large subunit rRNA. Phylogenetic analyses of basidiomycetous yeast using the 18S rRNA sequence and *Mrakia* spp. relationships by intergenic spacer (IGS) and internal transcribed spacer (ITS) region sequences of rDNA were subsequently carried out (Suh and Sugiyama, 1993; Diaz and Fell, 2000). Moreover, Fell et al. (2000) reported that basidiomycetous yeast species can be classified by phylogenetic analysis of their D1/D2 domain sequences of the 26S rRNA.

1-3. Cold adaptation strategies in fungi

Many living organisms, including fungi, have biochemical strategies to prevent themselves from freezing. Rossi et al. (2009) reported that cryophilic yeasts produce more polyunsaturated fatty acids (PUFA) in the fungal body than mesophilic yeasts. In some cryophilic yeasts, the PUFA concentration increases with decreasing ambient temperatures; however, the PUFA ratio decreases with increasing culturing temperatures (Singh et al., 2013). These yeasts have extracellular enzymes, e.g., amylase and cellulose, which activate even under low-temperature conditions. This indicates that PUFA and cold active enzymes are advantageous in cold environments.

Some fungi secrete extracellular antifreeze protein (AFP) to prevent freezing of the

cell when exposed to extreme cold. Fungal AFPs have been found in snow molds and are phytopathogenic in both wheat and grass (Snider et al., 2000; Hoshino et al., 2003; Hoshino et al., 2009). *Glaciozyma antarcticum* (syn. *Leucosporidium antarcticum*), a cryophilic yeast, forms colonies within frost columns and secretes AFP and extracellular polysaccharide (EPS)

when exposed to low temperatures both in the field and under laboratory conditions (Lee et al., 2010; Fujiu, 2010).

1-4. Biotechnological potential of the basidiomycetous yeast *Mrakia*

The conventional yeast *Saccharomyces cerevisiae* is well known in biotechnology and is used for various biotechnological processes such as beer, wine, and bread. In contrast, non-conventional basidiomycetous yeast, *Mrakia* spp., are relatively under-studied in their importance for biotechnology and environmental conservation.

When incubated at 20°C and 25°C for 3 hrs, *Mrakia frigida*, *M. gelida*, and *M. stokesii* all produce the heat shock proteins hsp70 and 90 and trehalose, which is mainly used for food and chemicals (Deegenaars and Watson, 1998).

Margesin et al. (2005) reported that *M. frigida* (strain PL) secretes high levels of cold active alkaline pectate lyase from 1–10°C. The optimal temperature of this enzyme is 25–30°C and its optimal pH is 8.5–9.0. The above authors reported a 60% reduction in relative and a 5–21% reduction in initial activity when incubated for 15 min at 30°C and 48 hrs at 2°C, respectively.

M. frigida strain 2E00797 possesses a cold active toxin lethal to the pathogenetic crab yeast *Metschnikowia bicuspidate* (Hua et al., 2010). Liu et al. (2012) carried out purification of the lethal toxin protein and reported its characteristics. After purification, 55.6-kDa single bands appeared by SDS-PAGE. The protein has about 35% sequence homology with a protein kinase; however, they did not determine its activity. The optimal temperature of the purified toxin protein was 16°C and the lethal toxin was stable at below 25°C, even after 60 min pre-incubation. The optimal pH for lethal toxin activity was 4.5 and the toxin was stable at pH 4.5 after 24 hrs pre-incubation. The lethal toxin was most active in the presence of 3% (w/v) NaCl.

1-5. Purpose of this study

Despite previous research on the Antarctic mycobiota, knowledge of fungal biodiversity there remains incomplete. Fungal diversity from the Skarvsnes ice-free area, located near the Syowa station, has not yet been studied. Moreover, the only applied study of fungi isolated from Antarctica is Xiao et al.'s patent (特許第 5397848 号) from the Japan Antarctica Research Expedition (JARE).

In this study, I first investigated fungal diversity in soil surrounding 16 lakes and in sediments from the Skarvsnes ice-free area, East Antarctica. Fungi were isolated and their physiological adaptations to cold temperatures were characterized. I also studied the biotechnological potential of *M. blollopis* under low-temperature conditions.

Chapter 2 describes fungal diversity in the surrounding soil and sediments of a lake in the Skarvsnes ice-free area. These fungi were tested for growth at temperatures ranging from −1 to 25°C, antifreeze activity, and extracellular polysaccharide (EPS), and their fatty acid profiles were determined.

In chapter 3, milk fat degradation under low-temperature conditions was investigated using the Antarctic basidiomycetous yeast *M. blollopis* SK-4. The SK-4 lipase—a major enzyme in wastewater milk fat degradation—was purified and characterized.

Chapter 4 describes the influence of initial pH on *M. blollopis* ethanol production. Little is known about either ethanol production or optimal pH and cell viability for ethanol fermentation by basidiomycetous yeasts. Therefore, I investigated ethanol fermentation at pH 2.0 and pH 11.0 at 10°C using *M. blollopis* SK-4 and recorded the influence of the initial pH on this process.

In chapter 5, *M. blollopis* SK-4 was employed for ethanol fermentation from glucose and

hydrolysates from lignocellulosic biomass, and direct ethanol fermentation (DEF) from lignocellulosic biomass under low-temperature conditions.

Chapter 6 describes the improvement of direct ethanol fermentation from woody biomasses. When SK-4 was fermented on mechanochemically treated Eucalyptus and Japanese cedar wood powders using the DEF technique with a non-ionic surfactant Tween 80, theoretical ethanol yields did not exceed 70%. Therefore, I tested DEF with a combination of Tween 80 and lipase at low temperatures to improve fermentation efficiency.

Finally, chapter 7 is a summary and general discussion.

Chapter 2

Biodiversity of fungi and its cold adaptation in the lake environments at Skarvsnes ice-free area, East Antarctica

2-1. Introduction

Cold environments cover a large portion of our planet, with many ecosystems permanently exposed to temperatures below 5°C (Feller and Gerday, 2003). Since microbes adapted to cold environments are capable of growth at temperatures below 0°C, it is expected that these microbes employ unique physiological tools, such as cold-adapted enzymes and antifreeze proteins (AFP), in order to survive (Buzzini et al., 2012). Antarctica is the southernmost landmass on Earth and has an area of approximately 14 million km^2, making it the fifth-largest continent in the world. Approximately 98 % of Antarctica is covered by ice and snow and temperatures in coastal areas usually range from 5°C to −35°C. Temperatures on Antarctic plateaus are much more extreme, ranging from approximately −25°C in summer to approximately −70°C in winter (Ravindra and Chaturvedi, 2011). The Skarvsnes ice-free area is located along the central Soya coast, East Antarctica, and contains many small oligotrophic freshwater lakes that constitute the only unfrozen water in the area (Imura et al., 1999). The surface of these lakes is covered by 1-2 m of ice for 11 months of the year (Tanabe et al., 2008); however, these lakes do not freeze below a depth of 3 m.

To our knowledge, over 1000 fungal species have been isolated and recorded from Antarctica (Bridge et al., 2012). Twelve ascomycetous and 4 basidiomycetous species were reported from around Syowa station, East Antarctica. (Soneda, 1961; Tsubaki, 1961a, b; Tsusbaki and Asano, 1965). Despite previous research into the mycoflora of Skarvesnes ice-free area, knowledge of fungal biodiversity in the area remains incomplete. The present study aimed to assess fungal biodiversity isolated from soil cultures obtained around lakes and lake sediments in the Skarvsnes ice-free area, and to determine any relevant adaptations to lake environments.

2.2 Materials and Methods

Ethics statement

All necessary permits were obtained for the field studies conducted and sample collection was performed with the permission of the Ministry of the Environment of Japan.

Sampling sites and sample collection

Samples were collected in and around lakes in the Skarvsnes ice-free area, which is located in the Lützow-Holm Bay, East Antarctica. Samples of soil surface from around lakes and of sediment at lake bottom were collected from lakes Abi-ike, Ageha-ike, Bosatsu-ike, Ebi-numa, Hyoutan-ike, Jizou-ike, Kuwai-ike, Kumogata-ike, Mago-ike, Naga-ike, Nisehyoutan-ike, Nyorai-ike, Ohgi-ike, Oyako-ike, Shimai-ike and Tokkuri-ike (Fig. 2-1). Sampling positions were recorded *in situ*. Collected samples were stored at −1°C prior to use.

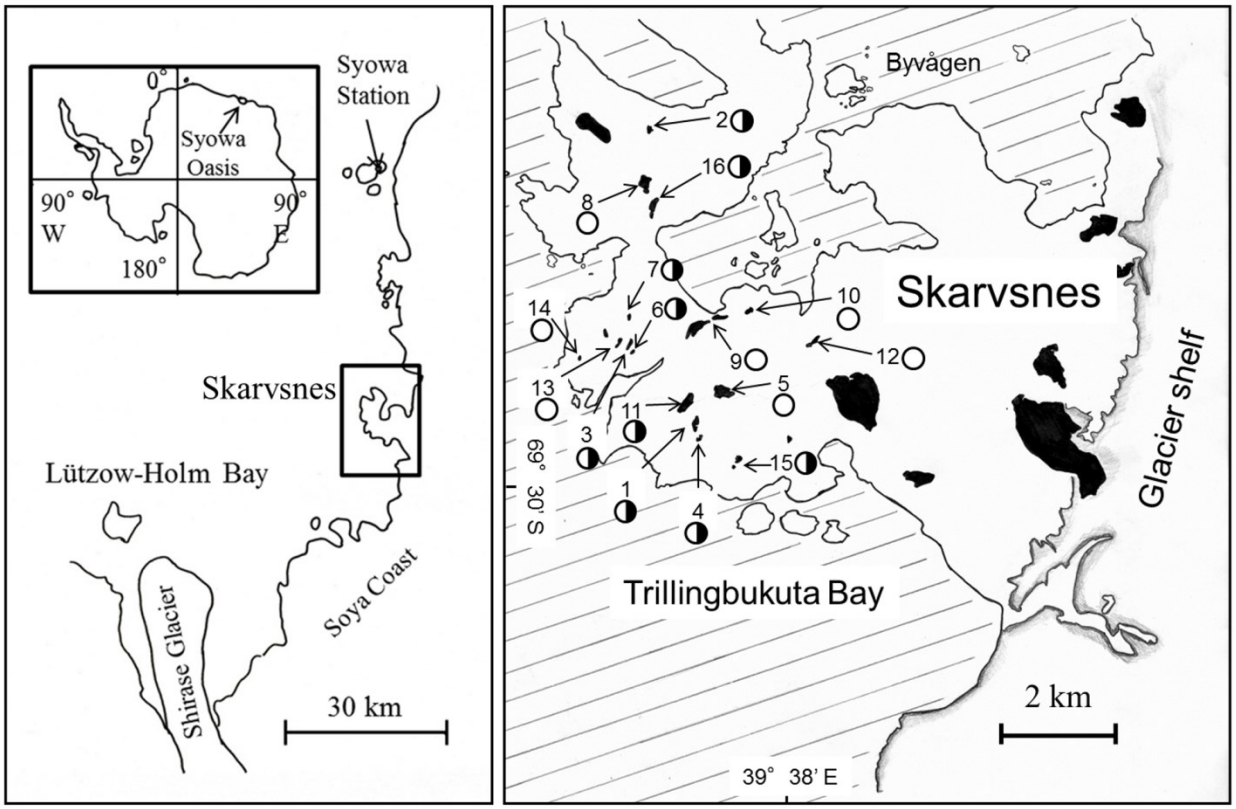

Fig 2-1. Location of the study site.

(From left) The location of the Skarvsnes ice-free area, Antarctica, and the locations of sampling sites within that area. Numbers and arrows indicate the locations of lakes in Skarvsnes, Syowa Oasis, Antarctica, that were examined in the present study (1. Abi-ike, 2. Ageha-ike, 3. Bosatsu-ike, 4. Ebi-numa, 5. Hyoutan-ike, 6. Jizou-ike, 7. Kuwai-ike, 8. Kumogata-ike, 9. Oyako-ike, 10. Mago-ike, 11. Naga-ike, 12. Nisehyoutan-ike, 13. Nyorai-ike, 14. Ohgi-ike, 15. Shimai-ike, 16. Tokkuri-ike). Open circles (○) indicate sites

from which samples were collected from the soil surrounding lakes, while half-filled circles (◑) indicate sites from which samples were taken both from the soil and from lake sediment.

Isolation of fungi

Each 0.1 g soil sample was placed in either a PDA or a Basidiomycetes selective agar (BSA) (15 g/L malt extract, 2 g/L yeast extract, 10 μg/L benomyl, 2 ml/L lactic acid and 15 g/L agar) containing 50 μg/mL chloramphenicol at 4°C for a period of up to 2 weeks. Fungal samples were chosen for isolation based on colony morphology. Each colony of a different morphology was purified by repeated streaking on PDA or BSA plates.

Sequencing analysis

DNA was extracted from colonies using an ISOPLANT II kit (Wako Pure Chemical Industries, Osaka, Japan) in accordance with protocols provided by the manufacturer. Extracted DNA was amplified through PCR using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). The ITS region of rDNA was amplified using the following primers: ITS1F (5'-GTAACAAGGTTTCCGT) (Gardes and Bruns, 1993) and ITS4 (5′-TCCTCCGCTTAT TGATATGC) (White et al., 1990). Sequences were determined using an ABI prism 3100

Sequencer (Applied Biosystems, Life Technologies Japan, Tokyo, Japan). rDNA ITS region sequences of isolates were deposited into the DNA Data Bank of Japan (DDBJ). Alignment was achieved using CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/) and corrected manually. Phylogenetic analyses with the neighbor-joining method of rDNA ITS regions containing 5.8S rRNA were performed using MEGA software version 5.0 (Tamura et al., 2011). Bootstrap analysis employing 1,000 replicates was also performed.

Determination of antifreeze activity

One loopful of pure culture was inoculated into 50 mL autoclaved PDB (potato dextrose broth) flask and incubated at −1°C and left standing without shaking for 2 months. 5 μL samples of culture broth were then collected and observed under 50× magnification using a Leica DMLB 100 photomicroscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a Linkam LK600 temperature controller (Linkam, Surrey, UK) in order to observe antifreeze activity. Samples were momently frozen at approximately −25°C, and then they were warmed up to 0°C on the sample stage in order to create ice crystal seeds in the solutions. Resulting solutions were then cooled to a temperature ranging from approximately

−1°C to −5°C and the growth of ice crystal seeds was monitored. Antifreeze activity of the isolate was determined by the shape of ice crystals. Hexagonal crystals indicated positive and rounded shape showed negative activity (Griffith et al., 1992).

Determination of EPS content and the effect of culture temperatures on growth

One loopful of each pure culture was inoculated onto PDA and incubated at either −1, 4, 10, 20 or 25°C for 2 weeks. Colonies of isolates were checked and the effect of temperature on each culture was determined.

In order to determine the presence of EPS, one loopful of pure culture was inoculated into 50 mL autoclaved PDB and the culture was incubated at 4°C for 4 weeks at 120 rpm. Biomasses were removed by centrifugation at 3,500 × g for 15 min at 4°C. Extracted supernatants were assumed to be crude EPS and were combined with 99% (v/v) ethanol to form solutions four-times the volume of the original supernatant. The resulting mixture was then centrifuged at 5,000 × g for 10 min at 4°C. Extracellular polysaccharide content of the resulting insoluble samples was determined using the phenol-sulfuric acid method (Dubois et al, 1956).

Analysis of whole-cell fatty acids

Whole-cell fatty acids were extracted from 100 mg freeze dried biomass using acid methanolysis and analyzed by gas chromatography equipped with a flame ionization detector (model GC353, GL Science) at an initial temperature of 140°C for 15 min. Temperature was then increased to 240°C at a rate of 4°C/min. Fatty acids were identified by comparison using fatty acid methyl ester (FAME) mix as a standard (Supelco 37 component FAME mix)

2-3. Results and Discussion

Samples were collected from a total 16 sites in the Skarvsnes ice-free area (Fig. 2-1). A total of 71 isolates were obtained and classified in ten genera based on rDNA ITS region sequences (Fig. 2-2 and 3a, b). In the results of ITS region sequences, average length of the read sequences was 517.6 bp, maximum length of those was 616 bp and minimum length of those was 400 bp. Twenty-three isolates were classified as ascomycetous fungi and assigned to five genera (*Embellisia*, *Phoma*, *Geomyces*, *Tetracladium*, *Thelebolus*). Forty-eight isolates were classified as basidiomycetes and assigned also to five genera (*Mrakia*, *Cryptococcus*, *Dioszegia*, *Rhodotorula*, *Leucosporidium*). Dominant isolates identified belong to the genera *Mrakia* (35.2%), *Cryptococcus* (22.5%), *Thelebolus* (11.3%) and *Tetracladium* (9.9%). These genera were collected from soils around lakes and lake sediments. Genera *Rhodotorula*, *Leucosporidium* and *Geomyces* were also collected from both the soil around lakes and lake sediments. Fungi of the genera *Phoma*, *Embelisia* and *Dioszegia* were isolated from soil surrounding lakes. No fungal species were exclusively isolated from the lake sediments.

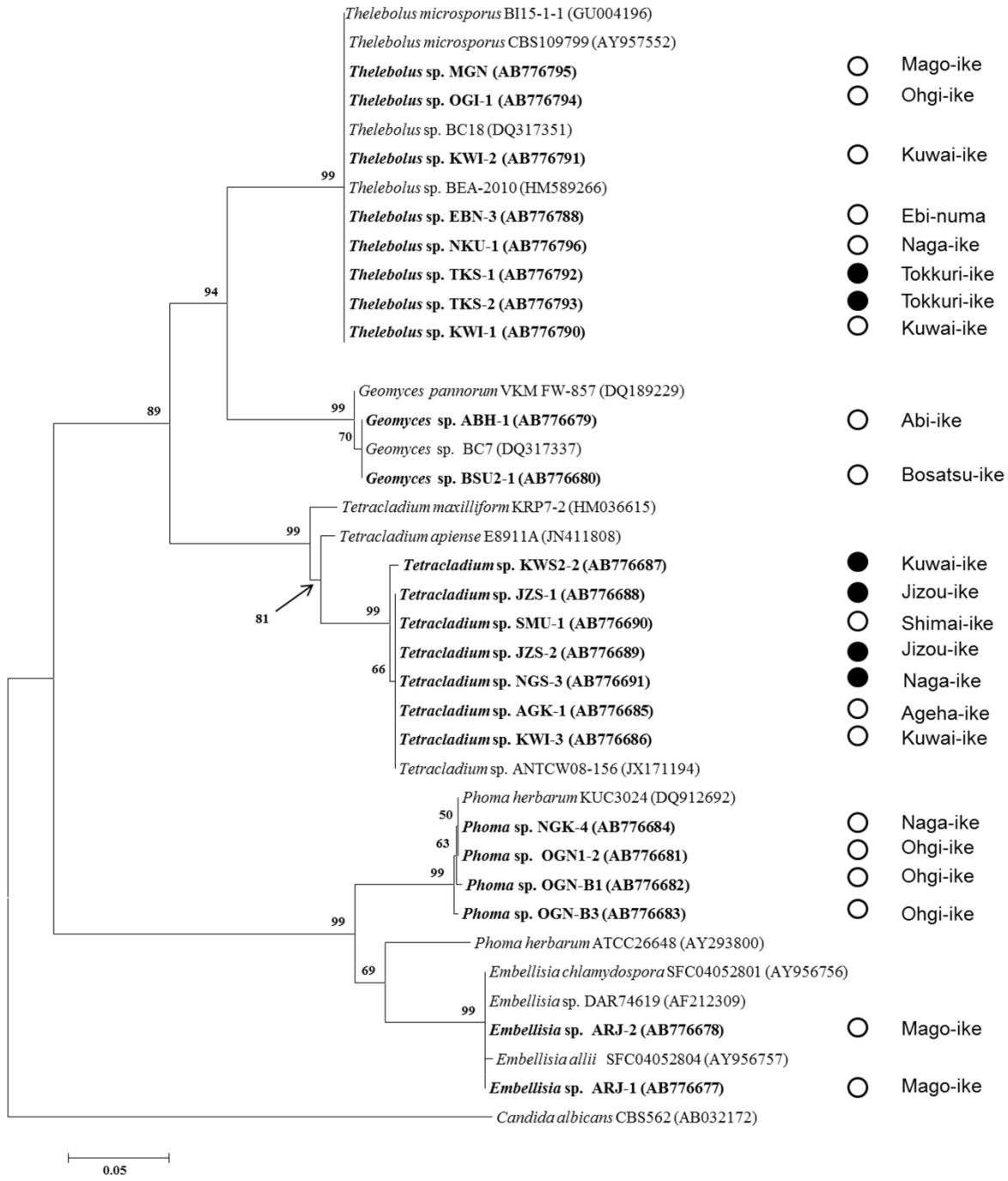
Thelebolus microsporus BI15-1-1 (GU004196)
Thelebolus microsporus CBS109799 (AY957552)
Thelebolus sp. MGN (AB776795)
Thelebolus sp. OGI-1 (AB776794)
Thelebolus sp. BC18 (DQ317351)
Thelebolus sp. KWI-2 (AB776791)
Thelebolus sp. BEA-2010 (HM589266)
Thelebolus sp. EBN-3 (AB776788)
Thelebolus sp. NKU-1 (AB776796)
Thelebolus sp. TKS-1 (AB776792)
Thelebolus sp. TKS-2 (AB776793)
Thelebolus sp. KWI-1 (AB776790)
Geomyces pannorum VKM FW-857 (DQ189229)
Geomyces sp. ABH-1 (AB776679)
Geomyces sp. BC7 (DQ317337)
Geomyces sp. BSU2-1 (AB776680)
Tetracladium maxilliform KRP7-2 (HM036615)
Tetracladium apiense E8911A (JN411808)
Tetracladium sp. KWS2-2 (AB776687)
Tetracladium sp. JZS-1 (AB776688)
Tetracladium sp. SMU-1 (AB776690)
Tetracladium sp. JZS-2 (AB776689)
Tetracladium sp. NGS-3 (AB776691)
Tetracladium sp. AGK-1 (AB776685)
Tetracladium sp. KWI-3 (AB776686)
Tetracladium sp. ANTCW08-156 (JX171194)
Phoma herbarum KUC3024 (DQ912692)
Phoma sp. NGK-4 (AB776684)
Phoma sp. OGN1-2 (AB776681)
Phoma sp. OGN-B1 (AB776682)
Phoma sp. OGN-B3 (AB776683)
Phoma herbarum ATCC26648 (AY293800)
Embellisia chlamydospora SFC04052801 (AY956756)
Embellisia sp. DAR74619 (AF212309)
Embellisia sp. ARJ-2 (AB776678)
Embellisia allii SFC04052804 (AY956757)
Embellisia sp. ARJ-1 (AB776677)
Candida albicans CBS562 (AB032172)
99
94
89
99
70
99
81
99
66
50
63
99
99
69
99
0.05
Mago-ike
Ohgi-ike
Kuwai-ike
Ebi-numa
Naga-ike
Tokkuri-ike
Tokkuri-ike
Kuwai-ike
Abi-ike
Bosatsu-ike
Kuwai-ike
Jizou-ike
Shimai-ike
Jizou-ike
Naga-ike
Ageha-ike
Kuwai-ike
Naga-ike
Ohgi-ike
Ohgi-ike
Ohgi-ike
Mago-ike
Mago-ike

Fig. 2-2 Phylogenetic tree of ascomycetes obtained from lakes in the Skarvsnes ice-free area, Antarctica.

