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Effects of Nitrogen Concentration and Culturing Temperatureon Lipase Secretion and Morphology of the Antarctic Basidiomycetous Yeast *Mrakiablollopis*

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ABSTRACT :The effect of nutrient concentration on Mrakiablollopis SK-4 colony morphology and the effects of nitrogen concentration on formation of a clear zone around colonies, which is indicative of lipase activity, and on morphology were examined on PDA and fresh cream agar at various culturing temperatures. When the yeast was inoculated on a eutrophic medium, it maintained its yeast form, while it showed an almost mycelial form on an oligotrophic medium regardless of culturing temperature. When grown on high-nitrogen fresh cream agar, the largest clear zone was formed around colonies at 4°C and the morphology was considered to be a yeast form. Morphology of SK-4 was changed by the nutrient condition under the colony on an agar plate. Secretion of lipase was increased by a high nitrogen concentration. SK-4 is thought to take the yeast form in aquatic environments, and this form may secrete more lipase than the mycelium form.
Keywords -*Cold-adapted yeast• Cryophilic fungi• Fluorescence in situ hybridization• Lipolytic enzyme• Mrakia*

I. INTRODUCTION

Lipase is known as one of the most important enzyme for industries. Cryophilic fungi [1] have been reported worldwide in cold environments including the polar regions. The cryophilic basidiomycetous yeast *Mrakia* spp. and *Mrakiella* spp. have been found in areas such as Antarctica, the Alps [2], Central Russia [3], Siberia and the Arctic [4].

diMenna [5] reported that *Mrakia* spp. accounted for about 24% of the culturable yeast in Antarctic soil. Furthermore, Fujiu reported that about 25% of culturable fungi isolated from lake sediments and soils of East Antarctica were *Mrakia* spp. (Master's thesis, Graduate School of Science, Hokkaido University, 2010). These reports indicated that *Mrakia* spp. are the dominant fungi in Antarctica and the most adaptive to the Antarctic environment.

The cryophilic yeast *M. blollopis* SK-4 was isolated from an algal mat from the oligotrophic lake Naga-ike, Skarvsnes ice-free area, East Antarctica [6]. When SK-4 was inoculated on agar plates containing fresh cream as the carbon source, this yeast formed the largest clear zone, formed by lipase activity, at 10°C [7]. However, there is little known about how SK-4 lipase production is influenced by nitrogen concentration, culturing temperatures or cell morphology. Moreover, there is little known about how *Mrakia* spp. lives under such oligotrophic conditions. Here, we report on the effects of nutrient concentration on SK-4 cell morphology, and the effects of nitrogen concentration on lipase secretion and cell morphology at different culturing temperatures. We also observed cell morphology on lipase production medium using fluorescence in situ hybridization (FISH).

II. MATERIALS AND METHODS

2.1. Media composition

Fresh cream agar (FCA) was composed of 3.2 g/l yeast extract (yeast extract containing 109 mg/g nitrogen and 163.3 mg/g carbohydrate), 5.4 g/l peptone (peptone consisting of 154 mg/g nitrogen and 13.3 mg/g carbohydrate), 5.4 g/l NaCl, 55 ml/l fresh cream (fresh cream containing 466 mg/ml milk fat and 23.5 mg/ml carbohydrate) and 20 g/l agar, pH 7.0. 2× FCA was identical to FCA except for 10.8 g/l peptone, and 5× FCA was identical to FCA except for 27.0 g/l peptone. 1/5 potato dextrose agar (PDA) was composed of 7.8 g/l PDA (DB Japan, Tokyo, Japan) and 14 g/l agar. 2× PDA was composed of 39 g/l PDA and 24 g/l potato dextrose

broth (PDB) (BD Japan, Tokyo, Japan). One thousand ml of PDA and PDB were composed of 35.2 g of carbohydrate and 20.0 g/l of dextrose.

2.2 Inoculum

M. blollopis SK-4 was grown on PDA at 15 ºC for 2 weeks. After 2 weeks, 10 µl (2.0 ×108 cells) of *Mrakiablollopis* SK-4 was used as inoculum.

