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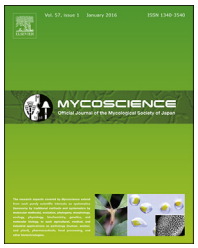

Full paper

Ethanol productivity of cryophilic basidiomycetous yeast *Mrakia* spp. correlates with ethanol tolerance

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ABSTRACT

Twenty-seven strains of basidiomycetous yeast *Mrakia* spp. were collected from lake sediment and soil surrounding the lakes in the Skarvsnes area, East Antarctica. Based on sequence similarity of their ribosomal internal transcribed spacer regions and D1/D2 domains, the isolated strains were classified into three species; *Mrakia blollopis* (18 strains), *M. gelida* (7 strains), and *M. robertii* (2 strains). All of the strains produced ethanol from glucose at 10 °C. Six strains selected from the 27 isolates were then tested for ethanol fermentation and ethanol tolerance at 100 g/l glucose. *Mrakia blollopis* SK-4 and *M. blollopis* NRI-4 completely consumed the glucose within 13 d and 6 d of fermentation respectively, and demonstrated high ethanol yields and ethanol tolerance. In contrast, *M. blollopis* AGK-3 and *M. blollopis* TKG1-2 exhibited low ethanol yields and poor ethanol tolerance. These results indicate that ethanol yields of different *Mrakia* species may depend on ethanol tolerance in individual strains.

1. Introduction

Cold environments cover a large portion of our planet. Approximately 85% of the biosphere is permanently exposed to temperatures below 5 °C (Feller and Gerday 2003) and approximately 14% of that is contributed by polar regions (Gunot 1999). To survive temperatures below 0 °C, microbes inhabiting cold environments have evolved unique physiological attributes, such as cold-adapted enzymes and antifreeze proteins (Buzzini et al. 2012). Antarctica is the fifth-largest continent and the southernmost landmass that covers approximately 14 million km^2. Approximately 98% of its area is covered by ice and snow and the temperatures in its coastal areas usually range from −35 °C to 5 °C. Temperatures on the Antarctic plateaus are much more extreme, ranging from −70 °C in winter to −25 °C in summer (Ravindra and Chaturvedi 2011). The Skarvsnes ice-free area is located along the central Soya Coast of East Antarctica, and contains many small oligotrophic freshwater lakes that constitute the only unfrozen water in the area (Imura et al. 1999). The surfaces of these lakes are covered by 1–2 m of ice over 11 mo of

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the year (Tanabe et al. 2008), but the lakes remain unfrozen below 3 m depth.

Cryophilic yeasts (Hoshino and Matsumoto 2012) of the genera *Mrakia* and *Mrakiella* have been found in the Arctic, Siberia, Alaska, Alps, Apennines, Patagonia and Antarctica (Morgesin et al. 2005; de Garcia et al. 2007; Pathan et al. 2010; Thomas-Hall et al. 2010; de Garcia et al. 2012; Singh and Singh 2012; Tsuji et al. 2013b, 2015). di Menna (1966) reported that the genus *Mrakia* accounts for about 24% of the culturable yeasts in Antarctic soil. Moreover, we previously reported that *Mrakia* spp. constitute about 35% of the culturable fungi isolated from lake sediments and soils of East Antarctica (Tsuji et al. 2013b). These reports suggest that *Mrakia* spp. are the dominant culturable fungi in East Antarctica and are well-adapted to the environment of the Skarvsnes ice-free area.

Relatively, little is known about the ethanolic fermentation in basidiomycetous yeasts. Fermentative ability has been reported for *Mrakiella* spp. (Jones and Slooff 1966), *Xanthophyllomyces* spp. (Fell et al. 2011), and *Bandoniozyma* spp. (Valente et al. 2012). Other species, namely, *Rhodotorula mucilaginosa*, *R. minuta*, *R. pallida* and *Cryptococcus saitoi*, were originally reported as unable to ferment ethanol (Fonseca et al. 2011; Sampio 2011). However, Rao et al. (2008) reported that some strains of these species can ferment ethanol from xylose. In Rao's report, ethanol yields ranged from 0.12 to 0.22 g-ethanol/g-D-xylose.