Neighbor-joining tree based on the ITS region including the 5.8S rRNA gene sequence for all Ascomycetes species collected. Bootstrap percentages based on 1000 replications are displayed over branches. *Candida albicans* CBS562 was used as an outgroup. Open circles (○) indicate samples obtained from soil surrounding lakes, while closed circles (●) indicate samples obtained from lake sediment. Lake names on the far right of the diagram indicate the sampling location of each species.

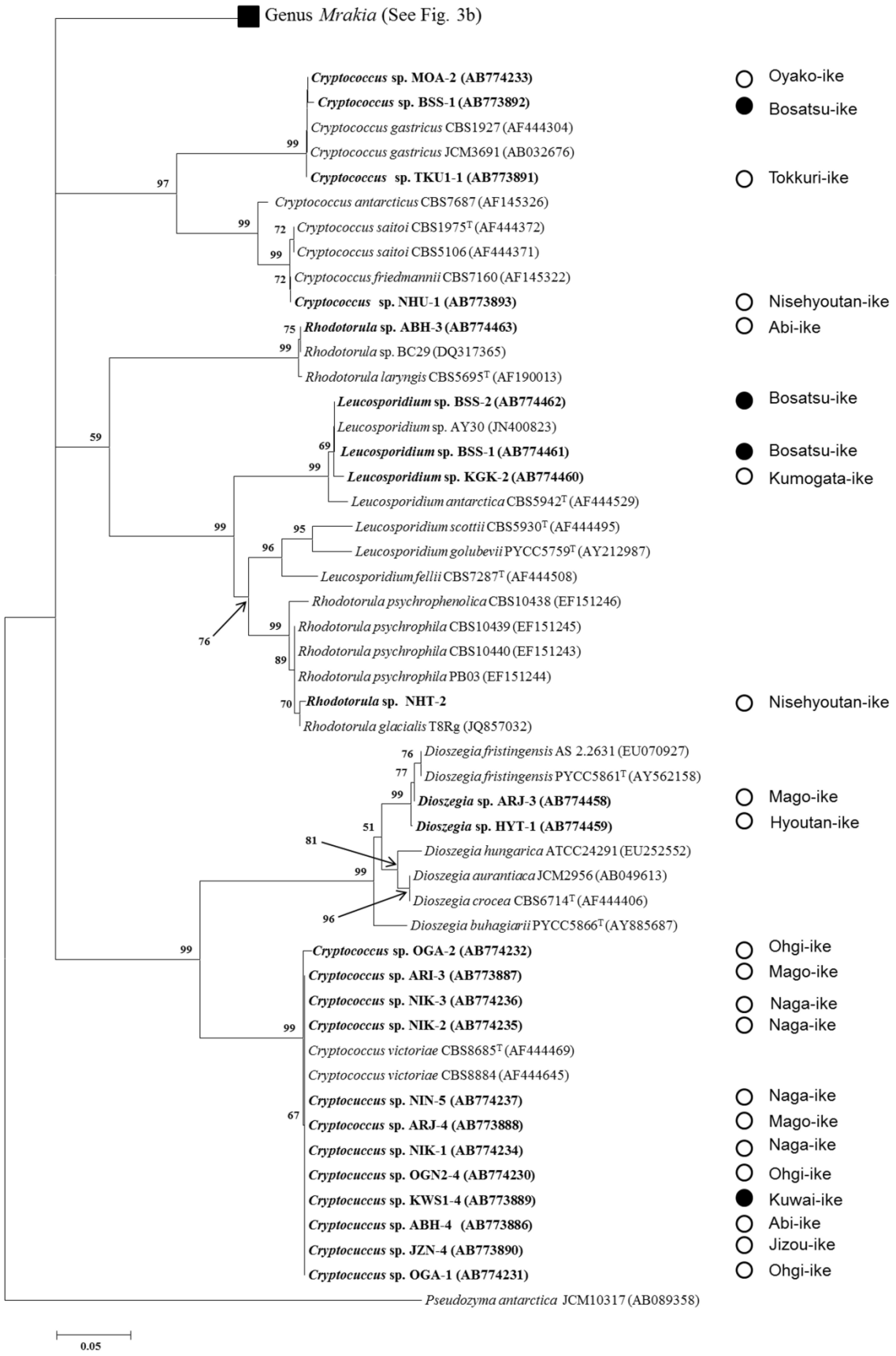
Genus *Mrakia* (See Fig. 3b)
Cryptococcus sp. MOA-2 (AB774233)
Cryptococcus sp. BSS-1 (AB773892)
Cryptococcus gastricus CBS1927 (AF444304)
Cryptococcus gastricus JCM3691 (AB032676)
Cryptococcus sp. TKU1-1 (AB773891)
Cryptococcus antarcticus CBS7687 (AF145326)
Cryptococcus saitoi CBS1975T (AF444372)
Cryptococcus saitoi CBS5106 (AF444371)
Cryptococcus friedmannii CBS7160 (AF145322)
Cryptococcus sp. NHU-1 (AB773893)
Rhodotorula sp. ABH-3 (AB774463)
Rhodotorula sp. BC29 (DQ317365)
Rhodotorula laryngis CBS5695T (AF190013)
Leucosporidium sp. BSS-2 (AB774462)
Leucosporidium sp. AY30 (JN400823)
Leucosporidium sp. BSS-1 (AB774461)
Leucosporidium sp. KGK-2 (AB774460)
Leucosporidium antarctica CBS5942T (AF444529)
Leucosporidium scottii CBS5930T (AF444495)
Leucosporidium golubevii PYCC5759T (AY212987)
Leucosporidium fellii CBS7287T (AF444508)
Rhodotorula psychrophenolica CBS10438 (EF151246)
Rhodotorula psychrophila CBS10439 (EF151245)
Rhodotorula psychrophila CBS10440 (EF151243)
Rhodotorula psychrophila PB03 (EF151244)
Rhodotorula sp. NHT-2
Rhodotorula glacialis T8Rg (JQ857032)
Dioszegia fristingensis AS 2.2631 (EU070927)
Dioszegia fristingensis PYCC5861T (AY562158)
Dioszegia sp. ARJ-3 (AB774458)
Dioszegia sp. HYT-1 (AB774459)
Dioszegia hungarica ATCC24291 (EU252552)
Dioszegia aurantiaca JCM2956 (AB049613)
Dioszegia crocea CBS6714T (AF444406)
Dioszegia buhagiarii PYCC5866T (AY885687)
Cryptococcus sp. OGA-2 (AB774232)
Cryptococcus sp. ARI-3 (AB773887)
Cryptococcus sp. NIK-3 (AB774236)
Cryptococcus sp. NIK-2 (AB774235)
Cryptococcus victoriae CBS8685T (AF444469)
Cryptococcus victoriae CBS8884 (AF444645)
Cryptococcus sp. NIN-5 (AB774237)
Cryptococcus sp. ARJ-4 (AB773888)
Cryptococcus sp. NIK-1 (AB774234)
Cryptococcus sp. OGN2-4 (AB774230)
Cryptococcus sp. KWS1-4 (AB773889)
Cryptococcus sp. ABH-4 (AB773886)
Cryptococcus sp. JZN-4 (AB773890)
Cryptococcus sp. OGA-1 (AB774231)
Pseudozyma antarctica JCM10317 (AB089358)
Oyako-ike
Bosatsu-ike
Tokkuri-ike
Nisehyoutan-ike
Abi-ike
Bosatsu-ike
Bosatsu-ike
Kumogata-ike
Nisehyoutan-ike
Mago-ike
Hyoutan-ike
Ohgi-ike
Mago-ike
Naga-ike
Naga-ike
Naga-ike
Mago-ike
Naga-ike
Ohgi-ike
Kuwai-ike
Abi-ike
Jizou-ike
Ohgi-ike
0.05

Fig. 2-3a Phylogenetic tree of basidiomycetes obtained from lakes in the Skarvsnes ice-free area, Antarctica.

Neighbor-joining tree based on the ITS region containing the 5.8S rRNA gene sequence for all Basidiomycetes species collected. Bootstrap percentages based on 1000 replications are displayed over branches. *Psudozyme antarctica* JCM10317 was used as an outgroup. Open circles (○) indicate samples obtained from soil surrounding lakes, while closed circles (●) indicate samples obtained from lake sediment. Lake names on the far right of the diagram indicate the sampling location of each species.

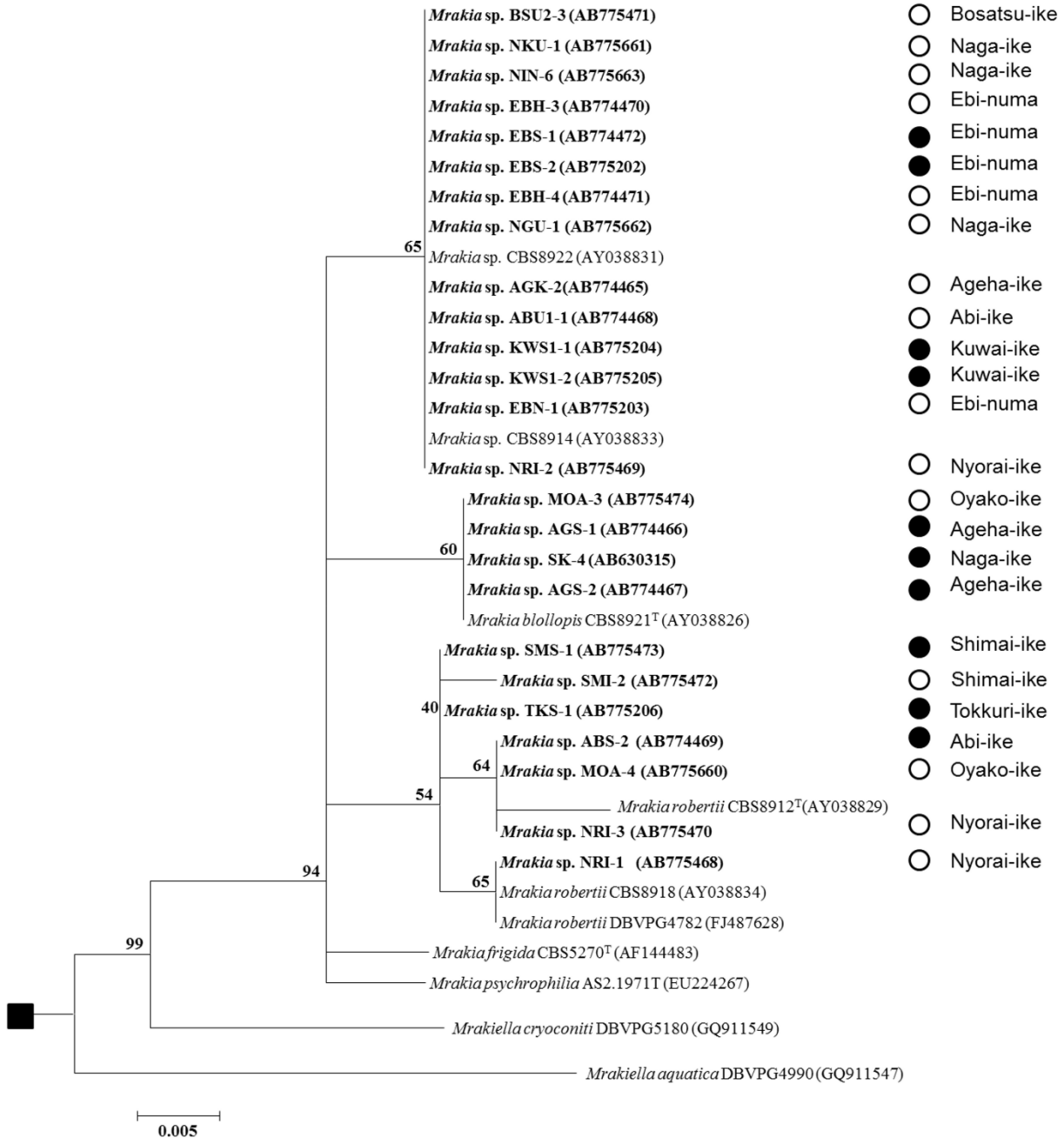

Fig. 2-3b Phylogenetic tree of basidiomycetous yeast *Mrakia* obtained from lakes in the Skarvsnes ice-free area, Antarctica. Neighbor-joining tree based on the ITS region containing the 5.8S rRNA gene sequence for all *Mrakia* species collected. Bootstrap percentages

based on 1000 replications are displayed over branches. *Mrakiella aquatic* DBVPG4990 was used as an outgroup. Closed squares (■) indicates that the link to the closed square in Figure 3a. Open circles (○) indicate samples obtained from soil surrounding lakes, while closed circles (●) indicate samples obtained from lake sediment. Lake names on the far right of the diagram indicate the sampling location of each species.

Species of the genus *Embellisia* have generally been isolated from higher plants, often as endophytes or pathogens, in areas of moderate temperature (Li and Nan, 2007). Brander et al. (2000) had previously isolated an *Embellisa* sp. from moss in Victoria Land, Antarctica. In the present study, two isolates of *Embellisia* sp. were collected from soil surface surrounding Lake Mago-ike. Genus *Phoma* have been reported from various cold environments (Fletcher et al., 1985). In Antarctica, this genus was isolated from Bunger Hill (Barker 1977), MacRobertson Land (Fletcher et al., 1985) and Victoria Land (Tosi et al., 2002). The majority of these samples were isolated from moss, which lead researchers to believe that the genus preferred moss as a substrate; however, Gonçalves et al. (2012) reported that a *Phoma* sp. was collected from water in a King George Island lake. The present study identified 4 isolates of *Phoma* sp. that were collected from soil surrounding lakes Ohgi-ike and Naga-ike. Isolates of the genus *Geomyces* have been obtained numerous times from soil samples taken from Kay Land, Surroun Base, and Cape King, Antarctica (Frate and Caretta, 1990). In the present study, only two isolates of *Geomyces* sp. were collected from the Skarvsnes ice-free area. Ruisi et al. (2007) determined that a *Thelebolus* sp. was one of the fungi most frequently isolated from the Antarctic continent.

Thelebolus colonies have been isolated from both littoral mats and under ice mats in Ace Lake, Lake Druzhby, and Highway Lake (Vestfold Hills); Organic Lake, and Red Lake on Manning Island (Larsemann Hills); and Lake Fryxell and Lake Hoare (McMurdo Dry Valleys) (Cöttlich et al., 2003). In this study, eight isolates of *Thelobolus* sp. were collected from both the soil around lakes and lake sediments in the Skarvsnes ice-free area. Seven isolates of *Tetracladium* sp. were isolated from soil and lake sediment taken from the Skarvsnes region. *Tetracladium* sp. had been previously isolated from soil taken from West Antarctica and plant matter taken from Admiralty Bay (Bridge and Newsham, 2009; Rosa et al., 2009). The author obtained total of 23 ascomycetous isolates from soil surrounding lakes and lake sediments. From lake sediments, I collected a total of six isolates from Tokkuri-ike, Kuwai-ike, Jizou-ike and Naga-ike. These isolates belonged to the genera *Thelebolus* spp. and *Tetracladium* spp.

These results indicate that there is a possibility of poor biodiversity of culturable ascomycetous fungus in the bottom of lakes at Skarvsnes ice-free area, compared to those in soil around lakes.

Sixteen of the 71 isolates obtained belong to *Cryptococcus* and 12 isolates out of 16 isolates possessed high sequence similarity with *Cryptococcus victoriae*, three isolates out of 16 isolates were closely related to *C. gastricus* and one isolate out of 16 isolates was related to *Cry. friedmannii*. *Cryptcoccus victoriae* had also been collected from soil samples taken from Victoria Land, Lichen Valley, Vestfold Hills and Davis Base (Montes et al., 1999; Thomass-Hall et al., 2002). Two isolates of *Rhodotorula* spp., three isolates of *Leucosporidium* spp., recently *L. antarcticum* was redefined as *Glaciozyma antarcticum* (Turchetti et al., 2011) and two isolates of *Dioszgia* spp. were also collected in the present study. Species of these genera have been identified in South Victoria Land and various other cold temperature environments (Fell et al., 1969; Buzzini et al., 2012). Furthermore, a *Leucospridium* sp. had also been isolated from meltwater stream sediment and glacial meltwater stream sediment (Vishiniac and Klingler, 1986; Klingler and Vishiniac, 1988).

di Menna (1966b) reported that approximately 24% of culturable yeasts from soil in Antarctica were *Mrakia* spp. Furthermore, *Mrakia* spp. had been previously identified in various polar regions, such as the Arctic, Siberia, the Alps and Antarctica (Margesin et al., 2005; Pathan et al.,

2010; Thomass-Hall et al., 2010; Singh and Singh, 2012). *Mrakia* spp. were the major mycobiota and one of the most adaptive fungi in the bottom of the lake at Skarvsnes ice-free area, East Antarctica.

Some fungi secrete extracellular AFP in order to prevent freezing of the cell when exposed to extreme cold. Fungal AFPs have been found in snow mold fungi, and have been known to be phytopathogen in both wheat and grass (Snider et al., 2000; Hoshino et al., 2003; Hoshino et al., 2009). Xiao et al. (2010a) tested 23 fungal species from Antarctica for antifreeze activity and determined that only two species demonstrated antifreeze activity. Xiao et al. (2010b) also reported that 13 of a total of 145 eukaryotic microorganisms (fungi and stramenopile) isolates obtained in Antarctica demonstrated antifreeze activity. Nine of the 13 isolates that demonstrated antifreeze activity were basidiomycetous yeast, of which seven were *L. antarcticum* (*Geociazyma antarcticum*) and two were *Rhodotorula glacialis*. In the present study, 18 isolates of eight genera were chosen from 71 total isolates and tested for antifreeze activity and capacity for growth at temperatures ranging from −1°C to 25°C. *Rhodotorula* sp. NHT-2 demonstrated high ITS region sequence homology with *R. glacialis*, and

Leucosporidium sp. BSS-1 demonstrated high rDNA ITS region sequence homology with *L. antarcticum* (*G. antarcticum*), and only these two isolates demonstrated antifreeze activity. Results of the present study as well as those of Xiao et al (2010a, b) indicated that Antarctic fungi rarely demonstrated antifreeze activity and that almost all fungi employed other strategies in order to survive in extreme environments. Eighteen of the isolates tested in the present study were capable of growth at −1°C. *Leucosporidium* sp. BSS-1 was not capable of growth at 20°C, while *Phoma* sp. NGK-4, *Tetracladium* spp. AGK-1 and JZS-2 and *Thelebolus* sp. EBN-3 were capable of growth at 25°C (Table 2-1).

Table 2-1. Antifreeze activity, presence of EPS and growth at various culture temperatures of all isolates.

Isolates	Antifreeze activity	EPS	Culture temperature				
			-1°C	4°C	10°C	20°C	25°C
Mrakia sp. AGK-2	-	w	+	+	+	w	-
Mrakia sp. EBN-1	-	w	+	+	+	w	-
Mrakia sp. KWS1-1	-	w	+	+	+	w	-
Mrakia sp. SK-4	-	w	+	+	+	w	-
Mrakia sp. SMS-2	-	w	+	+	+	w	-
Mrakia sp. TKS-1	-	w	+	+	+	w	-
Cryptococcus sp. OGA-1	-	w	+	+	+	+	-
Cryptococcus sp. NHU-1	-	w	+	+	+	+	-
Cryptococcus sp. MOA-2	-	w	+	+	+	+	-
Rhodotorula sp. ABH-3	-	w	+	+	+	+	-

Rhodotorula sp. NHT-2	+	w	+	+	+	+	-
Leucospridium sp. BSS-1	+	+	+	+	+	-	-
Dioszegia sp. ARJ-3	-	w	+	+	+	+	-
Phoma sp. NGK-4	-	w	+	+	+	+	+
Tetracladium sp. AGK-1	-	w	+	+	+	+	+
Tetracladium sp. JZS-2	-	w	+	+	+	+	+
Thelebolus sp. EBN-3	-	w	+	+	+	+	+
Thelebolus sp. TKS-1	-	w	+	+	+	+	+

'-' indicates a lack of antifreeze activity, a lack of EPS, or the inability to grow at a given temperature. '+' indicates positive antifreeze activity, considerable amounts of EPS (>0.05 mg/ml), or considerable growth at a given temperature. 'w' indicates weak antifreeze activity, the presence of EPS (<0.05 mg/ml), or a capacity for weak growth at a given temperature.

Table 2-2. Fatty acid composition of *Mrakia* spp., *R. glacialis, C. victoriae* and *Leucosporidium* sp.

Fatty acids	*M. blollopis* SK-4	*M. blollopis* CBS 8921^T	*M. frigida* CBS5270^T	*M. robertii* CBS8912^T	*R. glacialis* DBVPG4785	*C. victoriae* CBS8920	*L.* sp. BSS-1
Palmitic acid (C16:0)	8	19	13	16	14.5	10	12
Palmitoleic acid (C16:1)	8	2	0	1	1.4	ND	3
Oleic acid (C18:1n9)	22	16	8	30	39.4	50	58
Linoleic acid (C18:2)	55	33	52	35	21.2	30	13
Linolenic acid (C18:3n3)	2	30	27	18	15.4	7	5

Fatty acid analysis results for *M. blollopis* SK-4 and other species are shown. Fatty acid data were taken from Thomas-Hall et al. (2002, 2010), Rossi et al. (2009) and this study.

