Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant Issatchenkia orientalis

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A B S T R A C T
A newly isolated thermotolerant ethanologenic yeast strain, Issatchenkia orientalis IPE 100, was able to produce ethanol with a theoretical yield of 85% per g of glucose at 42 °C. Ethanol production was inhibited by furfural, hydroxymethylfurfural and vanillin concentrations above 5.56 g L⁻¹, 7.81 g L⁻¹, and 3.17 g L⁻¹, respectively, but the strain was able to produce ethanol from enzymatically hydrolyzed steam-exploded cornstalk with 93.8% of theoretical yield and 0.91 g L⁻¹ h⁻¹ of productivity at 42 °C. Therefore, I. orientalis IPE 100 is a potential candidate for commercial lignocelluloses-to-ethanol production.

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1. Introduction
Lignocellulosic biomass is abundant and low-cost, and has great potential to be exploited for ethanol production (Hahn-Hägerdal et al., 2006; Talebnia et al., 2010). However, pretreatment of the material is necessary to allow ethanologenic microorganism access to the fermentable sugars. Some of the pretreatments result in by-products that are inhibitory to fermentation (Heipieper et al., 1994). Additionally the fermenting organisms are subject to stresses from high concentrations of ethanol, pH changes, and temperature fluctuations (Keatig et al., 2006; Gibson et al., 2007; Liu et al., 2009; Babiker et al., 2010). Therefore, thermotolerant microorganisms that have a high tolerance for fermentation inhibitors are desirable.

Some wild-type and mutant strains of yeasts have already been reported to produce ethanol from lignocellullosics at temperatures above 40 °C with efficiencies of as much as 75% of theoretical yield (Banat et al., 1992; Abdel-fattah et al., 2000; Sridhar et al., 2002; Limtong et al., 2007; Wanapu et al., 2009), but strains achieving higher yields are still needed. The current study was undertaken to find a thermotolerant yeast strain that would have higher ethanol yields from lignocellullosics. Such a yeast strain was found and its ethanol productivity and susceptibility to fermentation inhibitors were determined.

2. Methods
2.1. Isolation and identification of thermotolerant yeast strain
Yeasts were isolated from cornstalk, sweet sorghum stalk, rice straw and crop compost from suburban farms near Beijing, China. Yeasts were enriched in the slightly modified M 9 medium (Abdel-fattah et al., 2000) containing 1 g L⁻¹ KH₂PO₄, 1 g L⁻¹ MgSO₄·7H₂O, 3 g L⁻¹ (NH₄)₂SO₄, 3 g L⁻¹ yeast extract and 20 g L⁻¹ glucose supplemented with 100 mg L⁻¹ ampicillin by culturing at 42 °C for 1–2 days. Yeast like colonies were selected and isolated based on morphological characteristics on plate and microscopy. The yeasts were purified by streaking on the same composition agar.

Single colonies were maintained on YPG agar slants (3 g L⁻¹ yeast extract, 5 g L⁻¹ peptone, 20 g L⁻¹ glucose, and 20 g L⁻¹ agar) at 4 °C. Thermotolerant yeast strains were selected based on their ability to grow and produce ethanol at 30, 35, 37, 40, 42, 45, and 50 °C in YPG medium, pH 5.5, containing 150 g L⁻¹ glucose. One isolated, IPE 100, was selected for taxonomic studies due to its highest growth rate and ethanol yield at 42 °C among all isolates. The isolate was characterized morphologically and biochemically as scribed by Van der Walt and Yarrow (1984). Phylogenetic analysis was carried out after sequencing of 18S rDNA. The DNA was amplified by PCR with forward primer NS1 (GTAGTCATATGCTTGTCTC) and reverse primer EF3 (TCCCTAAATGACCAAGTTTG) (O’Donnell, 1993). The sequence of the amplicon was compared with GenBank database sequences using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/).
Sequences were aligned using the ClustalW program (White et al., 1990). Neighbor-joining trees were constructed using MEGA version 4.0 [Molecular Evolutionary Genetics Analysis (http://www.mega-software.net)] with 1000 bootstrap replicates. The 185 rDNA sequences data of the strain IPE100 have been deposited in GenBank with the accession number GQ 166761.

