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SHORT COMMUNICATION

Ethanol Production Potential of Ethanol-Tolerant *Saccharomyces* and Non-*Saccharomyces* Yeasts

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Abstract

Four ethanologenic ethanol-tolerant yeast strains, *Saccharomyces cerevisiae* (ATKU132), *Saccharomycodes ludwigii* (ATKU47), and *Issatchenkia orientalis* (ATKU5-60 and ATKU5-70), were isolated by an enrichment technique in yeast extract peptone dextrose (YPD) medium supplemented with 10% (v/v) ethanol at 30°C. Among non-*Saccharomyces* yeasts, *Sd. ludwigii* ATKU47 exhibited the highest ethanol-tolerance and ethanol production, which was similar to *S. cerevisiae* ATKU132. The maximum range of ethanol concentrations produced at 37°C by *S. cerevisiae* ATKU132 and *Sd. ludwigii* ATKU47 from an initial D-glucose concentration of 20% (w/v) and 28% (w/v) sugarcane molasses were 9.46–9.82% (w/v) and 8.07–8.32% (w/v), respectively.

Key words: ethanol production, ethanol-tolerant, *Saccharomyces* yeast, non-*Saccharomyces* yeast

In recent years, ethanol used as a fuel, has become increasingly important because it is a renewable and environmentally friendly resource. The major requirements of industrial fuel ethanol production are high ethanol yield and low-cost production. Effective ethanol production depends on yeast fermentation capability and the ability to grow under industrial conditions, such as ethanol accumulation, high temperature, low pH and osmotic pressure from product and substrate sugars (Blieck *et al.*, 2007, Zhao and Bai, 2009). Among various stresses, ethanol concentration is the most important stress factors that can not be avoided during fermentation (Querol *et al.*, 2003). High ethanol concentration is well known to inhibit cell growth and viability (Pina *et al.*, 2004). Multiple study performed to date, focused interest on ethanol tolerance in yeasts based on the presumption that ethanol-tolerant yeast strains would have enhanced ethanol productivity and yield, and such strains can be used for the production of economically viable industrial fuel ethanol (Basso *et al.*, 2008, Hu *et al.*, 2007, Shi *et al.*, 2009).

In this study, we describe the screen for ethanologenic and ethanol-tolerant yeast strains from environmental samples. We also tested the capability of the selected yeast strains to produce ethanol from D-glucose and carbon substrates from sugarcane molasses and yeast cell viability in ethanol.

We assumed that soil samples in long-term exposure to ethanol might contain ethanol-tolerant microorganisms. To confirm this, soil samples from the drainage area of a winery at Kasetsart University, Kamphaeng Saen Campus were collected and enriched in YPD (1% yeast extract, 2% peptone, 2% D-glucose) medium supplemented with 10% (v/v) ethanol for 48–72 h at 30°C, and then plated onto YPD agar supplemented with 10% (v/v) ethanol using spread-plate technique. Each isolated ethanol-tolerant strain was grown in medium supplemented with 10% (v/v) ethanol in a test tube containing a Durham tube and incubated at 30°C for 72 h. The strains showing an accumulation of CO_2 gas in the Durham tubes were selected for screening for high ethanol-production in fermentative (FM) medium

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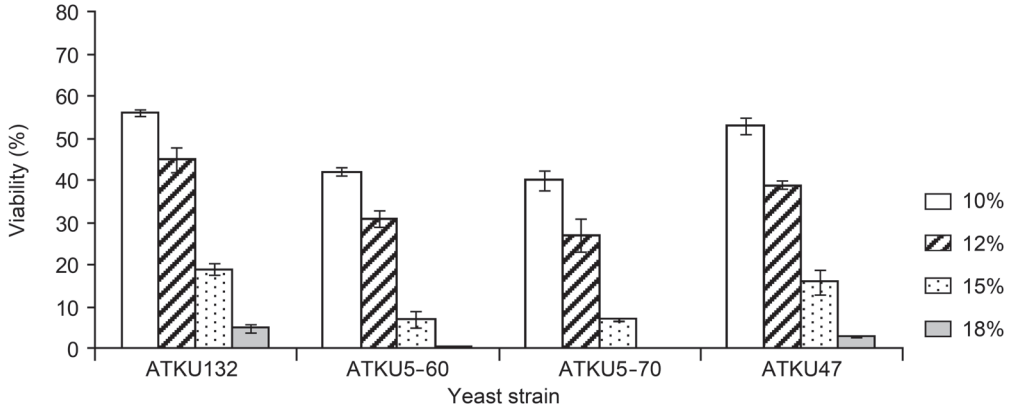

Fig. 1. Effect of increasing ethanol concentration on cell viability of the four selected ethanol-tolerant yeasts. Ethanol concentrations are given in % (v/v). Viability at each ethanol concentration is expressed as the % of the colony-forming units for the stress treatments compared with the untreated condition

(1% yeast extract, 2% peptone, 10% D-glucose, 0.6% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, pH 5.5) (Shi *et al.*, 2009) at 30°C with shaking at 100 rpm for 48 h. The ethanol concentration was analyzed by gas chromatography (GC) (Chrompack CP9001, Chrompack, The Netherlands) equipped with a capillary column type CP WAX 52 CB (Chrompack, The Netherlands) and fitted with a flame ionization detector. The temperature of the detector and injector were 275°C and 250°C, respectively.