2.3 Effect of the nutrient concentration for morphology of SK-4 on PDA

M. blollopis SK-4 was inoculated onto PDA with different nutrient concentrations, such as 1/5 PDA, 2x PDA and 5x PDA) at 4°C, 10°C or 15°C for 4 weeks. After 4 weeks, the percentage of yeast forms and mycelial forms in the colonies was measured.

2.4 Assay for decomposition ability of milk fat (DAMF)

Four weeks after inoculation on FCA, 2× FCA or 5× FCA at 4, 10 or 15ºC, DAMF was assessed by the formation of a clear zone around the colony. The diameters of the clear zone and the colony on each plate were measured.The DAMF was calculated by the following formula:

Decomposition ability of milk fat (DAMF)

= (Clear zone diameter – colony diameter) / colony diameter.

2.5 Lipase production and FISH analysis

M. blollopis SK-4 was inoculated in a lipase production medium (2 g/l KH_2PO_4, 2.9 g/l Na_2PO_4, 0.2 g/l NH_4Cl, 0.4 g/l $CaCl_2$, 0.01 g/l $FeCl_3$, 5 g/l yeast extract, and 10.0 g/l Tween 80) and incubated at 100 rpm at 10 °C. One-mL samples were collected and centrifuged at 4 ºC for 5 min at 15100 × g, and then lipase activity was determined. Cell density was monitored by absorbance at 600 nm with a BioSpectrometer (Eppendorf, Hamburg, Germany).Lipase activity was measured by a colorimetric method using p-nitrophenyl-palmitate as a substrate [9]. 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.

To confirm SK-4 cell morphology during lipase secretion, cells were stained by the FISH method. *M. blollopis* SK-4 was inoculated in lipase production medium (2 g/l KH_2PO_4, 2.9 g/l Na_2PO_4, 0.2 g/l NH_4Cl, 0.4 g/l $CaCl_2$, 0.01 g/l $FeCl_3$, 5 g/l yeast extract, and 10.0 g/l Tween 80) and incubated at 100 rpm at 10°C. After 2 months, cells were collected by centrifugation and washed twice with PBS buffer (1.37 M NaCl, 27 mMKCl, 81 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4) and then washed twice with hybridization buffer (20 mM Tris-HCl, pH 8.0, 0.9 M NaCl, 0.01% SDS, 9% paraformaldehyde). To detect *M. blollopis* SK-4, a specific probe targeting the 26S rRNA of *M. blollopis* was designed and its 5′ end (5′-TGG TAA TCT GTG TCA A-3′) was labeled with Alexa Fluor 555. Hybridization buffer (500 µL containing 100 pmol/ml probe) was added to the cell sample, which was incubated at room temperature overnight with continual shaking. After incubation, samples were washed twice with PBS and then cells were suspended in PBS containing 2.3% 1,4-diazabicyclo[2.2.2]octane (DABCO) and 4 nmol/ml 4,6-diamidino-2-phenylindole (DAPI). Fluorescence signals were observed under a fluorescence microscope.

III. RESULTS AND DISCUSSION

M. blollopis SK-4 was incubated on PDA for 4 weeks at 4, 10 or 15 °C. Colony morphology closely resembled a yeast form at 4 ºC (Fig. 1a, d). When this yeast was incubated at 10 or 15 ºC, the ratio of mycelial forms in the colony gradually increased with increasing in culturing temperature (Fig. 1b-c, Fig. 1e-g).