Robinson (2001) described that physiological characteristics such as cryoprotectant sugars, polyols, fatty acids, AFP and cold-active enzyme was profitably worked for surviving polar region. *Leucospridium antarcticum* (*G. antarcticum*), known as cryophilic yeast, (Hoshino and Matusmoto, 2012) has been known to form colonies within frost columns and secrete AFP under low temperature conditions (Lee et al., 2010). Moreover, the species has been shown to secrete both AFP and EPS when exposed to low temperatures (Fujiu 2010;). Despite the capacity of *L. antarcticum* (*G. antarcticum*) for survival at low temperatures, *Mrakia* spp. have been identified as the dominant fungi in the bottom of the lake environments at Skarvsnes ice-free area, East Antarctic, even though species of this genus have not demonstrated antifreeze activity, and very few of them have been known to secrete EPS. Pathan et al. (2010) determined that cold-adapted yeasts commonly contained high concentrations of unsaturated fatty acids, such as $C_{18:1}$ and $C_{18:2}$, and that these fatty acids were considered essential for survival at low temperatures. Results of fatty acids composition indicated that *Mrakia* spp. and *Cry. victoriae* had adapted to low temperature conditions by achieving high membrane fluidity (Turk et al., 2011; Singh et al., 2013). Moreover, *Mrakia blollopis* SK-4 has high concentration

of $C_{18:2}$, and total of $C_{18:1}$ and $C_{18:2}$, compared to those of *Mrakia blollopis* CBS8921^T (Table 2-2). It was therefore concluded that strain SK-4 was more highly adapted for survival in cold lake environments than other *Mrakia* spp.

This is an Author's Original Manuscript of an article published by Oxford University Press in FEMS Microbiology Letters on 01/09/2013, available online: http://dx.doi.org/10.1111/1574-6968.12217".

Chapter 3

An application of wastewater treatment in a cold environment and stable lipase production of Antarctic basidiomycetous yeast *Mrakia blollopis*

3-1. Introduction

Drainage from dairy parlors and milk factories produced in the process of cleaning transport pipes and milking tanks pollute rivers and groundwater with detergents, bactericides, mucus and milk fat (Healy et al., 2007). In low temperature conditions, the wastewater is treated by bio-filters (Shah et al., 2002) and a reed bed system (Biddlestone et al., 1991; Kato et al., 2010). However, the system is not used widely because of the high running cost and the necessity of a large space. Instead, an activated sludge (AS) system is now widely used for industrial treatment of dairy parlor wastewater (Ying et al., 2010) due to its advantages in maintenance and running cost. However, there is a problem in this system of low temperature conditions in winter having adverse effects on microbial functions.

The use of microorganisms living in polar regions for the purpose of removing nitrogen and phosphorus compounds from wastewater under low temperature conditions has been reported by Chevalier et al. (2000) and Hirayama-katayama et al. (2003), but it has not yet been applied for milk fat.

In our previous work, a total 305 isolates of fungi including eight ascomycetous and six basidiomycetous species collected from Antarctica were examined and it was found that they included fungi of the genus *Mrakia*, in psychrophilic basidiomycetous yeast, suggesting that *Mrakia* is a major mycoflora highly adapted to the Antarctic environment (Fujiu, 2010). Therefore, we screened our *Mrakia* isolates for their ability to decompose milk fat under low temperature conditions and evaluated their potential for application to an activated sludge system in a region with a cold climate. The results showed that 56 strains of *Mrakia* spp. exhibited a clear zone according to fat decomposition. Antarctic yeast strain SK-4 had physiological characteristics similar to those of *M. blollopis* (Shimohara et al., 2012).

Here in the chapter, it was examined that the potential activity of yeast strain SK-4 to remove milk fat BOD when they were mixed in AS. It was also described that identification of yeast strain SK-4 and the purification and characterization of lipase, a major enzyme to degrade milk fat in wastewater.

3-2. Materials and Methods

Sample isolation

Algal mat samples were collected from sediments of Naga-ike, a lake in Skarvsnes, located near Syowa station, East Antarctica. The isolate was cultivated on potato dextrose agar (PDA) (Difco™, BD Japan, Tokyo, Japan) at 4°C for 1 week. Yeast strain SK-4 was selectively picked for isolation on the basis of its morphology. Yeast strain SK-4 was maintained on PDA plates at 4ºC and long-term storage was performed in 40 % (w/v) glycerol at −80ºC.

Phylogenetic analysis

Phylogenetic analysis was done by sequencing the rDNA ITS region including 5.8S rRNA and D1/D2 domain of 26S rRNA. Cells were harvested from 2-weeks-old cultures. DNA was extracted with an ISOPLANT II kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocol. Extracted DNA was amplified by PCR using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). The D1/D2 domain was amplified using the following primers: NL1 (5'-GCATATCAATAAGCGGAGGAA AAG) and NL4 (5'-GGTCCGTGTTTCAAGACGG).

Sequences were obtained with an ABI prism 3100 Sequencer (Applied Biosystems, Life Technologies Japan, Tokyo, Japan) using an ABI standard protocol. The ITS region and D1/D2 domain sequences of yeast strain SK-4 are deposited in DNA Data Bank of Japan (BBDJ) (Accession numbers AB630315 and AB691134). Alignment was made using CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/) and corrected manually. Phylogenetic analysis was performed using MEGA software version 4.0 (Tamura et al., 2007) with neighbor-joining analysis of the rDNA ITS region containing 5.8S rRNA and maximum parsimony analysis of the D1/D2 domain of 26S rRNA. Bootstrap analysis (1000 replicates) was performed using a full heuristic search.

Physiological characterization

Assimilation of carbon was performed at 15ºC on modified Czapek-Dox agar composed by 6.7 g/L of yeast nitrogen base without amino acids (DifcoTM, BD Japan, Tokyo, Japan), 2.0g/L of sodium nitrate (Wako Pure Chemical Industries, Osaka, Japan), 30g /L of carbon source and 15.0 g/L of Agar (DifcoTM, BD Japan, Tokyo, Japan). Assimilation of nitrogen and other

physiological tests were carried out according to the protocols described by Yarrow (1998). All tests were performed at 15ºC after 2 and 4 weeks of inoculation.

Preparation of active sludge and measurement of biochemical oxygen demand

AS was cultivated at room temperature with aeration by using cow's milk as the substrate. After one month, the sludge was divided into two parts. One part of the AS was mixed with *M. blollopis* SK-4 (1.4 g/L, dry weight), and the other part was used as a control. Separated AS was prepared with Mixed Liquor Suspended Solids (MLSS, 3000 mg/L) and cow's milk at 10ºC with aeration. One week later, prepared activate sludge was added to cow's milk, and biochemical oxygen demand (BOD) of waste-treated water was measured after 24 hrs. BOD assay was carried out using a coulometer (Ohkura Electric, Saitama, Japan).

Inoculum

Mrakia blollopis SK-4 was grown in YPD liquid medium (1% yeast extract, 2% peptone, and 2% glucose) at 15ºC for 96 hrs at 120 rpm. After 96 hrs, *M. blollopis* SK-4 was collected by

centrifugation at 3500 × g for 15 min at 4°C. The pellet was transferred to fresh cream liquid medium (0.5% peptone, 0.5% NaCl, 5% fresh cream, pH 7.0) and incubated at 10°C for 14 days at 90 rpm. The resulting culture was used as inoculum.

Lipase production medium

Lipase production medium was composed of 0.2% KH_2PO_4, 0.29% Na_2PO_4, 0.02% NH_4Cl, 0.04% $CaCl_2$, 0.001% $FeCl_3$, 0.5% yeast extract, and 1% Tween 80. The yeast was cultivated at 10ºC for 324 hrs at 90 rpm. One mL samples were collected every 24 h and centrifuged at 4ºC for 10 min at 20000 × g, and then lipase activity was measured.

Assay of lipase activity

Lipase activity was measured by a colorimetric method using p-nitrophenyl-palmitate as a substrate (Berekaa et al., 2009). Forty mL of 50 mM sodium phosphate buffer (pH 7.0) containing 50 mg gum arabic and 0.2 g TritonX-100 was mixed with 3 mL 2-propanol containing 1 mM p-nitrophenyl-palmitate. Eight hundred µL of prepared substrate was added to

200 μL of enzyme solution. The enzyme reaction was carried out at 30°C for 30 min. The released p-nitrophenol was measured at A_{410}. One unit of lipase activity was defined as the activity required to release 1 μmol of free fatty acids per minute at 30°C.

Measurement of protein concentration

Protein concentration was measured by BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions using bovine serum albumin as a standard.

Purification of lipase

Mrakia blollopis SK-4 lipase was purified by ultrafiltration and Toyopearl-butyl 650M (Tosho, Tokyo, Japan) hydrophobic interaction chromatography. Four hundred mL of lipase production liquid medium was centrifuged at 4°C for 15 min at 3000 × g. The supernatant was filtered through a 0.45-μm of membrane filter (Advantec, Tokyo, Japan). The filtered medium was concentrated by ultrafiltration using an ultracel YM-30 membrane (Millipore, Billerica, MA,

USA). The concentrated sample was adsorbed to a Toyopearl butyl 650M column (2.5 x 20cm) containing 1 M sodium chloride and eluted with a linear gradient from 750 mM to 100 mM sodium chloride in 20 mM Tris-HCl buffer (pH 8.5) at a flow rate of 60 mL / hrs. Fractions of high lipase activity was pooled and concentrated and then stored at 4°C until use. Protein molecular weight was estimated by SDS-PAGE according to Laemmli (1970) and stained with CBB R-250. Precision plus protein unstained standards (Bio-Rad Laboratories Japan, Tokyo, Japan) were used as protein molecular weight makers.

Characterization of lipase

Substrate specificity was determined by using substrate as different p-nitrophenyl esters (C_4-C_{18}). For determining the effects of metal ions and EDTA on lipase activity, residual lipase activity assays were carried out under standard assay conditions with final concentrations of 1 mM of various bivalent metal ions and EDTA. Lipase activity assay in the absence of metal ions and EDTA was carried out as a control. Optimum pH was measured at 30°C for 30 min and determined at various pH values of 50 mM buffer as follows: sodium citrate (pH 3.0-5.0),

sodium phosphate (pH 6.0, 7.0 and 8.0), Tris-HCl (pH 7.5 and 8.5), glycine-NaOH (pH 9.0) and sodium carbonate (pH 9.3, 9.5 and 10.0).

Optimum temperature was measured in 50 mM sodium phosphate buffer (pH 7.0) for 30 min. To determine the pH stability of lipase, the enzyme was preincubated in various buffers for 15 hrs at 30°C and then adjusted to pH 8.5. The residual enzyme activity was measured by using p-nitrophenyl-palmitate as a substrate at 65°C for 30 min. For determining thermo-stability, lipase was preincubated for 30 min at different temperatures and the residual activity was measured at 65°C for 30 min in 50 mM Tris-HCl (pH 8.5). Effects of organic solvents on lipase activity were determined at 65°C for 30 min in 50 mM Tris-HCl (pH 8.5) containing various organic solvents at final concentration of 5% (v/v). Lipase activity assay in the absence of organic solvents was carried out as a control.

3-3. Results

Phylogenetic analysis

As a result of phylogenetic analysis of the rDNA ITS region and D1/D2 domain, yeast strain SK-4 was grouped with the clade of *Mrakia blollopis* CBS 8921^{T} (Fig.3-1 A and B). By comparison of the rDNA ITS region sequence containing 5.8S rRNA, yeast strain SK-4 showed high homology (>99.6%) with *M. blollopis* CBS8921^{T}. The D1/D2 domain sequence showed no variation with *M. blollopis* CBS 8921^{T}.

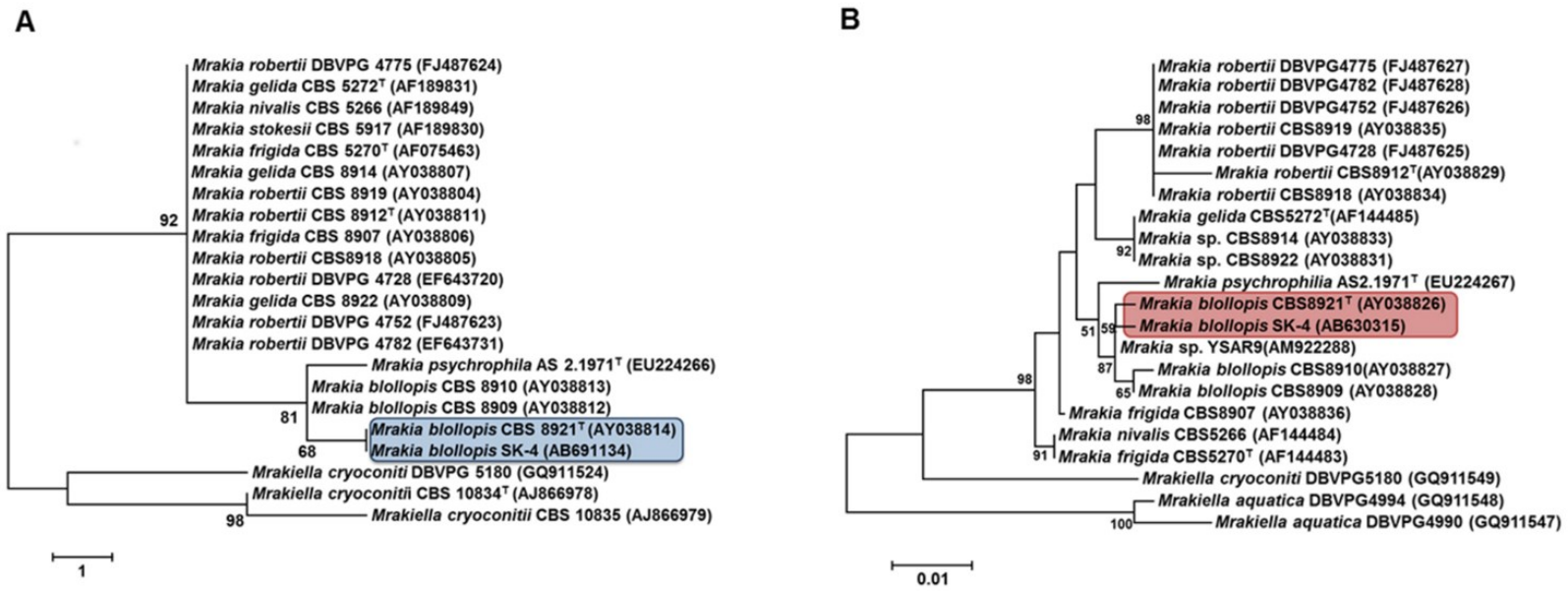

Figure 3-1. Phylogenetic tree of *M. blollopis* SK-4 and related species.

(A) Maximum parsimony analysis of the D1/D2 domain of the 26 rRNA gene sequence. Bootstrap percentages from 1000 replications are shown on the branches. *Mrakiella cryoconiti* CBS 10834^{T} and *Mrakiella cryoconiti* CBS 10835 were used as an out group.

(B) Neighbor-joining tree of the ITS region containing the 5.8S rRNA gene sequence. Bootstrap percentages from 1000 replications are shown on the branches. *Mrakiella aquatica* DBVPG4994 and *Mrakiella aquatica* DBVPG4990 were used as an out group.

Physiological characterization

Results of assimilation of carbon compounds and other physiological tests of *M. blollopis* SK-4 are shown in Table 3-1 with its type strain's of related species. Test data for *M. blollopis* SK-4 are compared with those for *M. blollopis* CBS8921^T (Thomass-Hall et al., 2010), *M. psychrophila* AS2.1971^T (Xin and Zhou, 2007), and *M. frigida* CBS5270^T (Fell et al., 1969). Maximum growth temperature of *M. blollopis* SK-4 was 22ºC. Maximum growth temperatures of other related species were lower than 20ºC. *Mrakia blollopis* SK-4 differed from the other strains in substrate utilization as well. Strain SK-4 could thrive well on lactose, D-arabinose, and inositol medium. Unlike other strains, this strain also grew on vitamin-free medium. A comparison of fermentabilities showed that *M. blollopis* SK-4 could ferment typical sugars such as glucose, sucrose, galactose, maltose, lactose, raffinose, trehalose and melibiose, while other related species were not able to strongly ferment such as various sugars (Table 3-1).

Table 3-1. Comparison of physiological characteristics of *M. blollopis* SK-4 and other *Mrakia* species.

Characteristic	*M. blollopis* SK-4	*M. blollopis* CBS8921^T	*M. psychrophhila* AS2.1971^T	*M. frigida* CBS5270^T
Maximum growth temperature	22ºC	20ºC	18ºC	17ºC
Assimilation of				
Lactose	+	w	+	v
Inositol	+	w/+	+	v
D-arabinose	+	w/-	+	v
Ethanol	w/-	+	+	+
Growth on 50% glucose	w/-	-	+	-
Growth on vitamin-free medium	+	w	+	-
Fermentation of				
Galactose	+	-	-	w

Lactose	+	-	-	-
Raffinose	+	-	-	w
Maltose	+	-	-	w

Main physiology test results for characteristics of *M. blollopis* SK-4 and related species are shown. Physiological data were taken from Fell et al. (1969), Xin and Zhou (2007), Thomas-Hall et al. (2010) and this study. +, positive; w, weak; -, negative; v, variable; nd, no data

Assessment of milk fat decomposition in model wastewater

Model wastewater containing cow's milk as a source of milk fat was prepared as an equivalent of BOD sludge loading in standard waste water treatment (0.35 kg-BOD/ kg-MLSS・day). AS containing *M. blollopis* SK-4 had a BOD removal rate of 83.1%, higher than that in the control (63.8%, Fig. 3-2A). When BOD volume load was adjusted to 1.5 fold of standard wastewater treatment (0.52 kg-BOD/ Kg-MLSS・day), BOD removal rate by AS containing *M. blollopis* SK-4 was 80.1%, higher than that in the control (65.2%, Fig. 3-2B). Regardless of BOD volume load, AS containing *M. blollopis* SK-4 had a 1.25-fold higher BOD removal rate than that of the control.

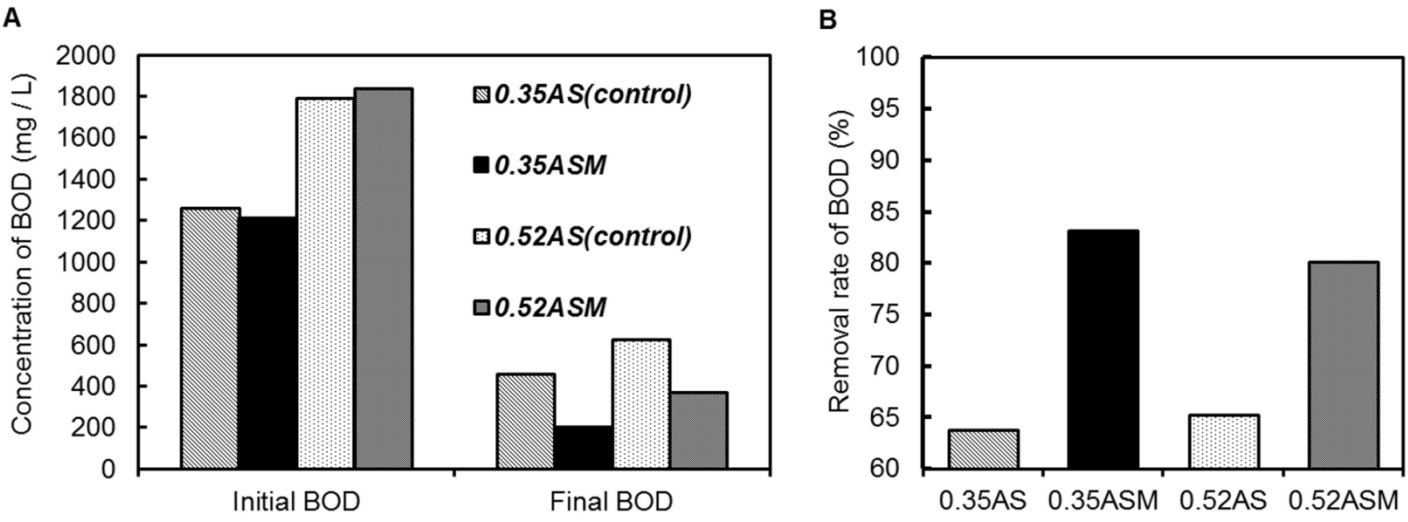

Fig. 3-2. Performance of AS system treating model milking parlor wastewater.

(A) Results of measurement of initial BOD and final BOD. (B) Effect of BOD removal rate by ASM treatment. (0.35AS) for 0.35 BOD loading rate of model parlor wastewater treated by AS (control), (0.35ASM) for 0.35 BOD loading rate of model parlor wastewater treated by AS containing *M. blollopis* SK-4, (0.52AS) for 0.52 BOD loading rate of model parlor wastewater treated by AS (control) and (0.52ASM) for 0.52 BOD loading rate of model parlor wastewater treated by activated AS *M. blollopis* SK-4.

Production of lipase from M. blollopis SK-4

Many microorganisms are known to produce lipase when Tween 80 is used as a substrate (Li et al., 2001; Taoka et al., 2011). Basidiomycetous yeast *M. blollopis* SK-4 also produced lipase. It is known that the production of lipase from *Candida rugosa* increased when yeast extract was used as a nitrogen source (Fadiloğlu and Erkmen, 2002). The same results as those for *M. blollopis* SK-4 were obtained. When 0.5% (w/v) yeast extract was used as a nitrogen source in the medium, lipase production by *M. blollopis* SK-4 increased dramatically after 180 hrs. Maximum lipase activity of 0.695 U/mL was obtained with 0.5% (w/v) yeast extract, as compared to that of 0.059 U/mL without yeast extract, after 324 hrs of inoculation; the accumulation of lipase markedly decreased (Fig. 3-3).

Morphology of *M. blollopis* SK-4 was observed by the fluorescence in situ hybridization (FISH) method during secretion of lipase. All of the morphology of *M. blollopis* SK-4 during secretion of lipase was yeast form.

