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Isolation of thermotolerant yeast *Pichia kudriavzevii* from *nuruk*

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Abstract Thermotolerant yeast strains were isolated from *nuruk*, a traditional Korean fermentation starter in which variety of microorganisms are present. Among the isolates, the MBY1358 identified as yeast *Pichia kudriavzevii* showed significantly higher growth rate (0.59 ± 0.00 1/h) at 44 °C than other strains. Maximum ethanol concentration of 8.35 ± 0.03 g/L was obtained from 20 g/L glucose with yield of 0.44 ± 0.01 g/g at 44 °C, which is 1.14 times ethanol production of the control strain of *P. kudriavzevii*. The MBY1358, which was significantly more thermotolerant than the control strain and fermented 200 g/L glucose to 107.33 ± 5.03 g/L ethanol at 44 °C, was deposited to Korean Collection for Type Cultures (KCTC) under the accession number 27654.

Keywords *Nuruk* · Thermotolerance · Yeast · *Pichia kudriavzevii* · Ethanol

Introduction

In recent years, there has seen the introduction of large-scale processing for the bioconversion of renewable biomass including feed stock, agricultural crops and animal wastes [1, 2]. The most important economic obstacles for the production of ethanol from biomass are the cost of the enzyme and loss of productivity due to inactivation of yeasts by various inhibitory conditions such as heat, acid, and fermentation inhibitors [3, 4]. In most cases, large-scale ethanol fermentation is performed at high concentrations of sugar and ethanol, and high temperatures, and these conditions cause stress to the yeast cells [5, 6]. Thus, tolerance to high temperature is one of the most desired properties of microorganisms of interest to fermentation industry. In particular, this property is advantageous for the reduction of cooling costs and contamination by other strains [7]. Simultaneous saccharification and fermentation (SSF) of biomass requires the use of microorganisms capable of working at high temperature [8]. The optimum temperature ranges is 40–60 °C for saccharification and 25–35 °C for fermentation [8].

Nuruk is a traditional Korean fermentation starter that contains a variety of microorganisms [9–11] including yeasts and other fungi, and bacteria [12]. Yeasts isolated from *nuruk* have been used to produce an alcoholic beverage and traditional fermented foods [13]. Non-conventional yeast species such as *Pichia stipitis* [14], *Kluyveromyces lactis* [15], *Candida thermophila* [16] and *Kluyveromyces marxianus* [17] have advantageous characteristics such as tolerance to acid or heat, or the ability to metabolize carbohydrates that *Saccharomyces cerevisiae* does not naturally metabolize [18]. Isono et al. [19] reported the ability of the yeast *Pichia kudriavzevii* to grow and produce ethanol at temperatures up to 43 °C.

In this study, thermotolerant *P. kudriavzevii* strains were isolated from *nuruks* and their growth and ethanol production properties were examined and compared with those of the control strain.

Materials and methods

Collection of *nuruk*

A variety of *nuruks* manufactured in different areas in Korea were purchased from local markets. A total of 12

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nuruks were collected from Busan (1), Jinju (2), Gwangju (2), Jeju (1), Sangju (1), Yesan (2), Chuncheon (1), and Yongin (2).

Strain isolation and growth conditions

One gram of *nuruk* was suspended in peptone water [20] and plated onto YEPD agar (1% bacto-yeast extract, 2% bacto-peptone, 2% glucose, and 2% bacto-agar, w/v) supplemented with chloramphenicol (0.01%, w/v) [20]. The plates were incubated at 30 °C for 48 h. As a result, 200 strains were isolated from *nuruk* and each colony was picked up, purified by streaking on YEPD agar, suspended in YEPD broth, supplemented with 15% glycerol, and stored at −80 °C. To evaluate cell growth rate and ethanol productivity, a colony was transferred from a YEPD plate into 10 mL of YEPD broth, incubated overnight at 30 °C, diluted to an OD_{600} of 0.2 with the same sterile medium, and cultivated to an exponential growth phase. Cells were then harvested, washed, inoculated into 100 mL of fresh YEPD in 500-mL shake flasks, and incubated at 30, 37, and 44 °C for 48 h. An API 20C kit (BioMérieux, France) was used examine the utilization of various substrates as carbon source. *Pichia kudriavzevii* KCTC17763 isolated from cabbage was used as control strain.