The selected ethanologenic ethanol-tolerant yeasts were identified by rDNA sequence analysis. The DNA region containing the D1/D2 domain of the 26S rRNA gene was amplified using the universal fungal primer pairs NL1 and NL4 (O'Donnell, 1993). PCR products were sequenced and compared with the sequences from the GenBank database using the BLAST tool (www.ncbi.nlm.nih.gov/BLAST/).

The viability of yeast cells in ethanol was tested in a medium containing ethanol. Yeast cells were grown in YPD medium at 30°C and were collected in log-phase of growth, cells were washed twice with YPD medium and then were transferred to YPD medium with 10–18% (v/v) ethanol and without ethanol as a control (final density 2×10^6 cells/ml). The cultures were incubated at 30°C with shaking at 100 rpm for 36 h. Serial dilutions of each yeast culture were plated on YPD agar and incubated at 30°C for 72 h.

Among the 82 tested ethanol-tolerant yeast strains, four ethanologenic ethanol-tolerant strains – ATKU132, ATKU47, ATKU5-60, and ATKU5-70, were selected for further studies. The strains exhibited the highest ethanol production (5–6% w/v), in FM medium containing 10% (w/v) D-glucose when grown at 30°C for 48 h. Based on the results of the BLAST comparison, the four ethanologenic ethanol-tolerant yeasts can be classified into two groups, *Saccharomyces* (*Saccharomyces cerevisiae* ATKU132) yeast and non-*Saccharomyces* yeasts (*Saccharomycodes ludwigii* ATKU47, and *Issatchenkia orientalis* ATKU5-60 and ATKU5-70). *S. cerevisiae* is one of the best known microorganisms used for industrial ethanol fermentation. This yeast exhibits higher ethanol tolerance than ethanol-producing bacteria and non-*Saccharomyces* yeasts (Ciani and Picciotti, 1995). Species of non-*Saccharomyces* yeasts generally are not tolerant to ethanol concentrations exceeding 5–6% (v/v) (Fleet, 2003, Gil *et al.*, 1996). The results of ethanol tolerance test revealed that the isolated non-*Saccharomyces* yeasts could survive in media containing 10–15% (v/v) ethanol (Fig. 1). When yeast cells were exposed to 18% (v/v) ethanol, *S. cerevisiae* ATKU132 and *Sd. ludwigii* ATKU47 exhibited cell viability in the range of 3–5%, while the others lost their viability. This is in an agreement with several reports which suggest that various species of non-*Saccharomyces* yeast, such as *Hanseniaspora*, *Candida*, *Saccharomycodes* and *Zygosacchromyces*, may have tolerance similar to the *Saccharomyces* species (Fleet, 2008; Pina *et al.*, 2004).

In addition, we found a correlation between cell viability and fermentation capability. The highest ethanol-tolerant strains, *S. cerevisiae* ATKU132 and *Sd. ludwigii* ATKU47 were producing ethanol with the highest efficiency. Table I presents the results of ethanol production in a medium containing 20% (w/v) D-glucose at 37°C with aeration (shaking), which is the commonly used temperature during ethanol fermentation in a tropical country. Maximal ethanol concentration produced by *Sd. ludwigii* ATKU47 was 9.82 ± 0.14% (w/v), productivity of 1.64 ± 0.02 g/l/h and a theoretical yield of 96.05%. The same values for *S. cerevisiae* ATKU132 were 9.46 ± 0.16% (w/v), 1.31 ± 0.02 g/l/h and 92.56%, respectively.