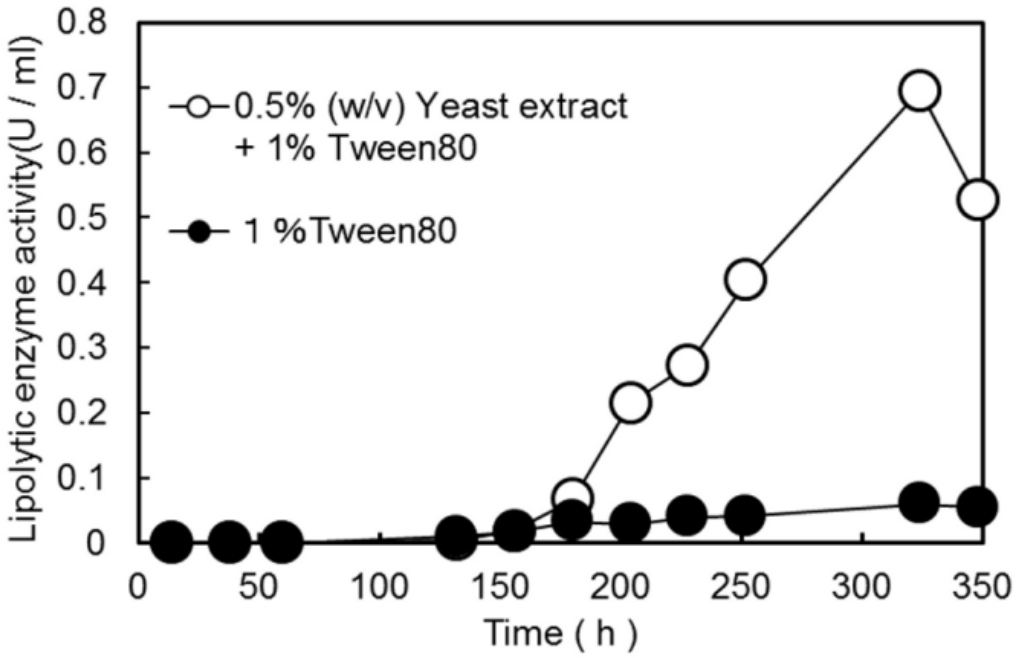

Fig.3-3. Effect of yeast extract on lipase production.

M. blollopis SK-4 was cultivated by lipase production medium (○) and lipase production medium without yeast extract (●).

Purification of lipase

Lipase production medium was centrifuged at 3000 × for 15 min at 4°C. The supernatant was filtered through a 0.45-μm membrane filter. The filtered solution was concentrated by ultrafiltration. After ultrafiltration, 72.3% of total lipase activities were recovered and 2.2-fold lipase specific activity was obtained. Then, enzyme solution was applied on a Toyopearl butyl-650M column (2.5 × 20 cm) and purified by single-step hydrophobic interaction chromatography. Finally, 9.4% of the enzyme was recovered and 20.1-fold of specific activity, compared to crude sample, was obtained with a specific activity of 51.7 U/mg (Table 3-2). The purified enzyme showed a single band on SDS-PAGE with a molecular mass of 60 kDa (Fig. 3-4).

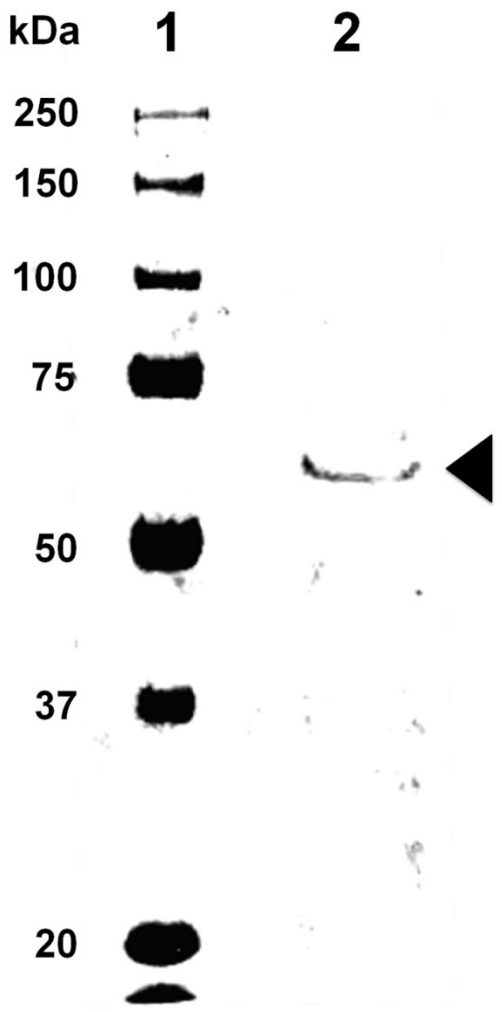

Fig. 3-4. SDS-PAGE of purified lipase from *M. blollopis* SK-4.

Lane 1. Molecular weight marker; 2. Purified lipase.

Table 3-2. Purification of lipase from *M. blollopis* SK-4

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold
Supernatant of culture medium	108.0	278.0	2.5	100.0	1.0
Ultrafilter concentration	32.2	200.9	6.2	72.3	2.2
Toyopearl Butyl-650M	0.5	26.2	51.7	9.4	20.1

Characterization of lipase

Optimum temperature of lipase activity was 60−65°C (k_{cat} =3.93 and 4.04 S^{-1}). At temperatures of 80°C and 95°C, 30.5% (k_{cat} = 1.23 S^{-1}) and 6.8% (k_{cat} = 0.27 S^{-1}) of enzyme activity were retained (Fig. 3-3A). The enzyme showed thermo-stability up to 65°C with 98.3% (k_{cat} = 5.12 S^{-1}) of residual enzyme activity even after 30-min preincubation. At 75°C, 80°C and 85°C, 48.2% (k_{cat} = 2.51S^{-1}), 41.8% (kcat = 2.18 S^{-1}) and 28.03% (k_{cat} = 1.46S^{-1}) of enzyme activity remained after 30-min preincubation (Fig. 3- 3A). Optimum pH range of lipase activity was between pH 8.0 and pH 9.0 (k_{cat} = 1.43 and 1.51 S^{-1}), whereas 57.0% (k_{cat} = 0.86 S^{-1}), 63.5% (k_{cat} = 0.96 S^{-1}) and 27.8% (k_{cat} = 0.42 S^{-1}) of the enzyme activity was retained at pH 7.0, pH 9.3 and pH 9.7 compared to 100% at pH 8.5 (Fig. 3-3B). The enzyme was quite stable over a wide pH range (4.0−10.0) and 45.5% (k_{cat} = 2.38 S^{-1}) of the enzyme activity remained at pH 3.0 even after preincubation for 15 hrs at 30°C (Fig.3-3B). Enzyme activity was affected by metal ions at a concentration of 1 mM, retaining relative activity higher than 80%. There was little inhibition of lipase activity in the presence of Cu^{2+} and Pb^{2+} ions. The metal-chelating agent EDTA did not affect lipase activity (Table 3-3). One of the most important characteristics of lipase was

substrate specificity toward various p-nitrophenyl esters (C_4−C_{18}). The substrate specificity was determined in the presence of 1 mM p-nitrophenyl esters as a substrate with 50 mM sodium phosphate (pH 7.0) at 30°C for 30 min. Relative activity toward C_4−C_{14} was more than 100% compared to 100% toward p-nitrophenyl-palmitate. C_{18} had relative activity of 71.6% (Fig. 3-6). Various organic solvents (ethanol, methanol, diethyl ether, dimethyl sulfoxide, hexane and *N, N*-dimethyl formamide) enhanced SK-4 lipase acidity. Solvents such as acetone and chloroform however, had only a slight inhibitory effect in the activity, with relative activities of 77.9% and 70.4%, respectively (Table 3-3). Organic solvents are known to be severely toxic to most enzymes including lipase (Iizumi et al., 1990).

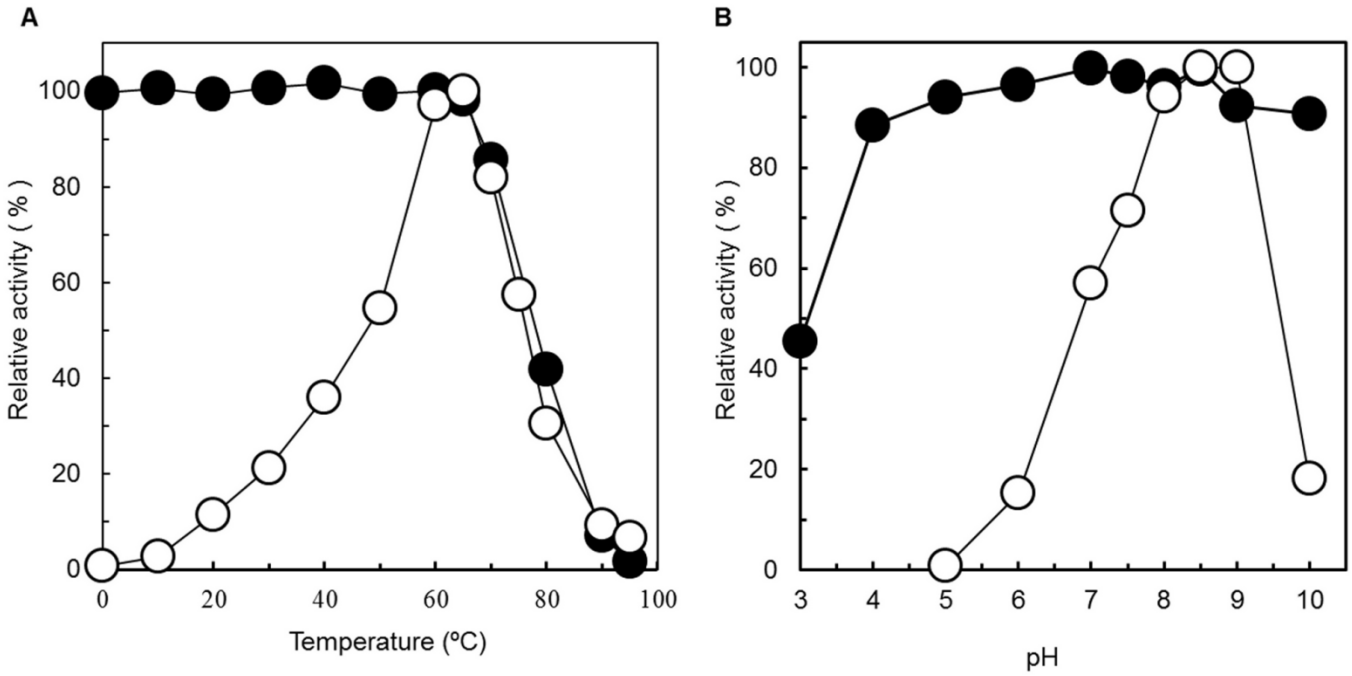

Figure 3-3. Effects of temperature and pH on SK-4 lipase activity.

(A) Effects of temperature on lipase activity (○) and thermostability of the lipase (●). For effect of temperature, lipase activity assay was performed at various temperatures in 50 mM sodium phosphate buffer (pH 7.0) for 30 min. For thermostability, lipase was preincubated at various temperatures for 30 min. Remaining lipase activity was examined at 65°C for 30 min in 50 mM Tris-HCl buffer (pH 8.5). The line was fitted by using the Eyring-Arrhenius equation.

(B) Effects of pH on lipase activity (○) and pH stability of lipase (●). For effect of pH, lipase activity assay was performed with various buffers at 30°C for 30 min using p-nitrophenyl-palmitate as a substrate. For pH stability, lipase was preincubated in various pH

buffers at 30°C for 15 h and then pH of the buffer was adjusted to 8.5. The remaining enzyme activity was examined at 65°C for 30 min using p-nitrophenyl-palmitate as a substrate. The line was fitted by Henderson- Hasselbalch equation.

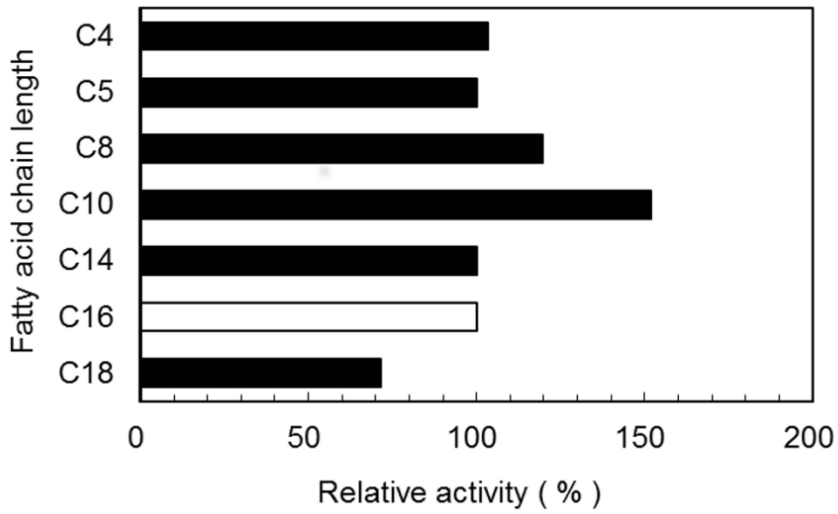

Fig. 3-4. Substrate specificity of lipase form *M. blollopis* SK-4.

Substrates were p-nitrophenyl esters. Relative activity for each substrate (■) is expressed as the percentage of activity toward p-nitrophenyl-palmitate (□).

Table 3-3.Comparison of the effects of various metal ions, EDTA and various organic solvents on *Mrakia blollops* SK-4 and *Cryptococcus* sp. S-2 lipase activity

Divalent cation or EDTA or	Relative activity (%)	
Organic solvents	*M. blollopis* SK-4	*Cryptococcus* sp. S-2
None	100.0	100.0
EDTA	106.7	ND
Metal salts		
1mM $MgCl_2$	102.2	>70
1mM $MnCl_2$	101.6	>70
1mM $FeCl_2$	90.0	>70
1mM $ZnCl_2$	83.6	ND
1mM $CoCl_2$	85.9	ND
1mM $CaCl_2$	112.8	>70
1mM $PbCl_2$	61.6	ND
1mM $CuCl_2$	67.2	<70

Ethanol	107.5	ND
Methanol	112.3	53.3
Diethyl ether	113.1	116.7
2 – Propanol	95.5	ND
Dimethyl sulfoxide(DMSO)	134.3	121.4
Hexane	104.9	95.2
Acetone	77.9	83.3
Chloroform	70.4	74.8
N,N- dimethylformamide (DMF)	122.3	92.4

The effects of various metal ions, EDTA and various organic solvents on *M. blollopis* SK-4 lipase and *Cryptococcus* sp. S-2 lipase data were taken from Iefuji et al. (1994) and this study.

3-4. Discussion

The Antarctic yeast strain SK-4, which was identified as *M. blollopis*, showed several unique characteristics; for example, it can use various carbon sources as nutrition, it prefers relatively high temperature conditions among allied species and it can be activated even in vitamin-free conditions. These results suggested that strain SK-4 is a potential candidate for a biological agent to decompose various sugars in milk parlor wastewater under low temperature conditions. This expectation was further reconfirmed by the application of strain SK-4 in AS; namely, addition of SK-4 to AS in the model parlor wastewater improved the BOD removal rate.

Improved BOD removal rate of the AS was attributed to lipase activity of SK-4 lower temperatures but stronger activity in middle to high temperature conditions compared to activities of those from the other psychrophilic fungi (Hoshino et al., 1997). *Mrakia blollopis* SK-4 lipase, in addition, was quite stable in wide ranges of temperature and pH conditions and was not affected by the existence of EDTA, various metals ions, or organic solvents. *Mrakia blollopis* SK-4 is thought to have acquired stable lipases by growing in extreme environments such as the Antarctica.

Table 3-4. Comparison of *M. blollopis* SK-4 lipase characteristics with other yeast lipase characteristics

Yeasts	MW in kDa	Optimum pH	pH stability range	Optimum temperature
Mrakia blollopis SK-4	60	8.0~9.0	4.0~10.0	60 – 65°C
Candida antarctaca	43 (lipaseA)	7.0	-	50°C
Candida rugosa ATCC1483	60	5.0	-	-
Cryptococcus sp. S-2	22	7.0	5.0-9.0	37°C
Geotrichum candidum	55	5.6~7.0	4.2~9.8	40°C
Yarrowia lipolytica	44 (lip)	8.0 (lip)	4.5~8.0	37°C
Kurtzmanomyces sp. L-11	49	1.9~7.2	Below 7.1	75°C
Trichosporon asteroids	37	5.0	3.0~10.0	50°C

Comparison of the lipase from *M. blollopis* SK-4 with that from *Cryptococcus* sp. S-2, which has actually been used in wastewater treatment (Iefuji et al., 1994; Kamini et al., 2000), revealed that the former was superior to the latter both in thermo-stability and pH stability (Table 3-4); i.e., the lipase from *M. blollopis* SK-4 retained 71.1% of the enzyme activity after 30 min at 60°C and was stable for 6 hrs at 30°C in the pH range from 5.0 to 9.0.

In conclusion, *M. blollopis* SK-4 has the ability to assimilate various carbon compounds and to use various sugars for fermentation. Moreover, the lipase is more tolerant in relatively higher temperature conditions and wider pH ranges, less sensitive to various metal ions and organic solvents, and highly reactive to various chain lengths of substrates. SK-4 lipase, therefore, is a promising biological agent for parlor wastewater treatment even in low temperature regions of the world.

This is an Author's Original Manuscript of an article published by Public Library of Science in PLOS ONE on 14/03/2013, available online: http://dx.doi.org/ 10.1371/journal.pone.0059376".

Chapter 4

Influence of initial pH on ethanol production by the Antarctic basidiomycetous Yeast *Mrakia blollopis*

4-1. Introduction

Mrakia bollopis SK-4, isolated from Naga-ike Lake in Skarvsnes, East Antarctica, was found to ferment for typical sugars such as glucose, sucrose, maltose, lactose, raffinose, and galactose at low temperatures (Tsuji et al., 2013b), but, little is known about ethanol production by basidiomycetous yeasts. Moreover, optimal pH and cell viability for ethanol fermentation have not yet been studied for basidiomycetous yeasts. Here in this chapter it was analyzed that the effects of pH on the ethanol fermentation and cell viability of the cryophilic basidiomycetous yeast *M. blollopis* SK-4.

4-2. Materials and Methods

Ten μL of *M. blollopis* SK-4 was inoculated in 400 mL of YPD liquid medium (10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of glucose) and inoculated at 120 rpm for 120 hrs at 10ºC. After 120 hrs, 400 mL of culture was collected by centrifugation at 3,500 × g for 10 min at 4°C.

The pellet was resuspended in 50 mL of distilled water, and the resulting culture (OD_{600} = 170) was used as an inoculum. OD_{600} = 1 of the *M. blollopis* SK-4 cells, cultured at 10°C after 120 h was 1.0×10^7 CFU/mL.

Experiments were performed in 28 -mL glass vials. The fermentation mixture consisted of 40 g/L of glucose, 5 g/L of yeast extract, 5 g/L of Bacto peptone, 2 g/L of NH_4Cl, 1 g/L of KH_2PO_4, and 0.3 g/L of $MgSO_4 \cdot 7H_2O$ in 50 mM of various buffers as follows: sodium citrate (pH 2.0-5.0), sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.5), and sodium carbonate (pH 9.0-11.0). A final concentration of 8.5×10^7 CFU/mL SK-4 was added to the sterilized fermentation mixture. Ten mL of each mixture was fermented at 120 rpm at 10°C. Six hundred μL of each culture was collected every 24 hrs, and the supernatants were used for measurement of glucose and ethanol concentrations. The collected samples were diluted in distilled water up to dilutions of 10^4-10^6 and then inoculated on YPD agar plates (Difco™, BD Japan, Tokyo), and incubated at 10°C for 5 days. The colonies that appeared after 5days of incubation were counted.

The glucose and ethanol concentrations in the fermentation solutions were measured by HPLC. All samples were analyzed by HPLC using an Aminex HP87 cation exchange column with both

UV and RI detection at 0.6 mL/min at 65°C or 80°C (Goshima et al., 2013). All experiments were carried out independently in three vials, and average results are given.

4-3. Results and Discussion

The results of ethanol fermentation at pH 2.0-11.0 are shown in Fig. 1. *M. blollopis* SK-4 fermented between pH 2.0 and pH 11.0. For maximum ethanol productivity using SK-4, the optimum pH was 8.0-10.0. At pH 2.0-4.0 and 10.5-11.0, SK-4 did not completely convert glucose to ethanol until 168 hrs of fermentation. When the yeast was fermented at pH 6.0 - 7.0, the glucose consumption speed was slower than that at pH 5.0 and glucose was completely consumed within 168 hrs. Moreover, when I performed a fermentation test, 3 times at pH 5.0, glucose was completely consumed by 168 hrs of fermentation. At pH 8.0-10.0, the ethanol fermentation rate was fastest. The details of maximum ethanol concentration ($EtOH_M$), ethanol yield based on total glucose content in the ethanol fermentation mixture ($Y_{E/G}$), theoretical ethanol yield ($Y_{E/EY}$) and ethanol production rate at 24 hrs of ethanol fermentation (Q_E) are summarized in Table 4-1. The theoretical yield of ethanol production was calculated as follows:

%Theoretical yield [$Y_{E/EY}$ (%)] = $EtOH_M$ / (Initial glucose concentration × 0.51).

Table 4-1. Summary of Ethanol Fermentation at Various pH Values by *M. blollopis*

	Initial glucose concentration (g/L)	$EtOH_M$ (g/L)	$Y_{E/G}$ (g/g)	$Y_{E/EY}$ (%)	Q_E (g/L·h)
pH 2.0	44.9	12.8	0.29	55.9	0.15
pH 3.0	43.0	15.7	0.36	71.5	0.17
pH 4.0	45.2	17.2	0.38	74.5	0.15
pH 5.0	44.0	19.1	0.43	85.3	0.19
pH 6.0	44.2	18.2	0.41	80.9	0.16
pH 7.0	43.8	17.9	0.41	80.2	0.18
pH 8.0	44.5	19.3	0.43	85.1	0.25
pH 8.5	45.9	20.4	0.44	87.0	0.20
pH 9.0	46.4	19.5	0.42	82.4	0.23
pH 10.0	45.3	19.2	0.42	83.0	0.23
pH 10.5	43.2	16.6	0.38	75.3	0.14

pH 11.0	43.2	15.9	0.38	72.1	0.13

$EtOH_M$, maximum ethanol concentration after 168 h of ethanol fermentation. $Y_{E/G}$, ethanol yield based on total glucose content in the ethanol fermentation mixture. $Y_{E/EY}$, The theoretical yield of ethanol is 0.51 g ethanol/g glucose. Q_E, volumetric ethanol production rate after 24 h of ethanol fermentation.