Strain identification

Genomic DNA was extracted from yeast cells grown in YEPD broth. Cells were collected by brief centrifugation, and genomic DNA was purified using a GenEx^{TM} genomic Sx kit (GeneAll, Korea) according to the manufacturer's instructions. To identify yeast strains, primers ITS1, ITS4 [21] were used to amplify the internal transcribed spacer (ITS) [22]. Polymerase chain reaction (PCR) was performed at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min [20]. PCR products were T-A cloned and sequenced; the sequences were analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Amersham Biosciences, USA). A high-performance liquid chromatograph LC-20 (Shimadzu, Japan) equipped with a Rezex ROA-Organic Acid H^+ column (Phenomenex, USA) and a refractive index detector (Shimadzu) was used to determine the concentrations of ethanol and glucose. The column was maintained at 65 °C. H_2SO_4 (5 mM) solution at a flow rate of 0.6 mL/min was used as a mobile phase.

Statistical analysis

Data were expressed as mean ± standard error of the mean using SigmaPlot 12 software (SPSS Inc., USA). A Duncan's multiple range test [23] was performed using PASW 18.0 (SPSS Inc.) to verify the group results.

Results and discussion

Isolation and identification of thermotolerant yeast strains

Several hundred colonies were isolated from *nuruks* purchased from local markets. To select thermotolerant yeast strains, single colonies were inoculated and cultivated in YEPD broth at 44 °C for 48 h. Five strains (MBY1325, 1330, 1337, 1357, and 1358) which showed rapid growth and high ethanol productivity were identified based on the ITS region of the rDNA gene. Interestingly, all strains were identified as *P. kudriavzevii*; their ITS regions shared over 99% similarity with that of the type strain (GenBank No. KM234478 or KF959839). MBY1325, 1330, 1337, 1357, and 1358 strains were isolated from the Yesan *nuruk*. MBY1358 showed approximately 99% homology with *P. kudriavzevii* (LC014798). Phylogenetic tree of ITS region of the rDNA gene with other various yeasts is shown in Fig. 1.

Carbon assimilation patterns of the isolated *P. kudriavzevii* strains were examined. All strains except MBY1325 assimilated glucose and glycerol. None of the tested strains utilized D-xylose, adonitol, xylitol, galactose, inositol, sorbitol, α-methyl-D-glucoside, cellobiose, lactose, maltose, sucrose, trehalose, or melezitose as sole carbon source. In addition, contrast to the control strain, *P. kudriavzevii* KCTC17763, all *P. kudriavzevii* isolates were unable to utilize raffinose as carbon source.

Growth characteristics of *P. kudriavzevii* isolates

Thermotolerance of *P. kudriavzevii* MBY1325, 1330, 1337, 1357, and 1358 were compared with that of KCTC17763. All strains showed the maximum growth rate at 37 °C (Table 1) and cell growth was retarded with the increase in cultivation temperature. MBY1325, 1337 and KCTC17763 were more sensitive to temperature than other strains, whereas MBY1358 showed thermotolerance at 44 °C.

Cell growth, glucose consumption, and ethanol production rates of the *P. kudriavzevii* isolates were examined in shake-flask cultures grown for 48 h at various temperatures (Table 1; Fig. 2). KCTC17763 produced 9.80 ± 0.32 g ethanol from 20 g glucose at 30 °C,

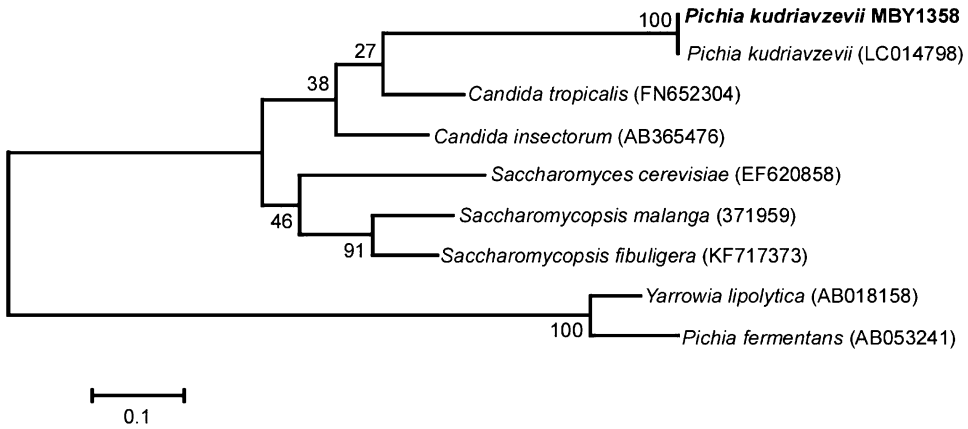

Fig. 1 The phylogenetic tree of the *P. kudriavzevii* ITS region. The tree was constructed with the neighbor-joining method. The *numbers* on the nodes correspond to the percentages, with which a cluster appears in a bootstrap test based on 1000 pseudoreplicates. The *bars* denote the relative branch length. The ITS region was identified by their GenBank accession number in parentheses