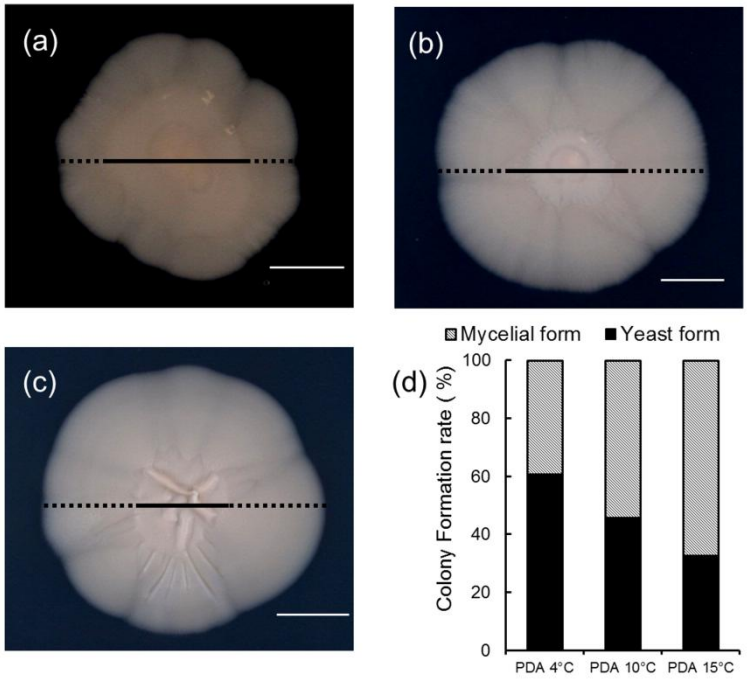

Figure 1.Colony morphology of *M blollopis* SK-4 colony on PDA.
(a) 4°C.(b) 10°C and (c) 15°C.Solid lines indicate yeast form and dashed lines indicate mycelial form. *Bars*: a-c, 1 cm. (d) Ratios of yeast colony morphology andmycelial colony morphology of *M .blollopis*SK-4 at 4 weeks after inoculation.

To confirm the effect of nutrient concentration on colony morphology, SK-4 was cultured on 1/5 PDA, 2× PDA at 4, 10, and 15 °C. When the yeast was inoculated on 1/5 PDA, colony morphology was more mycelial-like than yeast-like, and the extent of the mycelial form increased with increase in culturing temperature. (Fig. 2a-c). In contrast, the morphology of SK-4 colonies was maintained in the yeast form on the eutrophic medium regardless of culturing temperature (Fig. 2d-f). Based on the results of culturing on eutrophic and oligotrophic media, it was thought that the only utilized nutrients under the colony. Since SK-4 was not able to retain the colony type of the yeast form when utilizing only nutrients under each colony on an oligotrophic medium, SK-4 may undergo a change in morphology to obtain nutrients outside the colony boundary by germination and extension of mycelia. The optimal growth temperature of SK-4 is 15 °C. Therefore, colony morphology of SK-4 on PDA was thought to be related to growth speed and nutrient concentration under the colony.

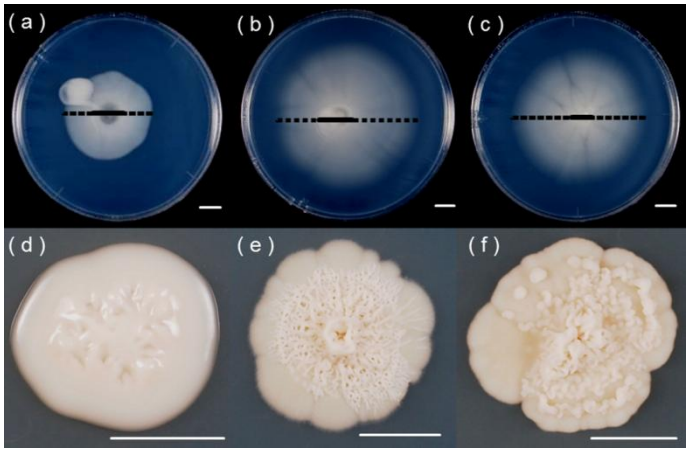

Figure 2.Colony morphology of *M. blollopis* SK-4 grown for 4 weeks on PDA of different nutrient concentrations.
(a) 1/5 PDA at 4 °C. (b) 1/5 PDA at 10 °C. (c) 1/5 PDA at 15 °C. (d) 2×PDA at 4 °C. (e) 2× PDA at 10 °C. (f)2× PDA at 15 °C. Solid lines indicate yeast form and dashed lines indicate mycelial form. Bars: a-f 1 cm.

When SK-4 was inoculated on FCA at 4, 10 or 15 ºC, the largest clear zone was unclearly formed at 10 °C (data not shown). However, in the case of 2× FCA and 5× FCA, the largest clear zone was formed at 4 °C (Fig. 3a-c). The DAMF score was higher at 4°C than at 10 °C regardless of nitrogen concentration, and the highest score was 1.95 at 4 ºC on 5 × FCA (Fig. 3e). At 15 ºC, a clear zone around the colony was not observed regardless of nitrogen concentration. Moreover, the ratio of mycelial form in SK-4 colonies increased with increasing in culturing temperature regardless of nitrogen concentration (Fig. 3d). The nutrient concentration was not sufficient for maintaining yeast form. SK-4 therefore germinated and extended mycelia for survival under a high temperature condition as was observed on PDA.