I also measured pH of the culturing mixture during fermentation. At pH 2.0-4.0, the pH of the mixtures did not change after 168 h of fermentation. However, in the cases of pH 5.0-7.0, 8.0-9.0 and 10.0-11.0, the pHs of fermentation mixtures were 7.0, 7.5 and 8.0-8.5 respectively after 24 hrs of fermentation, and these pH remained steady after 168 hrs of fermentation. The colony counts of SK-4 dramatically decreased during first 24 hrs under all fermentation conditions, and then they gradually decreased, through the fermentation time. At pH 5.0-10.0, the cell viability of the yeast was higher than that at other pH values, but I do not know why SK-4 cell viability dramatically decreased in the first 24 hrs of fermentation (Fig. 4-1).

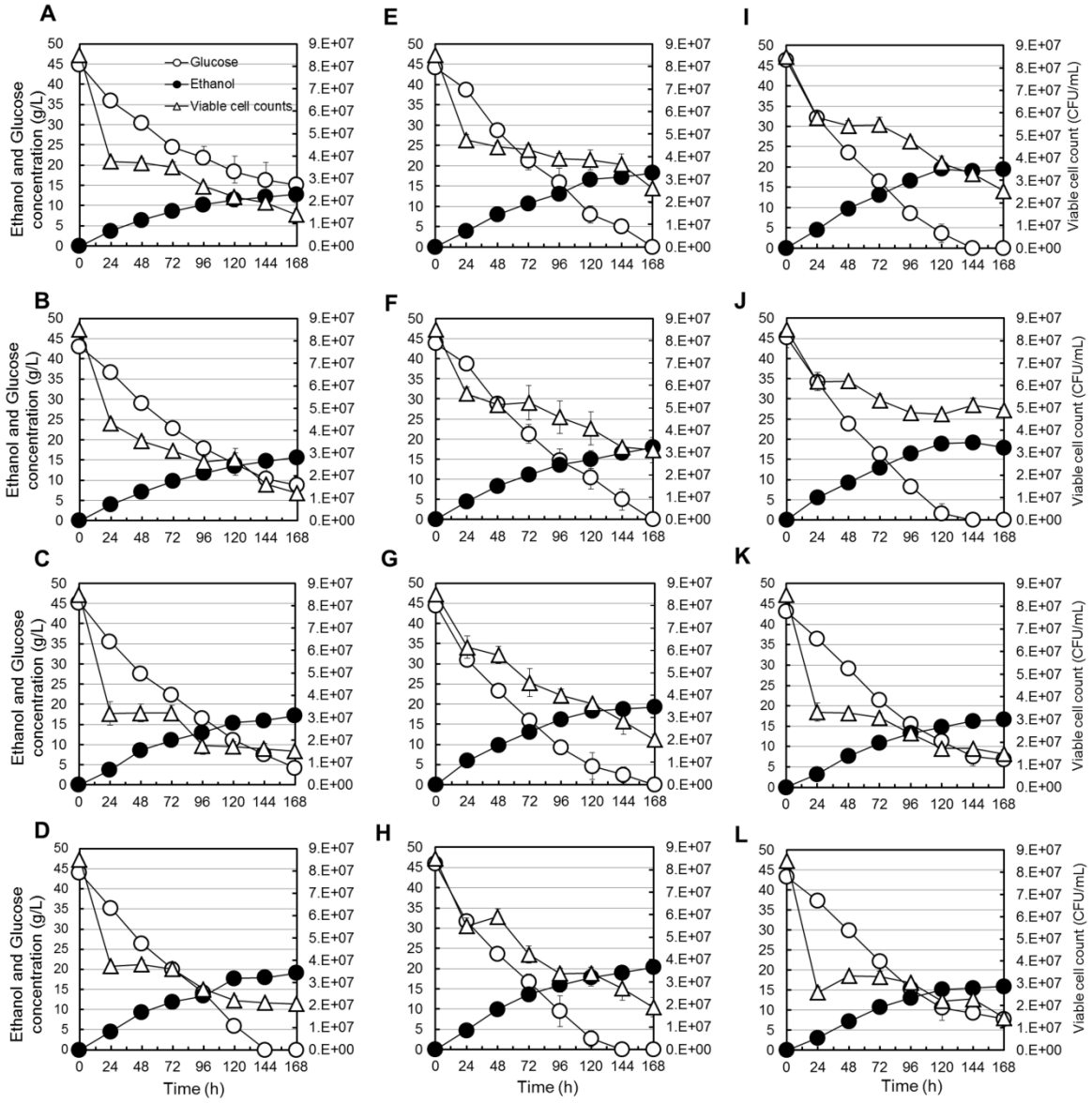

Fig.4- 1. Effect of Initial pH on Ethanol Production

A, pH 2.0; B, pH 3.0; C, pH 4.0; D, pH 5.0; E, pH 6.0; F, pH 7.0; G, pH 8.0; H, pH 8.5; I., pH 9.0; J, pH 10.0; K, pH 10.5; L, pH 11.0. All rounds of fermentation were performed at 10°C.

Triangles indicate viable cell counts, circles denote ethanol concentrations, and diamonds indicate glucose concentrations. Vertical axes denote glucose and ethanol concentrations (g/L) and second vertical axes indicate viable cell counts (CFU/mL). Horizontal axes indicate time (h). All experiments were carried out in triplicate and error bars indicate standard deviation.

To the best of my knowledge, little is known about the fermentation by basidiomycetous yeast. Some species have been reported to have fermentative ability, such as *Mrakiella* spp. (Jones and Slooff, 1966), *Rhodotorula* spp. (Rao et al., 2008), *Xanthophyllomyces* spp. (Fell et al., 2011), and *Bandoniozyma* spp. (Valente et al., 2012). Seven species of *Mrakia* have been reported: *M. frigida*, *M. gelida*, *M. stokesii*, *M. nivalis*, *M. psychrophila*, *M. robertii*, and *M. blollopis* (Thomass-Hall et al., 2010). Species in this basidiomycetous yeast genus are known for their ability to ferment sugars. Actually, all species could ferment glucose and sucrose. *M. frigida*, *M. blollopis*, *M. gelida*, and *M. robertii* were used for fermentation tests with a home brewing kit. Thomass-Holl et al. (2010) reported that all of those strains fermented sucrose, but did not completely convert sucrose to ethanol, and that cell growth was stopped at the ethanol concentration higher than 2% (v/v) ethanol. Strain SK-4 fermented raffinose, galactose, lactose, and maltose at low temperature, while *M. blollopis* CBS8921^{T} could not ferment raffinose, galactose, lactose, or maltose (Tsuji et al., 2013b). Moreover, it fermented, at –1°C to 20°C and the optimum ethanol fermentation temperature was 10-15°C.

I had little information about SK-4 fermentability, and hence I tested ethanol production by SK-4 at various pH values. When used for fermentation at pH below 4.0 and above 10.5, SK-4 did not completely convert glucose to ethanol. Buzás *et al.* (1988) reported the effect of pH on ethanol fermentation by *Saccharomyces cerevisiae* SC1. The optimal pH for strain SC1 was 4.0. When strain SC1 was used for fermentation at pH 2.0, fermentation capacity was 70% of that at pH 4.0. Ethanol production by SC1 dramatically decreased at pH values above 8.0. The halotolerant yeast *Debaryomyces nepalensis* NYC 3413 is known to survive pH 3.0-11.0, and the optimum fermentation pH of strain NYC 3413 is pH 6.0. When strain NYC 3413 was used for fermentation at pH 5.0 and 6.5, the fermentation capacity was 25% and 78% of that at pH 6.0 (Kumdam et al., 2013). *Mrakia blollopis* SK-4, on the other hand, had an ability for fermentation at pH 5.0 and 10.0. For maximum ethanol productivity with SK-4, the optimum pH was 8.0–10.0. It had high fermentation ability even at pH 5.0. Moreover, the fermentation capacity was about 63% and 78% of that of pH 8.5, at pH 2.0 and 11.0, respectively.

Strain SK-4 was isolated from an algal mat in lake sediment of Naga-ike, a lake in the Skarvsnes ice-free area of East Antarctica. Strain SK-4 secretes extracellular enzymes such as

cellulase, β-glucosidase, catalase, and amylase as well as lipase under low temperature conditions. SK-4 has a lipase stable against metal ions and organic solvents, and the optimum pH of SK-4 lipase is 8.5-9.0 (Tsuji et al., 2013b). Naga-ike is an oligotrophic lake, and pH is 8.5 (Tanabe, 2009). This is the first report on the influence of initial pH on ethanol fermentation by cryophilic basidiomycetous yeast at low temperature conditions.

This is an Author's Original Manuscript of an article published by Taylor & Francis Group in Bioscience, Biotechnology, and Biochemistry on 14/05/2014, available online: http://www.tandfonline.com/doi/abs/10.1271/bbb.130497".

Chapter 5

Direct ethanol fermentation from lignocellulosic biomass by Antarctic basidiomycetous yeast *Mrakia blollopis* under a low temperature condition

5-1. Introduction

Lignocellulosic biomass is mainly composed of heterogeneous complexes of cellulose, hemicellulose and lignin. Lignin tightly binds to fibers of cellulose and hemicellulose. These interactions impede enzymatic hydrolysis (Fengel and Wegener, 1984). Various pretreatments including mechanochemical (Millett et al., 1976; Endo et al., 2006), dilute acid (Esteghalin et al., 1997), alkali (Fan et al., 1987) and organosolv processes (Chum et al., 1988) have been used to split these bonds. However, end-products from lignocellulose have been shown to play a major role as inhibitors of the hydrolysis reaction (Andric et al., 2010). Glucose, cellobiose and ethanol have been shown to significantly inhibit β-glucosidase and cellulase (Holtzapple et al., 1990; Tengbord et al., 2001).

Direct ethanol fermentation (DEF) from cellulosic biomass was firstly reported by Takagi et al. (1977). In this technique, enzymatic hydrolysis and ethanol fermentation are carried out at the same time. In the presence of a high concentration of glucose, cellulase activity is considerably depressed. However, when yeast is mixed with an enzymatic hydrolysis reactor, glucose, formed

by cellulase activity from cellulolytic biomass, is maintained at a low concentration and is rapidly converted to ethanol via yeast. This technique is therefore expected to improve saccharification and ethanol fermentation rates. DEF using cryophilic yeasts, however, has not been reported. Moreover, little is known about fermentability of basidiomycetous yeast. Here I describe ethanol fermentation from glucose and hyfrolysates of lignocellulosic biomass, and DEF from lignocellilosic biomass by Antarctic yeast *M. blollopis* SK-4 under a low temperature condition.

5-2. Materials and methods

Inoculum

Ten µl of *M. blollopis* SK-4 was inoculated in 400 ml YPD liquid medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) and the culture was inoculated at 120 rpm for 120 hrs at 15ºC. After 120 hrs, 400 ml of culture was collected by centrifugation at 3500 × g for 10 min at 4°C. The pellet was dissolved in 50 ml of distilled water. The resulting culture (OD_{600} = 150) was used as the inoculum.

Enzymatic saccharification from lignocellulosic biomass

Mechanochemically treated Japanese cedar and Eucalyptus chips (Endo et al., 2006) were kindly provided by Dr. Tomoaki Minowa (BRRC, AIST). The enzymatic saccharification were performed in 2000 ml flasks. Twenty percent (w/v) of these chips were hydrolyzed by 6 FPU/g-dry substrate Acremozyme (Meiji Co., Ltd., Osaka, Japan) and 20 μL/g Optimash BG (Genencor Internatinal, USA) in 0.02 M citrate buffer (pH 5.0) at 120 rpm for 120 hrs at 37°C. After 120 hrs, the sacchairfied samples were centrifuged at 3000 × g for 10 min at 4°C. Then the supernatant was filtered through a 0.2-μm PES membrane filter (Merck Millipore, USA). Filtered samples were used as hydrolysates, and glucose concentrations in the solutions were measured.

Ethanol fermentation from glucose under a low temperature condition

The ethanol fermentation was carried out in 50 mL vials under sterile conditions. The ethanol fermentation mixture consisted of 40 g/L, 60 g/L or 120 g/L of glucose, 5 g/L yeast extract, 5 g/L Bacto peptone, 2 g/L NH_4Cl, 1 g/L KH_2PO_4 and 0.3 g/L $MgSO_4 \bullet 7H_2O$ in 20 mM citrate buffer (pH 5.0), and 50 mL/L of inoculum was also added. Strain SK-4 was fermented at 120 rpm at 10ºC. Six hundred μl of each samples was collected every 24hrs and centrifuged at 5000 × g for 10 min at 4ºC. The supernatant were used for measurement of glucose and ethanol concentrations.

Fermentation of hydrolysate from lignocellulosic biomass

The ethanol fermentation was carried out in 50 mL vials under sterile conditions. The ethanol fermentation mixture from the hydrolysate, described above, consisted of 1 L of hydrolysate, 5 g/L yeast extract, 5 g/L Bacto peptone, 2 g/L NH_4Cl, 1 g/L KH_2PO_4 and 0.3 g/L $MgSO_4 \cdot 7H_2O$, and 50mL/L of inoculum was also added. Strain SK-4 was fermented at 120 rpm at 10°C.

Fermentation samples were collected every 24 hrs and was centrifuged at 5000 × g for 5 min at 4°C. The supernatant was filtered through a 0.2-μm olefin membrane filter (GL Science, Tokyo, Japan). Filtered samples were used as fermentation solutions, and concentrations of residual glucose and ethanol in the solutions were measured.

DEF from filter paper and lignocellulosic biomass

DEF experiments were carried out in 50-mL vials. The DEF mixtures consisted of 5% (w/v) filter paper or 10% (w/v) lignocellulosic biomass, 5 g/L yeast extract, 5 g/L Bacto peptone, 2 g/L NH_4Cl, 1 g/L KH_2PO_4 and 0.3 g/L $MgSO_4 \cdot 7H_2O$ in 20 mM citrate buffer (pH 5.0), 50 FPU/g-dry substerate Acremozyme and 20 μL/g-dry substrate Optimash BG, and 50mL/L of inoculum were also added. The DEF mixtures were fermented at 180 rpm for 144 hrs at 10°C. Fermentation samples were collected every 24 hrs and centrifuged at 12100 × g for 5 min at 4°C. The supernatant were filtered through a 0.2-μm olefin membrane filter. Filtered samples were used as fermentation solutions, and residual glucose and ethanol concentrations in the solutions were measured.

DEF from lignocellulosic biomass in the presence of Tween 80 at a low temperature

DEF with Tween 80 was performed under the same conditions as those described above except for the addition of 1% (v/v) Tween 80 to the reaction mixtures. 'DEF with Tween 80' samples were collected every 24 hrs and centrifuged at 12100 × g for 5 min at 4°C. The supernatant was filtered through a 0.2-μm olefin membrane filter. Filtered samples were used as 'DEF with Tween 80' solutions, and residual glucose and ethanol concentration in the solutions were measured.

Analysis conditions

Glucose and ethanol concentrations in the ethanol fermentation solutions, hydrolysates and DEF without/with Tween 80 solutions were measured by high-performance liquid chromatography (HPLC). All samples were analyzed by HPLC using an Aminex HP87 cation exchange column using both UV and RI detection with 0.6 mL/min at 65°C or 80°C. The cell

density was monitored by the absorbance at 600 nm with a BioSpectrometer (Eppendorf, Hamburg, Germany).

5-3. Results

Low-temperature fermentation from glucose

From 40 g/L glucose, ethanol was rapidly formed from 24 hrs to 48 hrs of fermentation. Fermentation was almost completed after 72 hrs (Fig. 5-1A). When the yeast fermented 60 g/L glucose, the rate of ethanol conversion was almost the same as that in 40 g/L glucose up to 72 hrs fermentation, though the rate declined from 72 hrs to 120 hrs of fermentation (Fig. 5-1A). In the case of 120 g/L glucose, glucose was gradually consumed and ethanol was continuously produced until 19 days of fermentation (Fig. 5-1B). Finally, 14.4 g/L, 20.4 g/L and 48.2 g/L ethanol were obtained from 40 g/L, 60 g/L and 120 g/L glucose, respectively. At first, optical density (OD_{600}) of SK-4 continuously increased and then remained constant until the end of the reaction regardless of glucose concentration. (Fig. 5-1A, B).

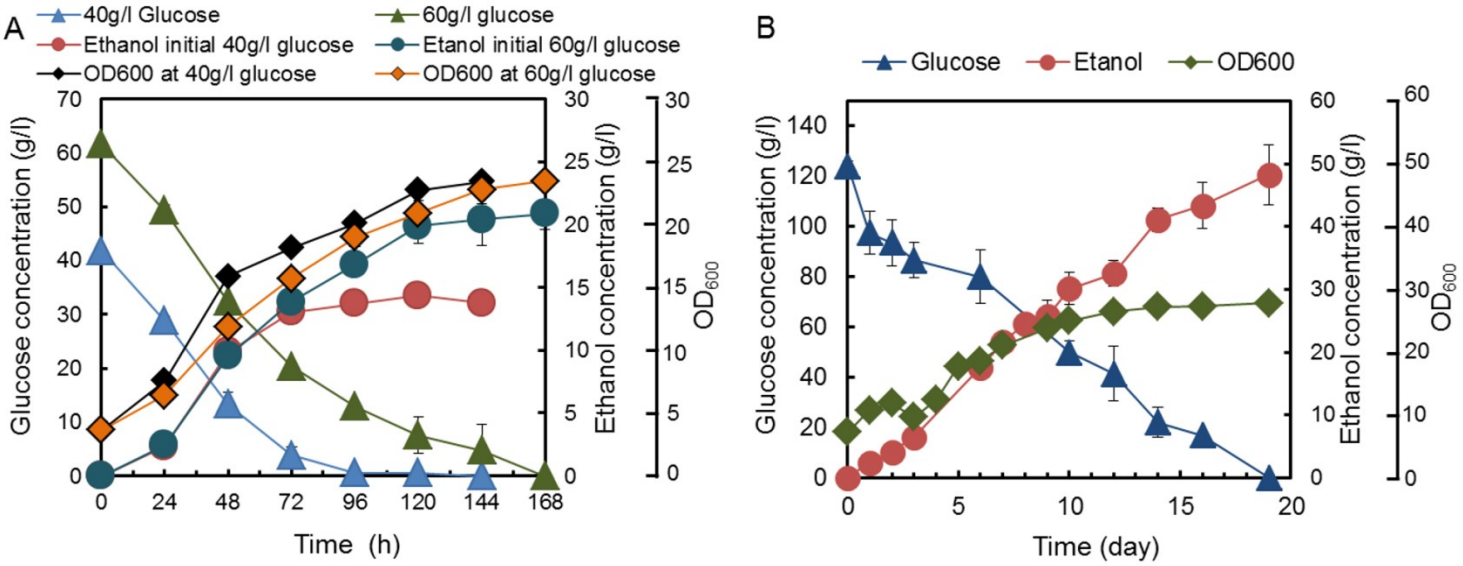

Fig. 5-1. Ethanol fermentation from various concentrations of glucose.

(A) 40 g/L glucose and 60 g/L glucose, (B) 120 g/L glucose

All ethanol fermentations were performed at 10°C. Triangles indicate glucose concentrations, circles denote ethanol concentrations and diamonds indicate OD_{600} values. All experiments were carried out in triplicate and error bars indicate standard deviation values.

Low-temperature fermentation of hydrolysates from lignocellulosic biomass

In hydrolysates from Japanese cedar, ethanol concentration dramatically increased between 24 hrs and 48 hrs of fermentation. Little ethanol was formed after 72 hrs to 120 hrs of fermentation. Glucose was completely digested after 72 hrs of fermentation (Fig. 5-2). The maximum concentration of ethanol formed from Japanese cedar hydrolysates was 17.4 g/L. OD_{600} value of SK-4 in the Japanese cedar hydrolysate continuously increased until the end of reaction (Fig. 5-2). On the other hand, SK-4 could not ferment the hydrolysate from eucalyptus and its OD_{600} value increased slowly until the end of reaction compared to that in the Japanese cedar hydrolysate (Fig. 5-2).

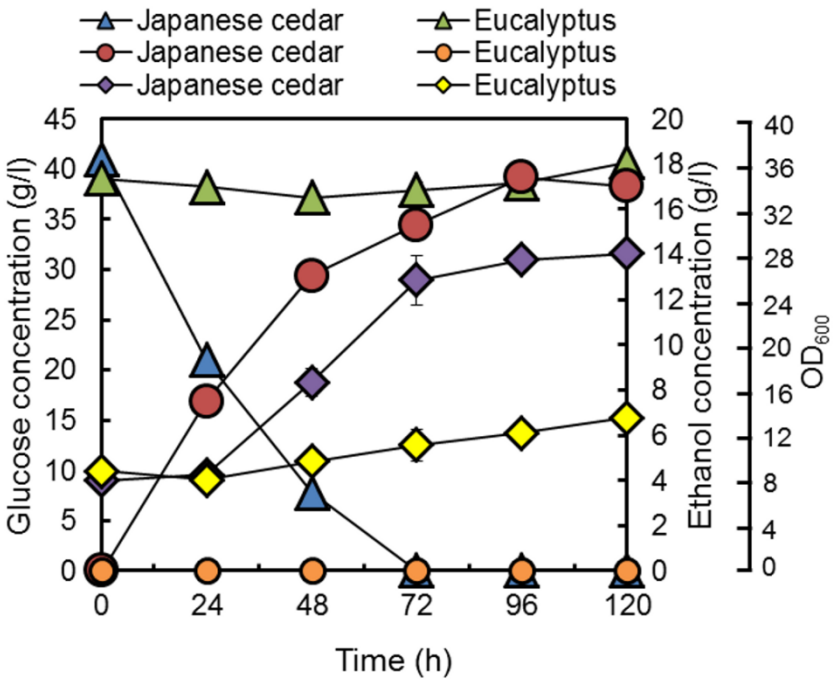

Fig. 5-2. Ethanol fermentation from hydrolysates of lignocellulosic biomass.

All ethanol fermentations were carried out at 10°C. Triangles denote glucose concentrations, circles denote ethanol concentrations and diamonds indicate OD_{600} values. All experiments were performed in triplicate and error bars show standard deviation values.