Table 1 Cell growth and ethanol production by *P. kudriavzevii* strains grown at various temperatures

Temperature (°C)	Strain	Cell mass (g/L)	Specific growth rate (1/h)	Ethanol (g/L)	Ethanol productivity (g/L h)	Ethanol yield (g/g)
30	KCTC17763	3.97 ± 0.18	0.41 ± 0.00	9.80 ± 0.32	0.81 ± 0.00	0.49 ± 0.00
	MBY 1325	4.23 ± 0.03	0.41 ± 0.00	9.14 ± 0.47	0.76 ± 0.01	0.47 ± 0.02
	MBY 1330	3.75 ± 0.05	0.38 ± 0.01	9.72 ± 0.20	0.81 ± 0.00	0.50 ± 0.01
	MBY 1337	4.75 ± 0.01	0.38 ± 0.01	9.36 ± 0.16	0.79 ± 0.00	0.42 ± 0.01
	MBY 1357	4.43 ± 0.12	0.60 ± 0.01	9.57 ± 0.06	0.80 ± 0.00	0.43 ± 0.00
	MBY 1358	4.74 ± 0.03	0.60 ± 0.02	9.60 ± 0.09	0.80 ± 0.00	0.43 ± 0.00
37	KCTC17763	2.81 ± 0.20	0.74 ± 0.01	8.08 ± 0.29	0.67 ± 0.01	0.44 ± 0.01
	MBY 1325	3.19 ± 0.07	0.51 ± 0.01	8.08 ± 0.52	0.67 ± 0.01	0.38 ± 0.02
	MBY 1330	3.48 ± 0.11	0.60 ± 0.02	8.43 ± 0.12	0.70 ± 0.00	0.40 ± 0.01
	MBY 1337	3.37 ± 0.01	0.62 ± 0.02	8.26 ± 0.21	0.69 ± 0.00	0.37 ± 0.01
	MBY 1357	3.77 ± 0.07	0.69 ± 0.01	8.43 ± 0.19	0.70 ± 0.00	0.32 ± 0.00
	MBY 1358	4.12 ± 0.06	0.70 ± 0.01	8.86 ± 0.28	0.74 ± 0.01	0.41 ± 0.01
44	KCTC17763	1.50 ± 0.02	0.51 ± 0.01	7.35 ± 0.00	0.61 ± 0.00	0.44 ± 0.00
	MBY 1325	1.44 ± 0.05	0.35 ± 0.00	7.07 ± 0.51	0.59 ± 0.01	0.37 ± 0.02
	MBY 1330	1.58 ± 0.03	0.42 ± 0.02	7.21 ± 0.53	0.60 ± 0.02	0.41 ± 0.07
	MBY 1337	1.51 ± 0.17	0.34 ± 0.01	7.23 ± 0.16	0.60 ± 0.00	0.39 ± 0.01
	MBY 1357	1.84 ± 0.08	0.48 ± 0.03	7.99 ± 0.18	0.66 ± 0.02	0.42 ± 0.04
	MBY 1358	2.19 ± 0.02	0.59 ± 0.00	8.35 ± 0.03	0.69 ± 0.00	0.44 ± 0.01

Averages and standard errors were determined from three independent shake flasks cultures. Concentrations of cell mass and ethanol were determined after 12 h inoculation. Specific growth rate of each strain was determined at an exponential growth phase

corresponding to an ethanol yield of 0.49 ± 0.00 g/g based on the amount of glucose consumed; this strain showed a specific growth rate of 0.41 ± 0.00 1/h. At 30 °C, MBY1357 (0.60 ± 0.01 1/h) and 1358 (0.60 ± 0.02 1/h) showed higher specific growth rates than KCTC17763, MBY1325 (0.41 ± 0.00 1/h), MBY1330 (0.38 ± 0.01 1/h), and MBY1337 (0.38 ± 0.01 1/h). In case the temperature was increased to 37 °C, cell growth of KCTC17763 was significantly decreased, as was the amount of ethanol formed from 20 g glucose (8.08 ± 0.29 g/L). At 37 °C, MBY1358 produced 8.86 ± 0.28 g ethanol from 20 g glucose with a productivity of 0.18 ± 0.01 g/L h. As the temperature was increased further, MBY1358 showed specific growth rate superior to those of the other strains and produced a maximum ethanol concentration of 8.35 ± 0.03 g/L; its ethanol production was 13.6% higher than that of KCTC17763. At 44 °C (Fig. 2), the specific growth rate of MBY1358 (0.59 ± 0.00 1/h) was 15.6% higher than that of KCTC17763 (0.51 ± 0.01 1/h).