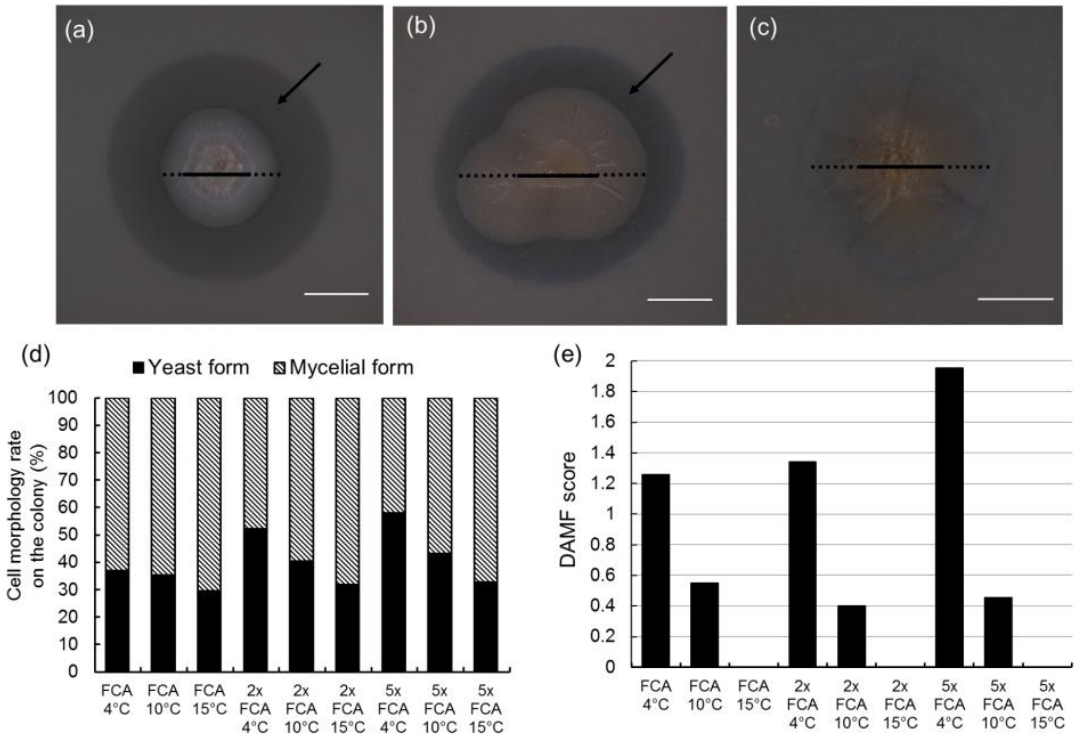

Figure 3.Effect of temperature on *M. blollopis* SK-4 grown for 4 weeks on FCA at various nitrogen concentrations.

(a) 5× FCA at 4°C. (b) 5× FCA at 10°C. (c) 5× FCA at 15°C. Solid lines indicate yeast form and dashed lines indicate mycelial form. Black arrows indicate clear zones. Bars: a-c 1 cm. (d) Ratio of the two colony morphologies of *M. blollopis* SK-4 at 4 weeks after inoculation. e. DAMF scores on FCA at various nitrogen concentrations.

In an early study, Shimohara et al. [7] found that this yeast formed the largest clear zone on FCA after 10 days at 10 °C. In this study, we obtained the same results on FCA. Pathan et al. [10] reported that *Mrakia* sp. YSAR-9, which had high sequence homology (99% >) with *M. blollopis* SK-4, showed stronger lipolytic reactivity using Tween 20 as a substrate at 22 ºC than at 8 ºC. However, when SK-4 was inoculated on agar plate with Tween 20 as a substrate, the yeast showed stronger reactivity at 4 ºC than at 10 ºC or 20 ºC (data not shown). Therefore, the relationship between the phylogenetic position and DAMF in the genus *Mrakia* remains unclear. In this study, the relationships between formation of a clear zone, nitrogen concentration and culturing temperature were also investigated. SK-4 inoculated on high-nitrogen media including 2× FCA and 5× FCA formed the largest clear zone at 4 °C. SK-4 colony morphology during formation of the largest clear zone correlated with the yeast form of the colonies. It has been shown that secretion of Candida rugosa lipase is increased at peptone concentrations up to 7% (w/v) [11]; similar results were obtained for SK-4 on the plate. However, we do not know why DAMF differs depending on nitrogen concentration and temperature. Further experiments, including comprehensive analysis and investigation of the effects of temperature, nitrogen source concentration and pH for DAMF, are needed to elucidate the DAMF of the genus*Mrakia*.