DEF from cellulosic biomass at a low temperature

Glucose, derived from digestion of filter paper, was formed at a low concentration after 24 hr and was gradually consumed until 72 hrs of fermentation. Glucose was then maintained at a very low concentration (Fig. 5-3A). Finally, about 12.2 g/L ethanol was generated after 144 hrs (Fig. 5-3B). Seven g/L of glucose was formed in the first 24 hrs of fermentation from DEF with Japanese cedar, and glucose was gradually converted to ethanol up to 120 hrs (Fig. 5-3A, B). The rate of ethanol conversion gradually decreased with fermentation time. Finally, 12.5 g/L ethanol was produced from DEF of Japanese cedar (Fig. 5-3B). When DEF from Eucalyptus was performed with SK-4, 11.9 g/L glucose was produced within 24 hrs (Fig. 5-3A). The concentration of glucose continuously increased and 14.2 g/L glucose was formed after 120 hrs of fermentation (Fig. 5-3A). After 48 hrs of fermentation, 5.4 g/L ethanol was formed from glucose. Maximally, 7.2 g/L ethanol was formed after 144 hrs (Fig.5- 3B).

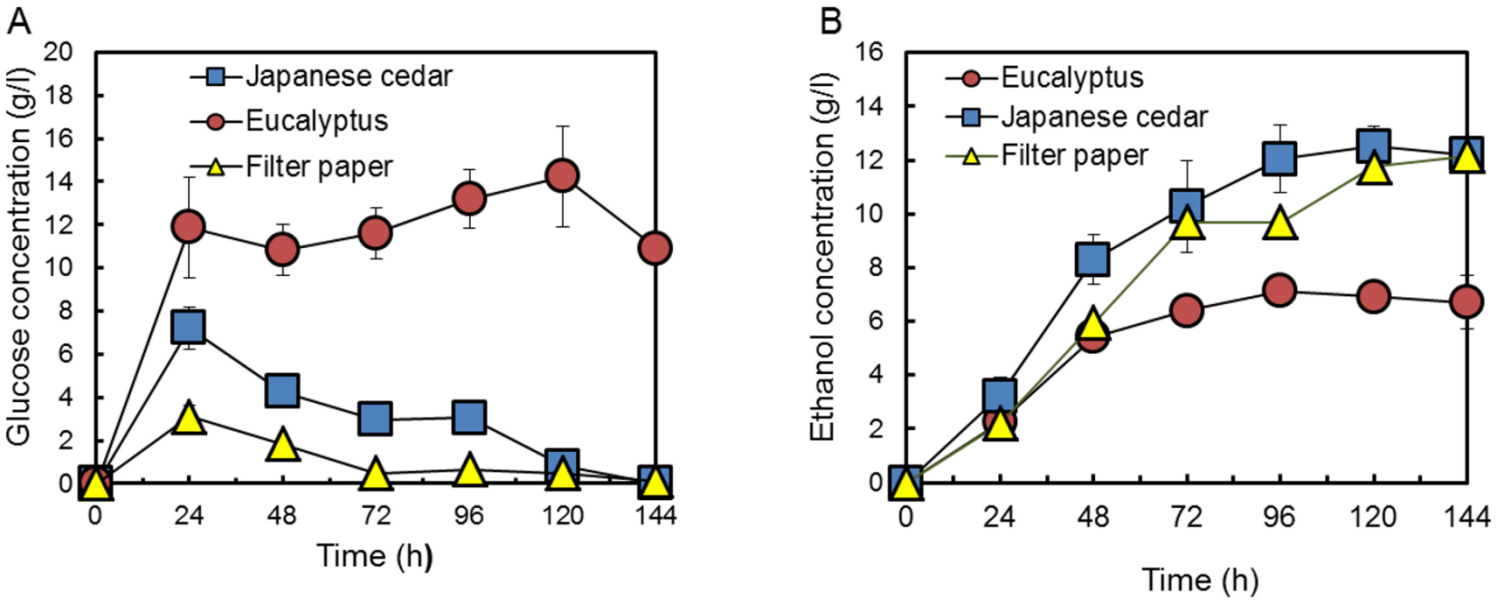

Fig. 5-3. DEF from cellulosic substrates.

(A) Glucose concentration (B) Ethanol concentration

All DEFs were carried out at 10°C. All experiments were performed in triplicate and error bars indicate standard deviation values.

Improvement of DEF with non-ionic surfactant

As a result of DEF from Japanese cedar with 1% (v/v) Tween 80, 1.5 g/l glucose was formed after 24 hrs of fermentation and glucose was maintained at a very low concentration. The rate of ethanol conversion was almost constant up to 96 hrs of fermentation. Finally, 14.1 g/L ethanol was produced after 144 hrs of fermentation (Fig. 5-4A, B). When eucalyptus was mixed with 1% (v/v) Tween 80, 7.3 g/L glucose was rapidly released from the biomass and was slowly converted to ethanol. After 144 hrs, 11.6 g/L of ethanol was formed, and 4.6 g/L of glucose remained at the end (Fig. 5-4 A, B).

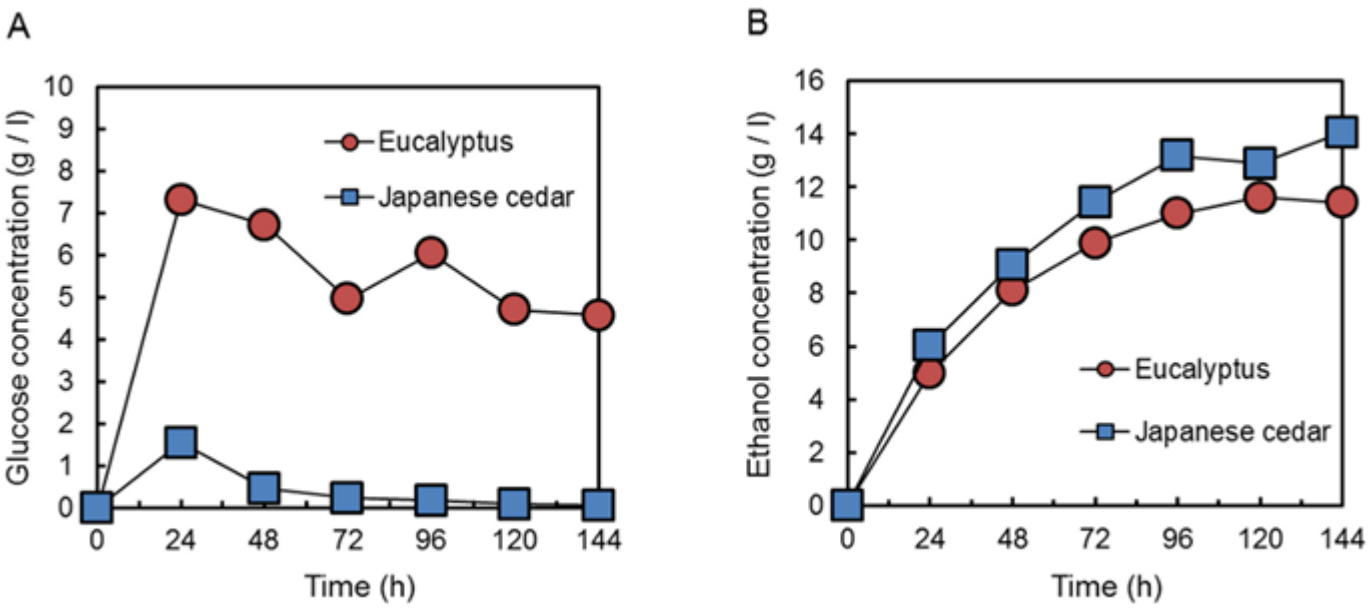

Fig. 5-4. DEF from lignocellulosic biomass with 1% (v/v) Tween 80.

(A) Glucose concentration (B) Ethanol concentration

All DEFs were carried out at 10°C. All experiments were carried out in triplicate and error bars denote standard deviation values.

5-4. Discussion

Strain SK-4 could ferment various sugars (Tsuji et al., 2013b). The author therefore tested the ethanol fermentation ability of SK-4 by using glucose at various concentrations. Strain SK-4 could strongly ferment 4% (w/v), 6% (w/v) and 12% (w/v) glucose and completely consumed all glucose. Maximally, 48.2 g/L ethanol was obtained from 12% (w/v) glucose (Fig. 5-1B).

Next, I tested the ability of SK-4 to ferment ethanol in hydrolysates from Japanese cedar and Eucalyptus. SK-4 completely consumed glucose derived from Japanese cedar cellulose. On the other hand, SK-4 could not utilize any glucose from eucalyptus hydrolysate. After 120 hrs of fermentation, the pellet was centrifuged from the fermentation mixture, washed twice with distilled water, and inoculated on YPD agar and PDA plates. After 4 days of incubation, yeast colonies clearly appeared on both agar plates indicating that SK-4 survived in the eucalyptus fermentation mixture. Eucalyptus hydrolysate was therefore considered to contain inhibitors of growth and fermentation for SK-4 strain more than those in Japanese cedar hydrolysate.

DEF from cellulosic substrates using SK-4 was successful under a low temperature condition (Fig. 5-3A, B). Various microbes have been reported to be utilized for DEF. Dwiarti et al. (2012)

performed DEF with *Taromyces cellulolyticus* cellulase and thermotolerant *Saccharomyces cerevisiae* at 42°C. Zhang et al. (2009) studied the relationship between substrate concentration and volume of enzymes using DEF technique on the corn stover. Cantarella et al. (2004) tried DEF from steam explosion Poplar wood and demonstrated inhibitors for the DEF process that were released from the substrate. A total of 27 yeasts were tested for ethanol production at 40°C - 45°C and then *Kluyveromyces marxianus* was tested for DEF at 42°C (Ballesteros et al., 1991). Ballesteros et al. (2004) performed DEF with *K. marxianus* CECT10875 using various lignocellulosic biomasses at 42°C.

However, DEF with cryophilic yeast has not yet been reported. For this reason, DEF was performed by using cryophilic yeast *M. blollopis* SK-4 from filter paper, Japanese cedar and Eucalyptus without Tween 80. DEF from Japanese cedar converted ethanol to glucose well, although DEF from eucalyptus remained unproductive even after 144 hrs of fermentation. Eucalyptus trees are known to maximally contain about 4% of oil content (Gupta et al., 1981). This oil covers the eucalyptus surface and impeded cellulase activity. Eriksson et al. (2002) suggested that cellulase was protected from absorption by the biomass in the presence of a

non-ionic surfactant and Tween 80 was also considered to combine with lignin. Since oil was considered to be able to disperse by addition of Tween 80, I therefore tested DEF with non-ionic surfactant Tween 80. Ethanol conversion rate was improved by about 1.1-1.6 fold by the addition of Tween 80 compared to the rate without Tween 80. This is the first report on DEF using a cryophilic yeast under a low temperature condition. I consider that *M. blollopis* SK-4 has good potential for ethanol fermentation and DEF in cold environments.

This is an Author's Original Manuscript of an article published by Elsevier Group in Cryobiology journal: http://dx.doi.org/ 10.1016/j.cryobiol.2013.06.003".

Chapter 6

Improvement of direct ethanol fermentation from woody biomasses by the Antarctic basidiomycetous yeast, *Mrakia blollopis*, under a low temperature condition

6-1. Introduction

DEF from cellulosic biomass was first reported by Takagi et al. (1977). In this technique, enzymatic hydrolysis and ethanol fermentation are carried out at the same time. In the presence of a high concentration of glucose, cellulase activity is considerably depressed. However, when yeast is mixed with an enzymatic hydrolysis reactor, glucose, formed by cellulase hydrolysis of cellulolytic biomass, is maintained at a low concentration and is rapidly converted to ethanol by the yeast. This technique is therefore expected to improve saccharification and ethanol fermentation rates. However, when SK-4 was used for fermentation of mechanochemically treated Eucalyptus and Japanese cedar wood meals using the DEF technique with the non-ionic surfactant Tween 80, theoretical ethanol yields from these biomasses were 51.2% and 65.1%. Since these fermentation rates did not exceed 70%, DEF from lignocellulosic biomass with *M. blollopis* SK-4 is required to improve fermentation efficiency. In this study, I tried to improve DEF by using the non-ionic surfactant, Tween 80, and lipase at 10ºC.

6-2. Materials and methods

Inoculum

Ten µl (2.0×10^8 cells) of *M. blollopis* SK-4 was inoculated into 400 ml YPD liquid medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) and cultured at 120 rpm for 120 hrs at 10 ºC, then 400 ml of the culture was collected by centrifugation at 3500 × g for 10 min at 4°C. The pellet was suspended in 50 mL of distilled water. The initial cell density in the DEF mixture was adjusted to $OD_{600} = 7.5$, which corresponds to approximately 6.0×10^7 cells/mL.

DEF from woody biomasses

Mechanochemically treated eucalyptus wood meal (Endo et al., 2006) and Japanese cedar wood meal were kindly provided by Dr. Tomoaki Minowa (BRRC, AIST). Eucalyptus wood meal was composed of 40.0% glucan, 10.4% xylan, and 28.8% lignin, and Japanese cedar wood meal was composed of 38.2% glucan, 6.0% xylan and 34.6% lignin. These woody biomasses were used as substrates for DEF.

DEF experiments were carried out in 28-mL vials. Each basal DEF mixture consisted of 10% (w/v) woody biomass, 5 g/L yeast extract, 5 g/L Bacto peptone, 2 g/L NH_4Cl, 1 g/L KH_2PO_4 and 0.3 g/L $MgSO_4 \cdot 7H_2O$ in 20 mM citrate buffer (pH 5.0), 50 FPU/g-dry substrate Acremozyme (Meiji Co., Ltd, Tokyo, Japan) and 20 μL/g-dry substrate Optimash BG (Genencor International, Rochester, NY, USA). The medium was inoculated with 50 ml/l of inoculum. All of the DEF experiments were carried out at 180 rpm for 144 hrs at 10°C.

DEF from woody biomasses with lipase or lipase and Tween 80

DEF with lipase was performed under the same conditions as those described for the basal DEF mixtures except for the addition of 5 U/g-dry substrate *Aspergillus niger* lipase (Wako Pure Chemical Industries, Osaka, Japan) to the basal reaction mixtures. 'DEF with lipase' samples were collected every 24 hrs and centrifuged at 12100 × g for 5 min at 4°C. The supernatant was used as 'DEF with lipase' solutions, and residual glucose and ethanol concentrations in the solutions were measured.

DEF with Tween 80 and lipase was performed under the same conditions as those described for the basal DEF mixtures except for the addition of 1% (v/v) Tween 80 and 5 U/g-dry substrate *Aspergillus niger* lipase to the reaction mixtures. 'DEF with Tween 80 and lipase' samples were collected every 24 hrs and centrifuged at 12100 × g for 5 min at 4 °C. The supernatant was used as 'DEF with Tween 80 and lipase' solutions, and residual glucose and ethanol concentrations in the solutions were measured.

Analysis conditions

Glucose and ethanol concentrations in the ethanol fermentation and DEF solutions without/with lipase, or without/with Tween 80 and lipase were measured by HPLC. All samples were analyzed by HPLC with an Aminex HP87 cation exchange column using both UV and RI detection at a flow rate of 0.6 mL/min at 65°C or 80°C. The methods used for analysis were described previously in detail by Goshima et al. (2013).

6-3. Results and Discussion

The ethanol yields for DEF from Eucalyptus and Japanese cedar were each calculated as a percentage of the maximum theoretical ethanol yield of 0.51 g ethanol per gram of glucose. The maximum ethanol concentration ($EtOH_M$), ethanol yield based on total glucose content in the ethanol fermentation mixture ($Y_{E/G}$), and theoretical ethanol yield ($Y_{E/EY}$) are summarized in Table 6-1.

Table 6-1. Summary of DEF assay from Eucalyptus and Japanese cedar with/without Tween 80, lipase, and Tween 80 and lipase by *M.blollopis.*

	$EtOH_M$ (g/l)	$Y_{E/G}$ (g/g)	$Y_{E/ET}$ (%)
Eucalyptus			
DEF	7.1 (±0.25)	0.16 (±0.01)	31.4 (±1.10)
T	11.6 (±0.14)	0.26 (±0.02)	51.2 (±0.49)
L	12.6 (±1.25)	0.28 (±0.03)	55.6 (±3.11)
T+L	17.3 (±0.54)	0.39 (±0.02)	76.4 (±1.72)
Japanese cedar			
DEF	12.5 (±0.76)	0.29 (±0.01)	57.8 (±1.50)
T	14.1 (±0.19)	0.33 (±0.04)	65.2 (±0.83)
L	14.6 (±0.96)	0.34 (±0.03)	67.5 (±2.27)
T+L	17.5 (±0.54)	0.41 (±0.01)	81.0 (±1.46)

DEF, DEF without 1% (v/v) Tween 80 and lipase; T, DEF with 1% (v/v) Tween 80; L, DEF with 5 U/g-dry substrate lipase; T+L, DEF with 1% (v/v) Tween 80 and 5 U/g-dry substrate lipase; $EtOH_M$, maximum ethanol concentration after 144 hrs of DEF; $Y_{E/G}$, ethanol yield based on total glucose content in the DEF mixture; $Y_{E/ET}$, theoretical yield of ethanol (0.51 g ethanol/g glucose). Upon hydrolysis, 1 g cellulose produces 1.11 g glucose.

As a result of DEF of Eucalyptus and Japanese cedar with 5U/g-dry substrate lipase, 13.8 g/L and 18.6 g/L glucose were formed, respectively, after 24 hrs of fermentation. Finally, 12.6 g/L ethanol from Eucalyptus and 14.6 g/L ethanol from Japanese cedar were produced after 144 hrs of fermentation (Fig. 6-1A, Fig. 6-2A). When Eucalyptus was mixed with 1% (v/v) Tween 80 and 5U/g-dry substrate lipase, 10.3 g/L glucose was rapidly released from the biomass and was continuously converted to ethanol for up to 120 hrs of fermentation (Fig. 6-1B). In the case of DEF from Japanese cedar with 1% (v/v) Tween 80 and 5U/g-dry substrate lipase, 6.1 g/L glucose was formed within 24 hrs and finally 17.5 g/L ethanol was produced after 144 hrs of fermentation (Fig. 6-2B).

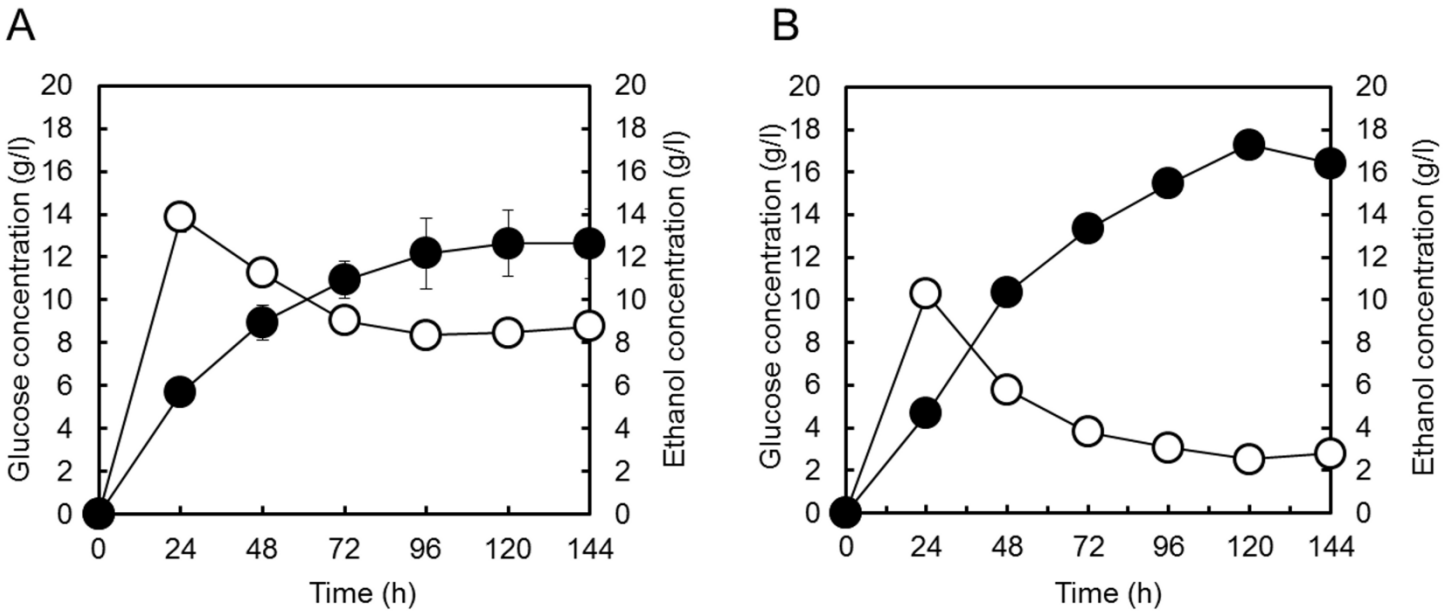

Fig. 6-1. DEF from eucalyptus wood meal with lipase or Tween 80 and liapse

(A) DEF with 5 U/g-dry substrate lipase and (B) DEF with 1% (v/v) Tween 80 and 5 U/g-dry substrate lipase. Closed circles denote ethanol concentrations and open circles indicate glucose concentrations.

All DEFs were carried out at 10°C. All experiments were carried out in triplicate and error bars denote standard deviation values.