Based on the ability to utilize and ferment D-sucrose of *S. cerevisiae* ATKU132 and *Sd. ludwigii* ATKU47 (data not shown), these yeast strains were selected to test their abilities to ferment sugarcane molasses, a by-product of sugar manufacturing, which con-

Table I
Ethanol fermentation by the ethanologenic ethanol-tolerant strains in 20% (w/v) D-glucose and 28% (w/v) sugarcane molasses at 37°C for 72 h

Strain	20% (w/v) D-glucose			28% (w/v) sugarcane molasses		
	Ethanol[1] % (w/v)	Productivity (g/l/h)	Theoretical yield (%)	Ethanol[1] % (w/v)	Productivity (g/l/h)	Theoretical yield (%)
S. cerevisiae ATKU132	9.46±0.16[c] (72 h)	1.31 ± 0.02[b]	92.56[c]	8.07 ± 0.10[a] (72 h)	1.12 ± 0.03[a]	92.67[a]
Sd. ludwigii ATKU47	9.82±0.14[c] (60 h)	1.64 ± 0.02[d]	96.05[d]	8.32 ± 0.07[a] (72 h)	1.15 ± 0.02[a]	95.54[b]
I. orientalis ATKU5-60	8.27 ± 0.30[b] (60 h)	1.38 ± 0.05[c]	80.93[b]	n.a.	n.a.	n.a.
I. orientalis ATKU5-70	7.30 ± 0.23[a] (60 h)	1.22 ± 0.04[a]	71.43[a]	n.a.	n.a.	n.a.

Results with the same letter are not significantly different ($p < 0.05$)
[1] The time points indicate the maximum ethanol concentrations producing by the yeast strains.
* The following theoretical values have been used for calculation (g ethanol/g sugar): sucrose, 0.538; maltose, 0.538; glucose, 0.511; fructose, 0.511; maltotriose, 0.548. The values of composition of fermentable sugars in molasses (78% (w/v)) have been used for calculation (% w/w): sucrose, 34.6; fructose, 13.5; glucose, 10.4; maltose, 0.11; maltotriose, 0.45. Theoretical yield of undiluted molasses = 0.311 g/g molasses.
n.a. – not analyzed

tains sucrose as a major fermentable sugar. Results presented in Table I show that *S. cerevisiae* ATKU132 and *Sd. ludwigii* ATKU47 were able to produce high amounts of ethanol from sugarcane molasses medium (28% soluble solid, 0.05% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.3). The amount of ethanol produced by *S. cerevisiae* ATKU132 and *Sd. ludwigii* ATKU47 were 8.07% (w/v) and 8.32% (w/v), respectively.

In summary, the results of this study indicated that the newly isolated ethanol-tolerant yeasts, especially *S. cerevisiae* ATKU132 and *Sd. ludwigii* ATKU47, are excellent strains that are promising candidates for large-scale ethanol production from sugarcane waste by-products. Further evaluation of these strains under scaled-up conditions and strain improvement for increasing ethanol yield are planned for future study.

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Literature

Basso L.C., H.V. De Amorim, A.J. De Oliveira and M.L. Lopes. 2008. Yeast selection for fuel ethanol production in Brazil. *FEMS Yeast Research*. 8: 1155–1163.

Blieck L., G. Toye, F. Dumortier, K.J. Verstrepen, F.R. Delvaux, J.M. Thevelein and P. Van Dijck. 2007. Isolation and characterization of brewer's yeast variants with improved fermentation performance under high-gravity conditions. *Appl. Environ. Microbiol.* 73: 815–824.

Ciani M. and G. Picciotti. 1995. The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making. *Biotechnol. Lett.* 17: 1247–1250.

Fleet G.H. 2003. Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86: 11–22.

Fleet G.H. 2008. Wine yeasts for the future. *FEMS Yeast Res*. 8: 979–995.

Gil J.V., J.J. Mateo, M. Jiménez, A. Pastor and T. Huerta. 1996. Aroma compounds in wine as influenced by apiculate yeasts. *J. Food Sci.* 61: 1247–1250.

Hu X.H., M.H. Wang, T. Tan, J.R. Li, H. Yang, L. Leach, R.M. Zhang and Z.W. Luo. 2007. Genetic dissection of ethanol tolerance in the budding yeast *Saccharomyces cerevisiae. Genetics.* 175: 1479–1487.

O'Donnell K. 1993. *Fusarium and its near relatives*, in *The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics*, Eds. D.R. Reynolds and J.W. Taylor, CAB International: Wallingford. p. 225–233.

Pina C., J.A. Couto and T. Hogg. 2004. Inferring ethanol tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts by progressive inactivation. *Biotechnol. Lett.* 26: 1521–1527.

Querol A., M.T. Fernandez-Espinar, M. del Olmo and E. Barrio. 2003. Adaptive evolution of wine yeast. *Int. J. Food Microbiol.* 86: 3–10.

Shi D.J., C.L. Wang and K.M. Wang. 2009. Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae. J. Ind. Microbiol. Biotechnol.* 36: 139–147.

Zhao X.Q. and F.W. Bai. 2009. Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. *J. Biotechnol.* 144: 23–30.