Note



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The Antarctic basidiomycetous yeast *Mrakia blollopis* SK-4 fermented ethanol between pH 5.0 and pH 10.0 with optimum pH at 8.0–10.0. Knowledge of ethanol fermentability as to the genus *Mrakia* remains incomplete. Further experiments are required to elucidate the ethanol fermentability of genus *e.g.*, as to optimum fermentation pH, optimum fermentation temperature, and cell viability during fermentation.

Key words: *Mrakia*; cryophilic yeast; ethanol fermentation; East Antarctica

Cold environments cover much of Earth including the deep sea, and most biospheres are permanently exposed to temperatures below $5 \,^{\circ}C^{(1)}$ Microbes adapted to such cold environments can grow at temperatures below 0°C and hence, they can be utilized in de novo bioprocesses.²⁾ Cryophilic yeasts,³⁾ Mrakia spp. and Mrakiella spp., have been found in the Arctic, Siberia, Alaska, CentralRussia, the Alps, the Apennines, Patagonia, and Antarctica.⁴⁾ Di Menna⁵⁾ reported that the genus Mrakia accounts for about 24% of culturable yeast in Antarctic soil. Moreover, we have reported that about 35% of culturable fungi isolated from lake sediment and soil of East Antarctica were Mrakia spp.⁶⁾ These reports suggest that Mrakia spp. are the dominant culturable yeasts in Antarctica and the most adapted to the Antarctic environment.

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,⁷⁾ 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 we report the effects of pH on the ethanol fermentation and cell viability of the cryophilic basidiomycetous yeasts *Mrakia blollopis* SK-4.

Ten μ L 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) at 120 rpm for 120 h at 10 °C. After 120 h, 400 mL of culture was collected by centrifugation at $3,500 \times g$ for 10 min at 4 °C. The pellet was dissolved in 50 mL of distilled water, and the resulting culture (OD₆₀₀ = 170) was used as an inoculum. OD₆₀₀ = 1 of the *Mrakia 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, 2g/L of NH₄Cl, 1g/L of KH₂PO₄, and 0.3g/L of MgSO₄•7H₂O 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 sample was collected every 24 h, 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 d. Then the colonies that appeared after 5 d were counted.

The glucose and ethanol concentrations in the fermentation solutions were measured by high-performance liquid chromatography (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 $65 \,^{\circ}\text{C}$ or $80 \,^{\circ}\text{C}$.⁸⁾ All experiments were carried out independently in three vials, and average results are given.

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 h of fermentation. When the

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Fig. 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.

yeast was fermented at pH 6.0–7.0, the glucose consumption speed was slower than at pH 5.0 and glucose was completely consumed at 168 h. Moreover, when we performed a fermentation test, 3 times at pH 5.0, glucose was completely consumed by 168 h 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 h of ethanol fermentation (Q_E) are summarized in Table 1. The theoretical yield of ethanol production was calculated as follows:

%Theoretical yield $[Y_{E/EY} (\%)]$

= $EtOH_M/(Initial glucose concentration \times 0.51)$.

We also measured the pH of 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 h of fermentation, and these pH remained steady after 168 h of fermentation (data not shown). The colony

Table 1. Summary of Ethanol Fermentation at Various pH Values by

 Mrakia blollopis

	Initial glucose concentration (g/L)	EtOH _M (g/L)	$\begin{array}{c} Y_{E/G} \\ (g/g) \end{array}$	Y _{E/EY} (%)	$\begin{array}{c} Q_E \\ (g/L \cdot h) \end{array}$
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 gethanol/g glucose. Q_E , volumetric ethanol production rate after 24 h of ethanol fermentation. counts of SK-4 dramatically decreased during first 24 h 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 we do not know why SK-4 cell viability dramatically decreased in 24 h of fermentation.

To the best of our knowledge, little is known about the fermentation by basidiomycetous yeast. Some species have been reported to have fermentative ability, such as Mrakiella spp.,⁹⁾ Rhodotorula spp.,¹⁰⁾ Xanthophyllomyces spp.,¹¹⁾ and Bandoniozyma spp.¹²⁾ Seven species of Mrakia have been reported: Mrakia frigida, Mrakia gelida, Mrakia stokesii, Mrakia nivalis, Mrakia psychrophila, Mrakia robertii, and Mrakia blollopis.⁴⁾ 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.⁴⁾ reported that all of those strains fermented sucrose, but did not completely convert sucrose to ethanol, and that cell growth was stopped in the presence of over 2% (v/v) ethanol. Strain SK-4 fermented raffinose, galactose, lactose, and maltose at low temperature, while CBS8921^T could not ferment raffinose, galactose, lactose, or maltose.⁷⁾ Moreover, it fermented, at -1-20 °C and the optimum ethanol fermentation temperature was 10-15 °C (data not shown). Maximally, 48.7 g/L of ethanol was produced from 120 g/L of glucose by SK-4 at 10 °C at 19 d fermentation.¹³⁾

We had little information about SK-4 fermentability, and hence we tested ethanol production by SK-4 at various pH values. When it was used for fermentation at below pH 4.0 and above pH 10.5, SK-4 did not completely convert glucose to ethanol. Buzás et al.¹⁴⁾ studied the effect of pH on ethanol fermentation with Saccharomyces cerevisiae SC1. The optimal pH for strain SC1 was 4.0. When it was used for fermentation at pH 2.0, it had 70% fermentation capacity as compared to 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 6.0. When NYC 3413 was used for fermentation at pH 5.0 and 6.5, it had 25% and 78% fermentation capacity as compared to pH 6.0^{-15} M. blollopis SK-4, on the other hand, fermented between pH 5.0 to 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, it had about 63% fermentation capacity at pH 2.0 and 78% fermentation capacity at pH 11.0 as compared to pH 8.5.

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 (data not shown). SK-4 has stable lipase against metal ions and organic solvents, and the optimum pH of SK-4 lipase is 8.5–9.0.7) Naga-ike is an oligotrophic lake, and pH is 8.5 (Tanabe, Ph. D. thesis, Graduate University for Advanced Studies, 2009). Knowledge as to ethanol fermentability of the genus Mrakia remains incomplete. Further experiments are required to elucidate the ethanol fermentability of this genus Mrakia, e.g., optimum fermentation pH, optimum fermentation temperature, and cell viability during fermentation. This is the first report on the influence of initial pH on ethanol fermentation by cryophilic basidiomycetous yeast at low temperature.

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