Seven *Mrakia* species are known; *M. frigida*, *M. gelida*, *M. stokesii*, *M. nivalis*, *M. psychrophila*, *M. robertii*, and *M. blollopis* (Thomas-Hall et al. 2010). Species in this basidiomycetous yeast genus are known for their ability to ferment sugars, and all can ferment glucose and sucrose. Sinclair and Stokes (1965) also noted that, at 10–15 °C, *M. frigida* CBS 5917 is a more efficient sugar fermenter than conventional yeast strains of *Saccharomyces cerevisiae*. Moreover, Thomas-Hall et al. (2010) tested the fermentation ability of *M. frigida*, *M. blollopis*, *M. gelida*, and *M. robertii* in a home brewing kit. Although all 4 strains fermented sucrose, their sucrose to ethanol conversion was incomplete and the cells ceased growing when the ethanol levels exceeded 2% (v/v).

Mrakia blollopis SK-4, isolated from Naga Ike Lake in Skarvsnes, East Antarctica, was found to ferment common sugars such as glucose, sucrose, maltose, lactose, raffinose, and galactose at 10 °C. At the same temperature, this species also converted 120 g/l of glucose to 48.7 g/l ethanol (Tsuji et al. 2013a,d), and completely converted glucose to ethanol over a wide pH range (4.0–10.0) (Tsuji et al. 2013c).

Although ethanol fermentation by members of the genus *Mrakia* has been investigated previously, the relationship between ethanol productivity and ethanol tolerance of *Mrakia* spp. remains uncertain. The present study investigated this relationship, and assessed the fermentation capacity of *Mrakia* spp. isolated from the Skarvsnes ice-free area.

2. Materials and methods

2.1. Sampling sites and sample collection

Samples were collected in and around lakes in the Skarvsnes ice-free area, located in the Lützow–Holm Bay area, East Antarctica. Samples of lake sediments and surrounding surface soils were collected from the following lakes: Ageha Ike, Bosatsu Ike, Ebine Ike, Hamagiku Ike, Hotoke Ike, Naga Ike, Nyorai Ike, Oyako Ike North Basin, Shimai Ike, Surikogi Ike, and Tokkuri Ike (Fig. 1). Collected samples were stored at −1 °C until used.

2.2. Mrakia strains

Twenty-seven *Mrakia* strains were investigated in this study. *Mrakia blollopis* SK-4 was previously isolated from Naga Ike lake; the other 26 strains were recently isolated from lake sediments and the soil surrounding lakes in the Skarvsnes ice-free area, East Antarctica (Tsuji et al. 2015).

2.3. Sequencing and phylogenetic analysis

DNA was extracted from yeast colonies using an ISOPLANT II kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocols. The extracted DNA was amplified by PCR using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). The internal transcribed spacer (ITS) region of the DNA samples was amplified using the following primers: ITS1F (5′-GTAACAAGGTTTCCGT) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC) (White et al. 1990). The D1/D2 domain of the ribosomal large subunit was amplified using another primer pair: NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5′-GGTCCGTGTTTCAAGACGG). The amplified DNA was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega KK, Tokyo, Japan). The purified DNA was sequenced at Macrogen Japan (Tokyo, Japan), and the most likely species identifications were determined by BLAST analysis (http://blast.ddbj.nig.ac.jp/blastn?lang=en). A strain was assigned to a species in the database if its sequence identity to that species exceeded 99%. The ITS region and D1/D2 domain sequences of *Mrakia* spp. were deposited into the DNA Data Bank of Japan (DDBJ) (Table 1). Because the ITS region and D1/D2 domain are contiguous overlapping, the two regions were used to combine into a concatenated sequence for each strain. The concatenated sequences were aligned by CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/), and manually adjusted. The alignment was deposited in TreeBASE (Submission ID: S17701). The combined ITS regions–D1/D2 domains sequences were used for phylogenetic reconstruction using the neighbor-joining method. The phylogenetic analysis was implemented with MEGA software version 6.05 (Tamura et al. 2013). Bootstrap analysis with 1000 replicates was used to the confidence the tree nodes.

2.4. Ethanol fermentation test

Ten μl aliquots ($OD_{600} = 25$) of each strain were inoculated in 15 ml YPD liquid medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) in a rotary shaker at 120 rpm for 120 h at 10 °C. After the 120 h of growth, cells from 15 ml of culture were collected by centrifugation at 3500 × g for 5 min at 4 °C. The pellet was resuspended in distilled water.