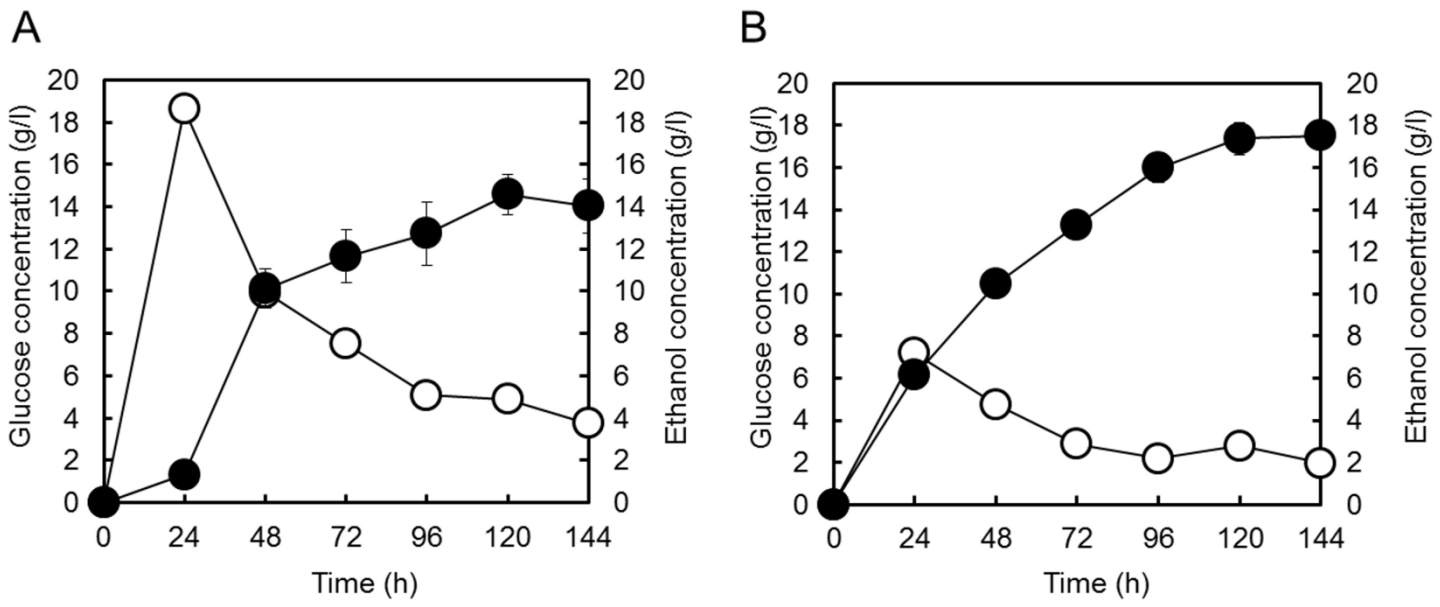

Fig. 6-2. DEF from Japanese cedar wood meal with Tween 80 and lipase

(A) DEF with 5 U/g-dry substrate lipase and (B) DEF with 1% (v/v) Tween 80 and 5 U/g-dry substrate lipase. Closed circles denote ethanol concentrations and open circles indicate glucose concentrations.

All DEFs were carried out at 10°C. All experiments were carried out in triplicate and error bars denote standard deviation values.

Ethanol fermentation under cold temperature conditions is generally used to produce wine and sake (Bakoyianis et al., 1992; Woo et al., 2013). Little is known about the fermentation ability of basidiomycetous yeast. Eight species of *Mrakia* have so far been reported: *M. frigida*, *M. gelida*, *M. stokesii*, *M. nivalis*, *M. curviuscula*, *M. psychrophila*, *M. robertii* and *M. blollopis* (Thomass-Hall et al., 2010). However, species in this basidiomycetous yeast genus are known for their ability to ferment sugars. Actually, 7 of the 8 species are able to ferment using glucose, but *M. curviuscula* lacking the ability (Fell et al., 2011).

M. blollopis SK-4, isolated from Naga-ike lake in the Skarvsnes ice-free area, could convert 120 g/l of glucose to 48.7 g/L ethanol at 10ºC, while other *Mrakia* spp. could not produce

SK-4 could completely convert glucose to ethanol at pH 4.0 to 10.0 at 10ºC (Tsuji et al., 2013c). The author previously reported DEF from lignocellulosic biomass using SK-4 at 10ºC. When DEF was carried out in the presence of 1% (v/v) Tween 80, the yield of ethanol was about 1.1 to 1.6-fold higher than that when DEF was carried out without Tween 80 (Tsuji et al., 2013d). However, in the case of DEF with 1% (v/v) Tween 80, ethanol conversion rates were only 51.2% and 65.1% of the theoretical maximum from Eucalyptus and Japanese cedar cellulose,

respectively (Table 6-1). Therefore, DEF from lignocellulosic biomasses with *M. blollopis* SK-4 is required to improve fermentation efficiency.

Eriksson et al. (2002) reported that enzymatic hydrolysis was improved by addition of non-ionic surfactants, Tween 80 and Tween 20, compared to the glucose concentration obtained by enzymatic hydrolysis without a non-ionic surfactant. This improvement in hydrolysis reaction was thought to be caused by prevention of the adsorption of cellulase on the biomass. Moreover, Eucalyptus and Japanese cedar trees are known to maximally contain about 4% oil content (Gupta et al., 1981).

The cell structures of these biomasses were completely destroyed by mechanochemical treatment (Endo et al., 2006). It was speculated that oils covering the surfaces of Eucalyptus and Japanese cedar wood meals may impede the contact of cellulase to the biomass. Since the oils can be decomposed by the addition of lipase and since the adsorption of cellulase on biomass can be prevented by the addition of Tween 80, it was examined that DEF in the presence of the non-ionic surfactant, Tween 80, and lipase.

When DEF was carried out with 5 U/g-dry substrate lipase, the yield of ethanol was approximately 1.2- to 1.8-fold higher than that when DEF was carried out without lipase. When DEF was carried out with 1% (v/v) Tween 80 and 5 U/g-dry substrate, the yield of ethanol was about 1.4- to 2.4-fold higher than that when DEF was carried out without Tween 80 and lipase. Theoretical ethanol yields were approximately 76.5% and 81.0% from Eucalyptus and Japanese cedar, respectively.

The author therefore believes that the use of DEF with Tween 80 and lipase is a good technique for the production of ethanol from woody biomass under cold temperature conditions. Moreover, if it becomes possible for cryophilic basidiomycetous yeasts to produce useful materials by genetic engineering, the combination of SK-4 and DEF with Tween 80 and lipase should be useful for the production of these materials from woody biomass under cold temperature conditions.

This is an Author's Original Manuscript of an article published by Elsevier Group in Cryobiology journal: http://dx.doi.org/10.1016/j.cryobiol.2013.12.008".

Chapter 7

Summary and General Discussion

In the present study, I first collected a total 71 fungal isolates from lake sediments and soil surrounding lakes in the Skarvsnes ice-free area, East Antarctica. Based on the sequence similarity of the rDNA ITS region, these isolates were classified into 10 genera. Twenty-three isolates were categorized as Ascomycetous fungi from five genera (*Embellisia, Phoma*, *Geomyces, Tetracladium*, and *Thelebolus*) and 48 isolates were categorized as basidiomycetous fungi from five genera (*Mrakia, Cryptococcus, Dioszegia, Rhodotorula*, and *Leucosporidium*). Thirty-five percent of culturable fungi were from the genus *Mrakia*, suggesting that they are the predominant species in this area. However, fungal biodiversity in the Skarvsnes ice-free area has not yet been fully researched, because there are still many lakes in the region whose soils and lake sediments remain untouched. Therefore, it would be interesting to conduct further investigations on fungal biodiversity in the Skarvsnes ice-free area using new high-throughput technologies such as next generation sequencing.

Eighteen isolates from the eight genera were selected and tested for both antifreeze activity and growth potential at cold temperatures ranging from −1 to 25°C. *Rhodotorula* sp. NHT-2 exhibited a high degree of sequence homology (99.8%) to *R. gracilis*, while *Leucosporidium* sp.

BSS-1 exhibited a high degree of sequence homology (100%) to *L. antarcticum* (*Glaciozyma antarctica*), and both isolates demonstrated antifreeze activity. All of the 18 isolates examined could grow at −1°C; however, no antifreeze activity was observed in *Mrakia* spp. and its ability to secrete extracellular polysaccharides was limited. Species of the genus *Mrakia* possessed high amounts of unsaturated fatty acids, suggesting that they have adapted to cold environments by increasing their membrane fluidity. Future research analyzing the molecular mechanisms behind *Mrakia*'s adaptations to the polar environment using metabolomics, whole genome sequencing, and transcriptome analysis is necessary.

I then examined the potential of the Antarctic basidiomycetous yeast *M. blollopis* as a bio-remediation agent under low-temperature conditions. Milk fat curdle in sewage is one of the refractory materials for active sludge treatment under low-temperature conditions. To address this, an Antarctic yeast, strain SK-4, isolated from algal mats on the sediments of Naga-ike Skarvsnes, was used as a bio-remediation agent. The yeast strain exhibited high nucleotide sequence homologies (>99.6%) to *M. blollopi*s CBS8921^{T} in rDNA ITS and D1/D2 sequences and had two unique characteristics when applied to an active sludge, i.e., it used various carbon

sources and grew under vitamin-free conditions. Indeed, its BOD removal rate was 1.25-fold higher than that of the control. It may be that the improved BOD removal rate when applying the strain SK-4 can be attributed to the unique activity and characteristics of lipase the strain possesses. Hence, the lipase was purified from strain SK-4 and characterized. As a result, the enzyme was found to be stable under a wide range of temperatures and pHs, even in the presence of various metal ions and organic solvents. Strain SK-4 is therefore a promising bio-remediation agent for cleaning up unwanted milk fat curdles from dairy milk wastewater under low-temperature conditions. The water temperature of actual dairy wastewater can be 3°C (the lowest) in winter and 25°C (the highest) in summer in northern Japan (ex. Hokkaido). Since SK-4s upper temperature for growth was 22°C in my analysis, it is necessary to check if strain SK-4 can survive at higher temperatures, such as 25°C, and evaluate if the strain can work in activated sludge to decrease BOD in dairy milk wastewater under practical temperature conditions.

Furthermore, the influence of initial pH on *M. blollopis* strain SK-4 ethanol production at 10°C was investigated. To the best of my knowledge, little is known about basidiomycetous

yeast fermentation. Species in the basidiomycetous yeast genus *Mrakia* are known for their ability to ferment sugars. In fact, all of the *Mrakia* species examined in this study could convert glucose and sucrose to ethanol by fermentation. *M. blollopis* strain SK-4 used raffinose, galactose, lactose, and maltose for fermentation at low temperatures. However, little is known about strain SK-4's fermentation ability, and hence SK-4 ethanol productivity was examined under various pH conditions. SK-4 produced ethanol at pHs ranging from 5.0-10.0, with the optimum being 8.0-10.0. SK-4 lipase is stable against metal ions and organic solvents, and its optimum pH was 8.5–9.0. Since Naga-ike is an oligotrophic lake and the pH is 8.5, it was assumed that the unique characters of SK-4 for fermentation under alkaline conditions might have been acquired for survival in the lake's extreme environment. Knowledge on ethanol fermentability in the genus *Mrakia* remains insufficient. More information and experimental data are necessary to elucidate the mechanisms of ethanol fermentability in the genus *Mrakia,* e.g., optimum fermentation pH, optimum fermentation temperature, and cell viability during fermentation under various pH and temperature conditions.

Strain SK-4 has the unique ability to ferment various sugars under low-temperature conditions. Hence, this yeast is a good ethanol-producing microbial agent candidate. For this purpose, strain SK-4's ability to process carbohydrates into ethanol was examined using glucose, hydrolysates, and lignocellulosic biomass. SK-4's ability for DEF from cellulosic biomass in the presence/absence of Tween 80 at 10°C was also examined. SK-4 produced as much as 48.2 g/L of ethanol from 12% (w/v) glucose solution, and 17.4 g/L ethanol was converted from Japanese cedar hydrolysate. However, the strain could not use glucose from Eucalyptus hydrolysate for DEF. During the DEF process, SK-4 converted filter paper, Japanese cedar, and Eucalyptus into 12.2 g/L, 12.5 g/L, and 7.2 g/L ethanol, respectively. In the presence of 1% (v/v) Tween 80, the final ethanol concentration increased by approximately 1.1-1.6-fold compared with that in the absence of Tween 80. This is the first report on DEF using cryophilic fungi under low-temperature conditions. *M. blollopis* strain SK-4 has good potential for ethanol fermentation in DEF technology in cold environments. However, when SK-4 was used to ferment mechanochemically treated Eucalyptus and Japanese cedar wood meals (i.e., lignocellulosic biomass) by DEF in the presence of non-ionic surfactant Tween 80, theoretical ethanol yields

were 51.2% and 65.1%, respectively. Since these fermentation rates did not exceed 70%, it was necessary to improve fermentation efficiency. By using lipase in addition to Tween 80, DEF with SK-4 was successfully improved to produce 17.3 g/L (fermentation rate = 76.4%) and 17.5 g/L (fermentation rate = 81.0%) ethanol at 10°C from Eucalyptus and Japanese cedar wood meals, respectively. In the presence of 1% (v/v) Tween 80 and 5 U/g dry substrate lipase, the ethanol concentration increased approximately 1.4–2.4-fold compared with that without Tween 80 and lipase. Consequently, DEF of lignocellulosic biomass with SK-4 in cold environments can be improved by adding Tween 80 and lipase. In this study, I performed the DEF with Acremonium cellulase.

More than 1,000 fungal species have been reported from Antarctica (Bridge et al., 2012), which includes 16 fungal species (12 ascomycetes and four basidiomycetes) from East Antarctica (Soneda, 1961; Tsubaki, 1961a, b; Tusbaki and Asano, 1965). In this study, samples were collected from a total 16 sites in the Skarvsnes ice-free area in East Antarctica and I reported a total of 71 fungal isolates from 10 genera. However, information on fungal biodiversity in the Skarvsnes ice-free area remains limited and only fragmented reports are

available. As described in this study, the cryophilic yeast genus *Mrakia* isolated from the Skarvsnes ice-free area has useful characteristics making it applicable as a microbial agent for various biotechnological purposes, such as bio-remediation and bio-ethanol production, especially in low-temperature environments. I believe that this study has shed light on the importance of the microbial resources inhabiting Antarctica; however, this is only a single example and many unknown promising species must still exist there. I believe that the results obtained in this study will contribute to the progress of the related research fields and hope that further investigation will offer many opportunities to obtain more valuable knowledge on the Antarctic microbes and their potential uses for human activities.

Acknowledgements

The present work was performed at Genomic Resources and Environmental Adaptation Research Group, Research Institute of Bioprocess department and Bio-conversion Research Team, Biomass Refinery Research Center in National Institute of Advanced Industrial Science and Technology (AIST). All the study has been planned by me and Dr. Tamotsu Hoshino, group leader of Genomic Resources and Environmental Adaptation Research Group, and team leader of Bio-conversion research team, AIST. The author appreciated all the researchers who provided samples of Mechanochemically treated woody biomasses, soil and moss samples. Especially Dr. Tomoaki Minowa, Biomass Refinery Research Center of AIST for the woody biomasses sample providing. The author is grateful to Mr. Yuji Yokota, Environmental Bio-function Research Group of AIST and Dr. Sakae Kudoh, National Institute of Polar Research for their helpful advice. The author also thanks to Dr. Shiv M Singh of National Centre of Antarctic and Ocean Research, India for his helpful advice. The authors are also grateful to Dr. Teruo Sano, Professor

in the Faculty of Agriculture and Life Science, Hirosaki University for useful suggestions. The author thanks to my parents for their support and encouragement.

References

1. Andrić P, Meyer AS, Jensen PA, Dam-Johansen K (2010) Effect and Modeling of Glucose Inhibition and In Situ Glucose Removal During Enzymatic Hydrolysis of Pretreated Wheat Straw. Appl Biochem Biotechnol **160**: 280-297.

2. Bab'eva IP, Lisichkina GA, Reshetova IS, Danilevich VN (2002) *Mrakia curviuscula* sp. nov.: A New psychrophilic yeast from forest substrates. Microbiology. **71**: 449-454.

3. Bakoyianis V, Kanellaki M, Kaliafas A, Koutinas AA (1992) Low-Temperature Wine Making by Immobilized Cells on Mineral Kissiris. J Agric Food Chem **40**: 1293-1296.

4. Ballesteros I, Ballesteros M, Cabaňas A, Carrasco J, Martín C et al. (1991) Selection of Thermotolerant Yeasts for Simltaneous Saccharification and Fermentation (SSF) of cellulose to Ethanol. Appl Biochem Biotechnol **28-29**: 307-315.

5. Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I (2004) Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochem **39**: 1843-1848.

6. Barker RJ (1977) A biological reconnaissance of the Bunger Hills. Aust Natl Antarct Res Exp Technical Memorandum **67**: 29-30.

7. Berekaa MM, Zaghloul TI, Abdel-Fattah YR, Saeed HM, Sifour M (2009) Production of a novel glycerol-inducible lipase from thermophilic *Geobacillus steaorothermophilus* strain-5. World J Microbiol Biotechnol. **25**: 287-294.

8. Biddlestone AJ, Gray KR, Job GD (1991) Treatment of dairy farm wastewaters in engineered reed bed systems. Process Biochem. **26**, 265-268.

9. Boomer E, Rousseau M (1900) Note préliminaire sur les champignons recueillis par l'Expedition Antarctique Belge. Bulletin de l'Académie Royale des Sciences de Belgique Classe des Sciences **8**: 640-646.

10. Branda E, Turchetti B, Diolaiuti G, Pecci M, Smiraglia C et al. (2010) Yeast and yeast-like diversity in the southernmost glacier of Europe (Calderone Glacier, Apennines, Italy) FEMS Microbiol Ecol **72**: 354-369.

11. Brander JR, Sidhun RK, Yee B, Skotnicki ML, Selkirk PM et al. (2000) A new microfungal isolate, *Embelissia* sp., associated with the Antarctica moss *Bryum argenteum*. Polar Biol **23**: 730-732.

12. Bridge PD, Newsham KK (2009) Soil fungal community composition at Mars Oasis, a southern maritime Antarctic site, assessed by PCR amplification and cloning. Fungal Ecol **2**: 66-74.

13. Bridge PD, Spooner BM, Roberts P (2010) List of non-lichenized fungi from the Antarctic region. http://www.antarctica.ac.uk//bas_research/data/access/fungi/. Accessed 1 February 2015.

14. Bridge PD, Spooner BM (2012) Non-lichenized Antarctic fungi: transient visitors or menbers of a cryptic ecosystem? Fungal Ecol; **5**: 381-394.

15. Budimir NJ, Jarić MS, Jaćimovic BM, Genić SB, Jaćimovic NB (2011) Rectified ethanol production cost analysis. Therm Sci **15**: 281-292.

16. Buzás Z, Dallmann K, Szajiáni B (1989) Influenc of pH on the growth and ethanol production of free and immobilized *Saccharomyces cerevisiae* cells. Biotechnol Bioeng **34**: 882-884.

17. Buzzini P, Branda E, Goretti M, Turchetti B (2012) Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiol Ecol **82**: 217-241.

18. Cantarella M, Cantarella L, Gallifuoco A, Spera A, Alfani F (2004) Effect of Inhibitor Released during Steam-Explosion Treatment of Poplar Wood on Subsequent Enzymatic Hydrolysis and SSF. Biotechnol Prog **20**: 200-206.

19. Chevalier P, Proulx D, Lessard P, Vincent WF, de la Noue J (2000) Nitrogen and phosphorus removal by high latitude mat-forming cyanobacteria for potential use in tertiary wastewater treatment. J Appl Phycol **12**: 105-112.

20. Chum HL, Johnsoon DK, Black S (1988) Organosolv pretreatment for enzymatic hydrolysis of poplars: I. Enzyme hydrolysis of cellulosic residues. Biotechnol Bioeng **31**: 643-649.

21. Deegenaars M, Watson K (1998) Heat shock response in psychrophilic and psychrotropic yeast from Antarctica. Extremophiles **2**: 41-49.

22. de Garcia V, Brizzio S, Libkind D, Buzzini P, van Broock M (2007) Biodiversity of cold adapted yeasts from glacial meltwater rivers in Patagonia Argentina. FEMS Microbiol Ecol **59**: 331-34

23. de Garcia V, Brizzio S, Broock MR (2012) Yeasts from glacial ice of Patagonia Andes, Argentina. FEMS Microbiol Ecol **82**: 540-550.

24. Diaz MR, Fell JW (2000) Molecular analyses of the IGS & ITS regions of rDNA of the psychrophilic yeasts in the genus *Mrakia*. Antonie van Leeuwenhoek **77**: 7-12.

25. di Menna ME (1966a) Three new yeasts from Antarctica soils: *Candida nivalis*, *Candida gelida* and *Candida frigida* spp. n. Antonie van Leeuenwoek **32**: 25-28.

26. di Menna ME (1966b) Yeasts in Antarctic soil. Antonie van Leeuwenhoek **32**: 29-38.

27. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem **28**: 350-356.

28. Dwiarti L, Boonchird C, Harashima S, Park EY (2012) Simultaneous saccharification and fermentation of paper sludge without pretoreatment using cellulase from Acremonium cellulolyticus and thermotolerant *Saccharomyces cerevisiae* Biomass Bioenerg **42**: 114-122.

29. Endo T, Tanaka N, Sakai M, Teramoto Y, Lee SH (2006) Enhancement mechanism of enzymatic saccharification of wood by mechanochemical treatment. Proceedings of the Third Biomass-Asia workshop: 105.

30. Eriksson T, Börjesson, Tjerneld F (2002) Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb Technol **31**: 353-364.

31. Esteghlalin A, Hashimoto AG, Fenske JJ, Penner MH (1997) Modeling and optimization of dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. Bioresour technl **59**: 129-136.

32. Fadiloğlu S, Erkmen O (2002) Effect of carbon and nitrogen sources on lipase production by *Candida rugosa*. Turk J Eng Env Sci. **26**: 249-254.

33. Fan LT, Gharpuray MM, Lee YH (1987) Cellulose Hydrolysis Biotechnology Monograph, Springer, Berlin, pp149-187.

34. Fell JW, Statzell AC, Hunter IL, Phaff HJ (1969) *Leucosporidium* gen. n., the heterobasidiomycetous stage of several yeasts of the genus Candida. Antonie van Leeuwenhoek **35**: 433-462.

35. Fell JW, Kurtzman CP (1990) Nucleotide Sequence Analysis of a Variable Region of the Large Subunit rRNA for Identification of Marine-Occurring Yeasts. Curr Microbiol **21**: 295-300.

36. Fell JW, Boekhout T, Fonseca A, Scoretti G, Statzelll-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int J Syst Evol Micr **50**: 1351-1371.

37. Fell JW, Scorzetti G, Connell L, Craig S (2006) Biodiversity of micro-eucaryotes in Antarctic Dry Valley soil with <5% soil moisture. Soil Biol Biochem **38**: 3107-3119.

38. Fell JW, Johnson EA, Scorzetti G (2011) The Yeast, a Taxonomic Study 5th edn, Elsevier, Amsterdam, pp.1595-1599.

39. Feller G, Gerday C (2003) Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol **1**: 200-208.

40. Fengel D, Wegener G (1984) Wood: chemistry, ultrastructure, reactions, De Gruyter, Berlin and New York, pp.132-167.

41. Fletcher LD, Kerry EJ, Weste GM (1985) Microfungi of MacRobertson and Enderby Lands, Antarctica. Polar Biol **4**: 81-88.