Cell growth and ethanol production by MBY1358 and control strain were further examined in shake-flasks cultures using an elevated glucose concentration. The MBY1358 consumed 200 g/L glucose for 40 h to produce 107.33 ± 5.03 g/L ethanol (Fig. 3). However, KCTC17763 did not utilize glucose completely, i.e.,

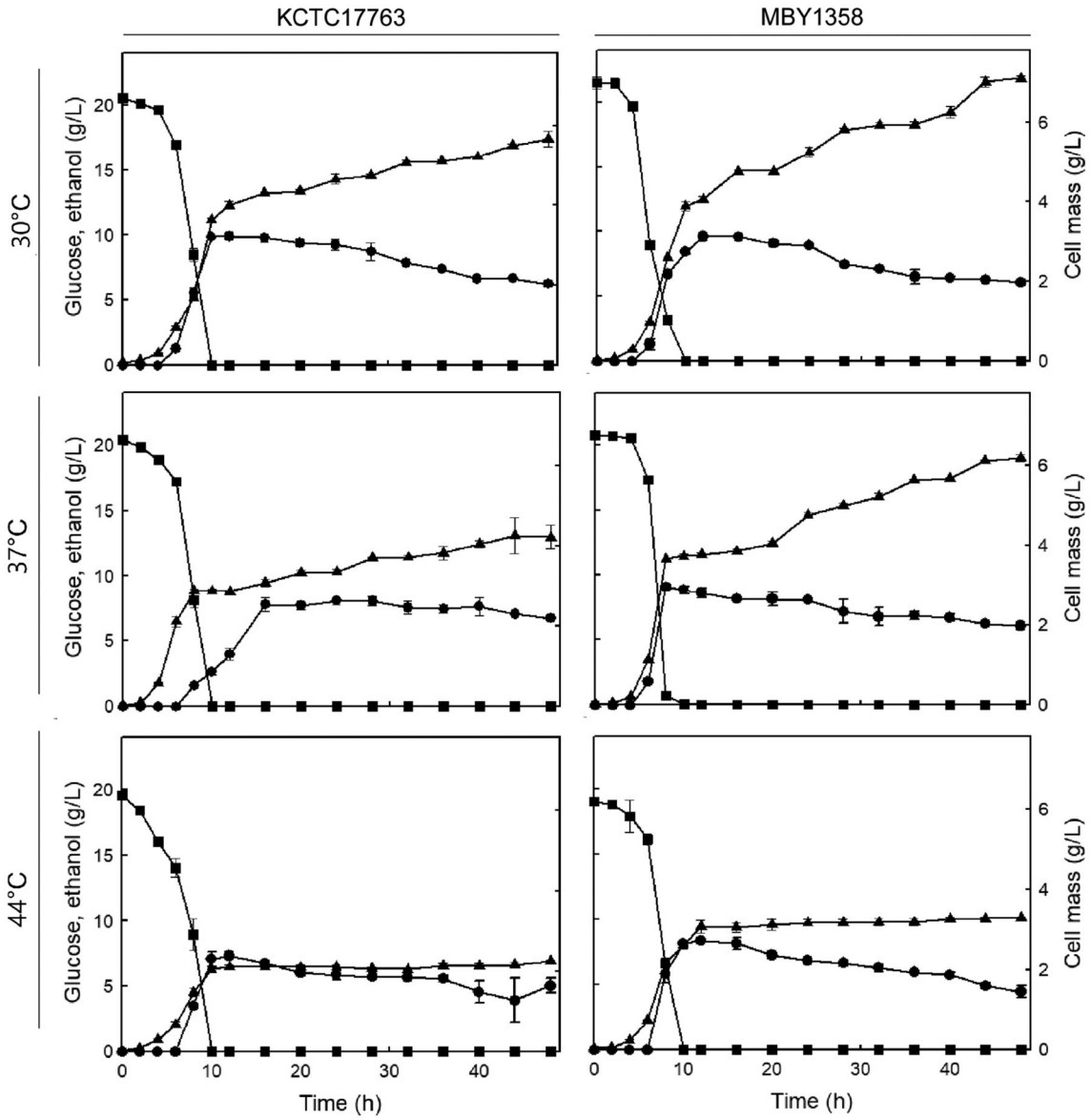

Fig. 2 Profiles of cell growth (*filled triangle*), glucose consumption (*filled square*), and ethanol production (*filled circle*) by *P. kudriavzevii* KCTC17763 and MBY1358 grown in YEPD at various temperatures (30, 37, 44 °C) for 48 h. Averages and standard errors determined from three independent cultivations are shown