One of our aims was to confirm *M. blollopis* SK-4 cell morphology when the yeast is actively secreting lipase. We therefore examined cell morphology of SK-4 during secretion of lipase using FISH and optical microscopy. The amount of SK-4 lipase was dramatically increased after 13 days of incubation, and maximally 0.64 U/mL of the activity was recorded after 24 days of incubation (Fig. 4).

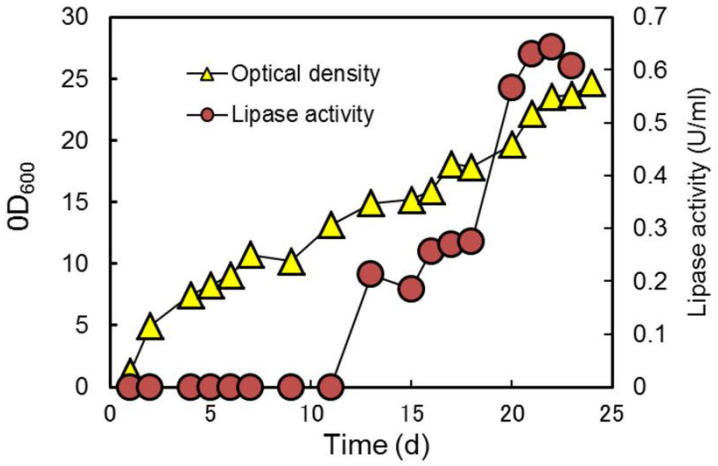

Figure 4.Time course of cell growth and lipase production in lipase production medium. Triangle represents cell density at OD600 and circle denotes lipase activity (U/ml).

The cells were then observed by optical microscopy. A *M. blollopis*-specific probe labeled at the 5′ end with Alexa Fluor 555 gave positive results in lipase production medium (Fig. 5b). The cell morphology of SK-4 during secretion of lipase was exclusively the yeast form (Fig. 5a). The morphology of this yeast was yeast-like in the field of view of the microscope throughout our observations.

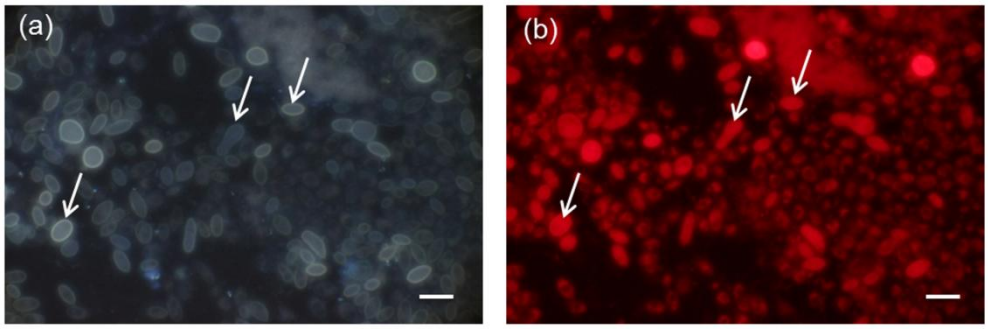

Figure 5.Cell morphology of *M. blollopis* SK-4 during lipase secretion determined by DAPI and FISH. (a) DAPI staining of *M. blollopis* SK-4. (b) FISH analysis of M. blollopis SK-4. White arrows indicate M. blollopis SK-4 cells. Bars: a, b 10 μm.

IV. CONCLUSION

In conclusion, *M. blollopis* SK-4 adopted a yeast rather than a mycelial form under low temperature. Secretion of lipase was increased by high nitrogen concentration. SK-4 is considered to take the yeast form in aquatic environments and this form may secrete more lipase than the mycelial form.

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