42. Frate GD, Caretta G (1990) Fungi isolated from Antarctic material. Polar Biol **11**: 1-7.

43. 藤生誠一 (2010) 凍結状態で霜柱状コロニーを形成する南極産担子菌酵母の環境適応に関する研究. 北海道大学大学院理学院　生命理学専攻　修士論文.

44. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes -

application to the identification of mycorrhizae and rusts. Mol Ecol **2**: 113-118.

45. Gonçalves VN, Vaz ABM, Rosa CA, Rosa LH (2012) Diversity and distribution of fungal communities in lakes of Antarctica. FEMS microbial Ecol **82**: 459-471.

46. Goshima T, Tsuji M, Inoue H, Yano S, Hoshino T et al. (2013) Bioethanol production from lignocellulosic biomass by a novel Kluyveromyces marxianus strain, Biosci Biotech Bioch **77**: 1505-1510.

47. Göttlich E, de Hoog GS, Genilloud O, Jones BE, Marinelli F (2003) Antarctic biology in a global context, Backhuys Publishers, Leiden, pp. 251-254.

48. Griffith M, Ala P, Yang DS, Hon WC, Moffatt BA (1992) Antifreeze protein produced endogenously in winter rye leaves. Plant Physiol **100**: 593-596.

49. Gupta PK, Mascarenhas AF, Jagannathan V (1981) Tissue culture of forest trees* - - Clonal propagation ofmature trees of *Eucalyptus citriodora* Hook, by tissue culture Plant Sci Lett **20**: 195-201.

50. Healy MG, Rodgers M, Mulqueen J (2007) Treatment of dairy wastewater using constructed wetland and intermittent sand filter. Bioresour Technol. 98: 2268-2281.

51. Hirayama-Katayama K, Koike Y, Kobayashi K, Hirayama K (2003) Removal of nitrogen by Antarctic yeast cells at low temperature. Polar Biosci **16**: 43-48.

52. Holtzapple M, Cognata M, Shu Y, Hendrickson C (1990) Inhibition of *Trichoderma reesei* cellulase by sugars and solvents Biotechnol Bioeng **36**: 275-287.

53. Hoshino T, Tronsmo AM, Matsumoto N, Sakamoto T, Ohgiya S et al (1997) Purification and characterization of a lipolytic enzyme active at low temperature from Norwegian *Typhula ishikariensis* groupIII strain. Eur J plant Pathol **103**: 357-361.

54. Hoshino T, Kiriaki M, Nakajima T (2003) Novel thermal hysteresis proteins from low temperature basidiomycete, *Coprinus psychromorbidus*. Cryo letters **24**: 135-142.

55. Hoshino T, Xiao N, Tkachenko OB (2009) Cold adaptation in the phytopathogenic fungi causing snow molds. Mycoscience **50**: 26-38.

56. Hoshino T, Matsumoto N (2012) Cryophilic fungi to denote fungi in cryosphere. Fungal Biol Rev **26**: 102-105.

57. Hua MX, Chi Z, Liu GL, Buzdar MA, Chi ZM (2010) Production of a novel and cold-active killer toxin by *Mrakia frigida* 2E00797 isolated from sea sediment in Antarctica. Extremophiles **14**: 515-521.

58. Iefuji H, Iimura Y, Obata T (1994) Isolation and characterization of a yeast *Cryptococcus* sp. S-2 that produces raw starch-digesting α-amylase, xylanase and polygalacturonase. Biosci Biotechnol Biochem **58**: 2261-2262.

59. Iizumi T, Nakamura K, Fukase T (1990) Purification and characterization of a thermostable lipase from newly isolated *Pseudomonas* sp. KWI-56. Agric Biol Chem **54**: 1253-1258.

60. Imura S, Bando T, Saito S, Seto K, Kanda H (1999) Benthic moss pillars in Antarctic lakes. Polar Biol **22**: 137-140.

61. Jones EBG, Slooff WC (1966) *Candida aquatica* sp.n. isolated from water scums. Antonie Van Leeuwenhoek **32**: 223-228.

62. Kamini NR, Fujii T, Kurosu T, Iefuji H (2000) Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus* sp. S-2. Process Biochem. **36**: 317-324.

63. Kato K, Ietsugu H, Koba T, Sasaki H, Miyaji N, et al. (2010) Design and performance of hybrid reed bed systems for treating high content wastewater in the cold climate., 12th International Conference on Wetland Systems for Water Pollution Control. http://reed-net.com/pdf/katoICWS2010.pdf. Accessed 7 April 2014.

64. Klingler J, Vishniac HS (1988) A contribution of Antarctic ecology to yeast systematics. Polarforschung **58**: 83-92.

65. Kumdam H, Murthy SN, Gummadi SN (2013) Production of ethanol and arabitol by *Debaryomyces nepalensis*: influence of process parameters AMB Express **3**: 23.

66. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

67. Lee JK, Park KS, Park S, Park H, Song YH, et al. (2010) An extracellular ice-binding glycoprotein from an Arctic psychrophilic yeast. Cryobiology **60**: 222-238.

68. Li CY, Cheng CY, Chen TL (2001) Production of *Acinetobacter radioresistens* lipase using Tween 80 as the carbon source. Enzyme Microb Technol **29**: 258-26318.

69. Li YZ, Nan ZB (2007) Symptomology and etiology of a new disease, yellow stunt, and root

rot of standing milkvetch caused by *Embellisia* sp. in Northern China. Mycopathologia **163**: 327-334.

70. Liu GL, Wang K, Hua MX, Buzdar MA, et al. (2012) Purification and characterization of the cold-active killer toxin from the psychrotolerant yeast *Mrakia frigida* isolated from sea sediments in Antarctica. Process Biochem **47**: 822-827.

71. Margesin R, Fauster V, Fonteyne PA (2005) Characterization of cold-active pectate lyases from psychrophilic *Mrakia frigida.* Lett Appl Microbiol **40**: 453-459.

72. Margesine R, Fell JW (2008) *Mrakiella cryoconiti* gen. nov., sp. nov., a psychrophilic, anamorphic, basidiomycetous yeast from alpine and arctic habitats. Int J Syst Evol Microbiol. **58**: 2977-2982.

73. Montes M J, Belloch C, Galiana M, Garcia MD, Andrés, et al. (1999) Polyphasic Taxonomy of a Novel Yeast Isolated from Antarctic Environment; Description of *Cryptococcus victoriae* sp. nov. Syst Appl Microbiol **22**: 97-105.

74. Millett MA, Baker AJ, Satter LD (1976) Physical and chemical pretreatments for enhancing cellulose saccharification. Biotechnol Bioeng Symp **6**:125-153.

75. Panikov NS, Sizova MV (2007) Growth kinetics of microorganisms isolated from Alaskan soil and permafrost in solid media frozen down to -35°C. FEMS Microbiol Ecol **59**: 500-512.

76. Pathan AAK, Bhadra B, Begum Z, Shivaji S (2010) Diversity of Yeasts from Puddles in the Vinicity of Midre Lovénbreen Glacier, Arctic and Bioprospecting for Enzymes and Fatty acids. Curr Microbiol **60**: 307-314.

77. Pegler DN, Spooner BM, Smith RIL (1980) Higher fungi of Antarctica, the subantarctic zone and Flkland Islands. Kew Bulletin **35**: 499-562.

78. Rao RS, Bhadra B, Shivaji S (2008) Isolation and characterization of ethanol-producing yeasts from fruits and tree barks Lett. Appl Micro **47**:19-24.

79. Ravindra R, Chaturvedi A (2011) "Antarctica" Encyclopedia of snow, ice and glaciers, Springer, Netherland, pp 45-53.

80. Robinson CH (2001) Cold adaptation in Arctic and Antarctic fungi. New Phytol **151**: 341-353.

81. Rosa LZ, Vaz ABM, Caligiorne RB, Campolina S, Rosa CA (2009) Endophytic fungi

associated with the Antarctic grass *Deschampsia antarctica* Desv. (*Poaceae*). Polar Biol **32**: 161-167.

82. Rossi M, Buzzini P, Cordisco L, Amaretti A, Sala M et al. (2009) Growth, lipid accumulation, and fatty acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts. FEMS Microbiol Ecol **69**: 363-372.

83. Ruisi S, Barreca D, Selbmam L, Zucconi L, Onofri S (2007) Fungi in Antarctica. Rev Environ Sci Biotechnol **6**: 127-141.

84. Shah SB, Bhumbla DK, Basden TJ, Lawrence LD (2002) Cool temperature performance of a wheat straw biofilter for treating dairy wastewater. J Environ Sci Health B **37**: 493-505.

85. Shimohara K, Fujiu S, Tsuji M, Kudoh S, Hoshino T, et al. (2012) Lipolytic activities and their thermal dependence of *Mrakia* species, basidiomycetous yeast from Antarctica. J water waste (in Japanese) **54**: 691-696.

86. Sinclair N.A, Stokes JL (1965) Obligately Psychrophilic Yeasts from the Polar Regions. Can J Microbiol **11**: 259-269.

87. Singh P, Singh SM (2012) Characterization of yeast and filamentous fungi isolated from cryoconite holes of Svalbard, Arctic. Polar Biol **35**: 575-583.

88. Singh P, Tsuji M, Singh SM, Roy U, Hoshino T (2013). Taxonomic characterization, adaptation strategies and biotechnological potential of cryophilic yeasts from ice cores of Midre Lovénbreen glacier, Svalbard, Arctic. Cryobiology **66**: 167-175.

89. Snider CS, Hsiang T, Zhao G, Griffith (2000) Role of ice nucleation and antifreeze activities in pathogenesis and growth of snow molds. Phytopathology **90**: 354-361.

90. Soneda M (1961) On some yeasts from the Antarctic region. Biol Res Jpn Res Expedition **15**: 3-10.

91. Suh SO, Sugiyama J (1993) Phylogeny among the basidiomycetous yeasts inferred from small subunit ribosomal DNA sequence. J Gen Microbiol **139**: 1595-1598.

92. Takagi M, Abe S, Suzuki S, Emert GH, Yata N (1977) A method for production of alcohol directly from cellulose using cellulase and yeast In; Bioconversion symposium proceedings. IIT, Delhi: 551-571.

93. 田邊 優貴子 (2009) 南極湖沼における藻類群衆の光生理・生態学的研究. 総合研究大学院大学複合科学研究科極域科学専攻 博士論文.

94. Taoka Y, Nagano N, Okita Y, Izumida H, Sugimoto S et al (2011) Effect of Tween 80 on the growth, lipid accumulation and fatty acid composition of *Thraustochytrium aureum* ATCC 34304. J Biosci Bioeng **111**: 420-424.

95. Tanabe Y, Kudoh S, Imura S, Fukuchi M (2008) Phytoplankton blooms under dim and cold conditions in freshwater lakes of East Antarctica. Polar Biol **31**: 199-208.

96. Tamura K, Dudley J, Nei M, Kumar S (2007) Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24**; 1596-1599.

97. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol **28**: 2731-2739.

98. Tengbord C, Galbe M, Zacchi G (2001) Reduced inhabitation of enzymatic hydrolysis of steam-pretreated softwood Enzyme Microb Technol 28: 835-844.

99. Thomas-Hall SR, Watson K, Scorzetti G (2002) *Cryptococcus stzelliae* sp. nov. and three novel strains of *Cryptococcus victoriae*, yeasts isolated from Antarctica soils. Int J Syst Evol Microbiol **52**: 2303-2308.

100. Thomas-Hall SR, Turchetti B, Buzzini P, Branda E, Boekhout T, et al. (2010) Cold-adapted yeasts from Antarctica and Italian alps-description of three novel species: *Mrakia robertii* sp. nov., *Mrakia blollopis* sp. nov. and *Mrakiella niccombsii* sp. nov. Extremophiles **14**: 47-59.

101. Toshi S, Casado B, Gerdol R, Caretta (2002) Fungi isolated from Antarctic mosses. Polar Biol **25**: 262-268.

102. Tsubaki K (1961a) On some fungi isolated from the Antarctic materials. Biol Results JARE **14**: 1- 9.

103. Tsubaki K (1961b) Note on some fungi and yeasts from Antarctica. Antarctic Reco (Tokyo) **11**: 161-162.

104. Tsubaki, K, Asano, I (1965) Additional species of fungi isolated from the Antarctic

materials. Japan. Antarct Exp Rep, Series E **27**: 1-12.

105. Tsuji M, Fujiu S, Xiao N, Hanada Y, Kudoh S,et al. (2013a) Cold adaptation of fungi obtained from soil and lake sediment in the Skarvsnes ice-free area, Antarctic , FEMS Microbiol Lett **346**: 121-130.

106. Tsuji M, Yokota Y, Shimohara K, Kudoh S, Hoshino T (2013b) An Application of Wastewater Treatment in a Cold Environment and Stable Lipase Production of Antarctic Basidiomycetous Yeast *Mrakia blollopis*. PLOS ONE **8**: e59376.

107. Tsuji M, Goshima T, Matsushika A, Kudoh S, Hoshino T (2013c) Direct ethanol fermentation from lignocellulosic biomass by Antarctic Basidiomycetous yeast *Mrakia blollopis* under a low temperature condition, Cryobiology **67:** 241-243.

108. Tsuji M, Singh SM, Yokota Y, Kudoh S, Hoshino T (2013d) Influence of initial pH on ethanol production by Antarctic Basidiomycetous yeast *Mrakia blollopis*, Biosci Biotech Bioch **77**: 2483-2485.

109. Turchetti B, Buzzini P, Goretti M, Branda E, Diolaiuti G et al. (2008) Psychrophilic yeasts in glacial environments of Alpine glaciers FEMS Microbiol Ecol **63**: 73-83.

110. Turchetti B, Hall SRT, Connell L B, Branda E, Buzzini P et al. (2011) Psychrophilic yeasts from Antarctica and European glaciers: description of *Glaciozyma* gen. nov., *Glaciozyma martinii* sp. nov. and *Glaciozyma watsonii* sp. nov. Extremophiles **15**, 573-586.

111. Turk M, Plemenitaš A, Gunde-Cimerman N. (2011) Extremophilic yeasts: plasma-membrane fluidity as determinant of stress tolerance. Fungal Biol **115**: 950-958.

112. Valente P, Boeckhout T, Landell MF, Crestanil J, Pagnocca FC, Sette LD et al.(2012) *Bandoniozyma* gen. nov., a Genus of Fermentative and Non-Fermentative Tremellaceous Yeast Species PLoS ONE, **7**, e46060.

113. Vaz ABM, Rosa LH, Vieira MLA, de Garcia V, Brandão LR, et al. (2011) The diversity, extracellular enzymatic activities and photoprotective compounds of yeasts isolated in Antarctica. Braz J Microbiol **42**, 937-947.

114. Vishniac HS, Klingler J (1986) Perspectives in microbial ecology, Ljubljana, Slovenia, pp. 46-51.

115. Vishniac HS (1996) Biodiversity of yeasts and filamentous microfungi in terrestrial Antarctic ecosystems. Biodivers Conserv **5**: 1365-1378.

116. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications **18**: 315-322.

117. Woo H, Zheng X, Araki Y, Sahara H, Takagi H, et al. (2013) Global Gene Expression Analysis of Yeast Cells during Sake Brewing, Appl Environ Microb, **72**: 7353-7358

118. Xiao N, Suzuki K, Nishiyama Y, Kondo H, Miura A, et al. (2010a) Comparison of functional properties of two fungal antifreeze proteins from Antarctomyces pschrotrophicus and Typhula ishikariensis FEBS J **277:** 394-403.

119. Xiao N, Inaba S, Tojo M, Degawa Y, Fujiu S, et al. (2010b) Antifreeze activities of various fungi and Stramenophilia isolated from Antarctica. North American Fungi **5**: 215-220.

120. Xin M, Zhou P (2007) *Mrakia psychrophila* sp. Nov., a new species isolated from Antarctic soil. J Zhejiang Univ-Sc B **8**(4): 260-265.

121. Yamada Y, Komagata K (1987) *Mrakia* gen. nov., a heterobasidiomycetous yeast genus for the Q8-equipped, self-sporulating organisms which produce a unicellular metabasidium, formerly classified in the genus *Leucosporidium* J Gen Appl Microbiol **33**: 455-457.

122. Yarrow D (1998) The yeast, a taxonomic study, 4th edn,. Elsevier, Amsterdam, pp77-100.

123. Ying C, Umetsu K, Ihara I, Sakai Y, Yamashiro T (2010) Simultaneous removal of organic matter and nitrogen form milking parlor wastewater by a magnetic activated sludge (MAS) process. Bioresour Technol **101**: 4349-4353.

124. You KM, Rosenfield CL, Knipple DC (2003) Ethanol Tolerance in the Yeast *Saccharomyces cerevisiae* Is Dependent on Cellular Oleic Acid Content Appl. Environ. Microbiol **69**: 1499-1503.

125. Zhang J, Chu D, Huang J, Yu Z, Dai G et al. (2009) Simultaneous Saccharification and Ethanol Fermentation at High Corn Stover Solids Loading in a Helical Stirring Bioreactor Biotechnol Bioeng 105: 718-728.

Publication lists (Peer-reviewed)

1. Tsuji M, Fujiu S, Xiao N, Hanada Y, Kudoh S,et al. (2013) Cold adaptation of fungi obtained from soil and lake sediment in the Skarvsnes ice-free area, Antarctic , FEMS Microbiol Lett **346**: 121-130.

2. Tsuji M, Yokota Y, Shimohara K, Kudoh S, Hoshino T (2013) An Application of Wastewater Treatment in a Cold Environment and Stable Lipase Production of Antarctic Basidiomycetous Yeast *Mrakia blollopis*. PLOS ONE **8**: e59376.

3. Tsuji M, Goshima T, Matsushika A, Kudoh S, Hoshino T (2013) Direct ethanol fermentation from lignocellulosic biomass by Antarctic Basidiomycetous yeast *Mrakia blollopis* under a low temperature condition, Cryobiology **67:** 241-243.

4. Tsuji M, Singh SM, Yokota Y, Kudoh S, Hoshino T (2013) Influence of initial pH on ethanol production by Antarctic Basidiomycetous yeast *Mrakia blollopis*, Biosci Biotech Bioch **77**: 2483-2485.

5. Tsuji M†, Yokota Y, Kudoh S, Hoshino T (2014) Improvement of direct ethanol fermentation from woody biomasses by Antarctic basidiomycetous yeast *Mrakia blollopis* under a low temperature condition, Cryobiology, **68**: 303-305. (†: Corresponding author)

Patent Lists

1）特許願 2011-167225 号「乳脂肪分解能を有する南極産担子菌酵母及びその利用方法」、星野保、横田祐司、辻雅晴、湯本勲、工藤栄、 2011 年 7 月

2) 特許願 2012-235103 号「酵素阻害物質を除去する工程を経由する糖の製造方法」、 星野保、辻雅晴、矢島由佳、横田祐司、吉宗一晃、2012 年 10 月

3) 特許願 2013-064750 号「南極産酵母による低温下でのエタノール製造方法」、星野保、辻雅晴、工藤栄、2013 年 3 月

4) 特許願 2013-169775 号「脂質分解酵素及び非イオン性界面活性剤添加によるリグノセルロース系バイオマスからの酵素糖化反応および同時糖化発酵の改善方法」、星野保、辻雅晴

Publication lists of related articles (Peer-reviewed)

1. Li SF, Guo R, Tsuji M, Sano T (2006) Two grapevine viroids in China and the possible detection of a third**.** Plant Pathology, **55**:564.

2. Li SF, Su Q, Guo R, Tsuji M, Sano T (2006) First report of *Coleus blumei* viroid from coleus in China. Plant Pathology, **55**: 565.

3. He YH, Isono S, Shibuya M, Tsuji M, Adkar-Purushothama CR, et al. (2012) Oligo-DNA custom macroarray for monitoring major pathogenic and non-pathogenic fungi and bacteria in the phyllosphere of apple trees. PLoS ONE **7**: e34249.

4. 下原広大，藤生誠一，辻雅晴，工藤栄，星野保，横田祐司（2012）南極産 *Mrakia* 属担子菌酵母の乳脂肪分解能とその温度依存性，用水と排水, **54**: 691-696.

5. Jiang D, Sano T, Tsuji M, Araki H, Kyota, CRA Purushothama et al. (2012) Comprehensive diversity analysis of viroids infecting grapevine on China and Japan. Virus Resarch, **169**: 237-245.

6. Singh P, Tsuji M, Singh SM, Roya U, Hoshino T. (2013) Taxonomic characterization, adaptation strategies and biotechnological potential of cryophilic yeasts from ice cores of Midre Lovénbreen glacier, Svalbard, Arctic. Cryobiology, **66**: 167-175.

7. Goshima T*, Tsuji M*, Inoue H, Yano S, Hoshino T, et al. (2013) Bioethanol production from lignocellulosic biomass by a novel *Kluyveromyces marxianus* strain, Biosci Biotech Bioch, **77**: 1505-1510 (* These authors contributed equally to this work.).

8. Goshima T, Negi K, Tsuji M, Inoue H, Yano S, et al. (2013) Ethanol fermentation from xylose by metabolically engineered strains of *Kluyveromyces marxianus*, J Biosci Bioeng, **116**: 551-554.

9. 横田祐司、辻雅晴、湯本勲、星野保 (2013) 南極産担子菌酵母を利用した低温下でのパーラー排水の活性汚泥処理. 用水と排水, **55**: 831-835.

10. Singh P, Singh S.M, Tsuji M, Prasad G. S, Hoshino T (2014) *Rhodotorula svalbardensis* sp. nov., a novel yeast species isolated from cryoconite holes of Ny-Ålesund, Arctic. Cryobiology, **68**: 122-128.

11. Tsuji M†, Yokota Y, Kudoh S, Hoshino T (2014) Effects of nitrogen concentration and culturing temperatureon lipase secretion and morphology of the Antarctic basidiomycetous yeast *Mrakia blollopis*. Int J Res Eng Sci, **2**: 49-55 (†: Corresponding author).

12. Tsuji M†, Kudoh S, Hoshino T (2015) Draft genome sequence of cryophilic basidiomycetous yeast *Mrakia blollopis* SK-4, isolated from an algal mat of Naga-ike Lake in the Skarvsnes ice-free area, East Antarctica. Genome Announc **3**(1):e01454-14 (†: Corresponding author).