31.10 ± 0.34 g/L glucose was present after 48 h. Ethanol concentration of 54.00 ± 3.80 g/L was obtained by control strain KCTC17763. Dhaliwal et al. [24] reported *P. kudriavzevii*, isolated from sugarcane, produced 71.9 g/L ethanol from sugarcane juice at 40 °C. *Pichia kudriavzevii* PBB511-1 produced 42.4 g/L ethanol for 48 h at 45 °C from 180 g/L reducing sugar obtained from cassava [25]. These results indicate that MBY1358 might be a promising thermotolerant *P. kudriavzevii* strain capable of fermenting glucose to produce ethanol.

Strains able grow at high temperatures are industrially useful, especially in bioethanol production, because the risk of contamination by other microorganisms and the cost of reactor cooling are reduced [26, 27]. For bioethanol production from lignocellulosic biomass, SSF is performed by enzymatic hydrolysis, usually followed by yeast fermentation. Optimal temperature for substrate degradation by cellulolytic enzymes is around 55 °C [26, 27], which means that the availability of a thermotolerant yeast strain is crucial for SSF-based bioethanol production. A number of studies reported thermotolerant yeasts such as *Kluyveromyces marxianus* [17], *Hansenula polymorpha* [28], *Debaryomyces hansenii* [29], and *P. kudriavzevii* [30]. Koutinas et al. [31] reported that *P. kudriavzevii* KVMP10

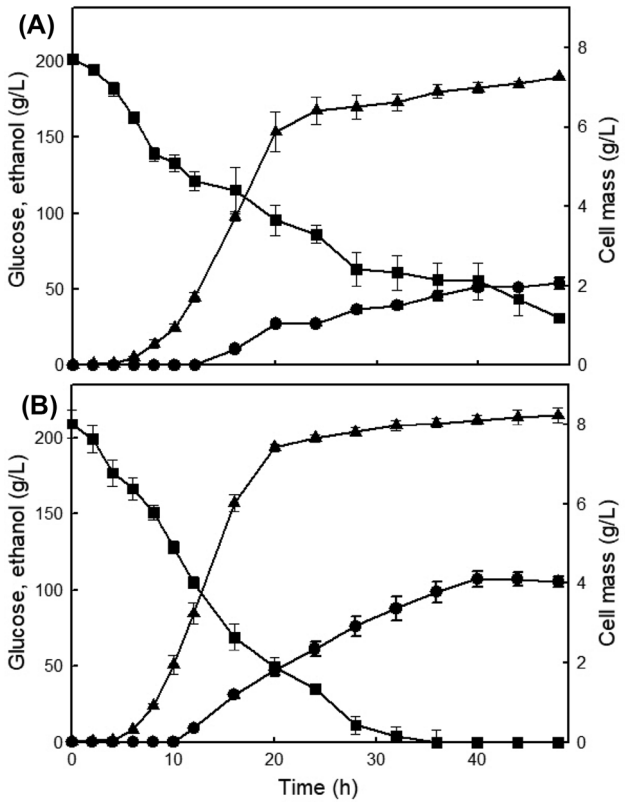

Fig. 3 Profiles of cell mass (*filled triangle*), glucose consumption (*filled square*), and ethanol production (*filled circle*) by *P. kudriavzevii* KCTC17763 (**A**) and MBY1358 (**B**). Shake-flask cultures were grown in YEPD medium containing 200 g/L glucose at 44 °C. Averages and standard errors determined from three independent cultivations are shown

produces ethanol from glucose at 42 °C. *Pichia kudriavzevii* DMKU 3-ET15 [32] was reported to produce 7.35% (w/v) ethanol with a volumetric productivity of 2.23 g/L h. *Pichia kudriavzevii* was also engineered to produce value-added organic acids such as lactic acid [33], succinic acid [34], and xylonic acid [30].

In conclusion, *P. kudriavzevii* strain MBY1358 showing significantly higher thermotolerance and ethanol productivity than the control strain was isolated from *nuruk* in this study. Further studies including improvement of stress tolerance and construction of gene expression vectors, are underway to enable utilization of this promising yeast strain.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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