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 at 30°C. Among non-Saccharomyces yeasts, Sd. ludwigii ATGU47 exhibited the highest ethanol-tolerance and ethanol production, which was similar to S. cerevisiae ATGU132. The maximum range of ethanol concentrations produced at 37°C by S. cerevisiae ATGU132 and Sd. ludwigii ATGU47 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
(1% yeast extract, 2% peptone, 10% D-glucose, 0.6% \( \text{NH}_4 \)\text{SO}_4, 0.15% KH\text{PO}_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 \( \times \) 10⁶ 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, \textit{Saccharomyces} (\textit{Saccharomyces cerevisiae} ATKU132) yeast and non-\textit{Saccharomyces} yeasts (\textit{Saccharomycodes ludwigii} ATKU47, and \textit{Issatchenka orientalis} ATKU5-60 and ATKU5-70). \textit{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-\textit{Saccharomyces} yeasts (Ciani and Picciotti, 1995). Species of non-\textit{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-\textit{Saccharomyces} yeasts could survive in media containing 10–15% (v/v) ethanol (Fig. 1). When yeast cells were exposed to 18% (v/v) ethanol, \textit{S. cerevisiae} ATKU132 and \textit{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-\textit{Saccharomyces} yeast, such as \textit{Hanseniaspora}, \textit{Candida}, \textit{Saccharomyces} and \textit{Zygosaccharomyces}, may have tolerance similar to the \textit{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, \textit{S. cerevisiae} ATKU132 and \textit{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 \textit{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 \textit{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 \textit{S. cerevisiae} ATKU132 and \textit{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-
contains 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% (NH₄)₂SO₄, 0.05% KH₂PO₄, 0.05% MgSO₄·7H₂O, 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


### Table I

<table>
<thead>
<tr>
<th>Strain</th>
<th>20% (w/v) D-glucose</th>
<th>28% (w/v) sugarcane molasses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol% (w/v)</td>
<td>Productivity (g/l/h)</td>
<td>Theoretical yield (%)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> ATKU132</td>
<td>9.46±0.16⁺ (72 h)</td>
<td>1.31±0.02⁺</td>
</tr>
<tr>
<td><em>Sd. ludwigii</em> ATKU47</td>
<td>9.82±0.14⁺ (60 h)</td>
<td>1.64±0.02⁻</td>
</tr>
<tr>
<td><em>I. orientalis</em> ATKU5-60</td>
<td>8.27±0.30⁻ (60 h)</td>
<td>1.38±0.05⁻</td>
</tr>
<tr>
<td><em>I. orientalis</em> ATKU5-70</td>
<td>7.30±0.23⁻ (60 h)</td>
<td>1.22±0.04⁺</td>
</tr>
</tbody>
</table>

Results with the same letter are not significantly different (p<0.05)

1 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; maltose, 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

The time points indicate the maximum ethanol concentrations producing by the yeast strains.

Theoretical values of composition of fermentable sugars in molasses (78% (w/v)) have been used for calculation (% w/w): sucrose, 34.6; maltose, 13.5; glucose, 10.4; maltose, 0.11; maltotriose, 0.45. Theoretical yield of undiluted molasses = 0.311 g/g molasses.