2.2. Seed culture preparation

The isolated IPE100 was maintained on fresh YPG solid medium slants and restreaked once every 2 months. Three small pieces of the IPE 100 cultures were transferred from the YPG slant into a slants and restreaked once every 2 months. Three small pieces of the strain IPE100 cultures were transferred from the YPG slant into a 250-mL Erlenmeyer flask containing 100 mL of YPG liquid medium (3 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) peptone, 20 g L\(^{-1}\) glucose). The cultures were grown on a rotary shaker at 150 rpm for 16 h at 42°C, and 4\% (v/v) of the cultures was used for inoculation of fermentation media.

2.3. Growth and fermentation characteristics of the isolated yeast strain

The influence of temperature on cell growth and ethanol production was investigated in foam-plugged 250-mL Erlenmeyer flasks containing 150 mL of YPG liquid medium at 30–50°C. The effect of ethanol on cell growth was determined at 42°C with ethanol (1–10%, w/v) added to the YPG liquid medium. Batch fermentation experiments at 42°C were also performed in a 5-L bioreactor (G8JT-5C, East Biotech Equipment and Technology Co., Ltd., ZhenJiang, China) using 3.5 L YPG liquid medium with 150 g L\(^{-1}\) glucose. Fermentation was conducted with an agitation speed of 150 rpm and an aeration rate of 0.2 vvm for the first 12 h. Samples were withdrawn every 6 h.

2.4. Effect of lignocellulosic inhibitory compounds on cell growth and ethanol fermentation

The effects of inhibitory compounds were investigated in foam-plugged 250 mL Erlenmeyer flasks containing 150 mL YPG liquid medium (3 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) peptone, and 150 g L\(^{-1}\) glucose) with various concentrations of furfural (0.25–5.0 g L\(^{-1}\)), 5-HMF (0.25–7.0 g L\(^{-1}\)) and vanillin (0.25–4.0 g L\(^{-1}\)). Flasks were incubated in a rotary shaker at 150 rpm and 42°C for 72 h, and samples were withdrawn every 2 or 4 h to determine the concentration of biomass, glucose and ethanol.

2.5. Ethanol fermentation from enzymatic hydrolysate of steam-exploded cornstalk

Steam-exploded cornstalk containing (%, w/w, on dry weight basis) cellulose, 31.35; hemicellulose, 14.5; and lignin, 25.03; was used for fed-batch enzymatic hydrolysis as described by Yang et al. (2010). The hydrolysate from the washed steam-exploded cornstalk contained 95.70 g L\(^{-1}\) glucose, 17.97 g L\(^{-1}\) xylose, 13.21 g L\(^{-1}\) cellobiose, 0.05 g L\(^{-1}\) furfural and 0.15 g L\(^{-1}\) 5-HMF. The enzymatic hydrolysate from unashed steam-exploded cornstalk contained 60.60 g L\(^{-1}\) glucose, 10.45 g L\(^{-1}\) xylose, 7.23 g L\(^{-1}\) cellobiose, 0.153 g L\(^{-1}\) furfural and 0.215 g L\(^{-1}\) 5-HMF. The enzymatic hydrolysate was centrifuged at 16099 g for 20 min, and the supernatants were used as fermentative substrates. Fermentation of the enzymatic hydrolysate was conducted in the foam-plugged 250-mL Erlenmeyer flasks with 150 mL working volume. Flasks were incubated for 60 h at 42°C on a rotary shaker at 150 rpm. Samples were withdrawn every 6 h. 2 mL aliquots were immediately centrifuged at 16,099 g for 10 min at 4°C, and cell-free supernatants were frozen at -20°C for sugar, ethanol, and inhibitory compound analyses by GC and HPLC.

2.6. Calculation of fermentation parameters

Ethanol productivity, defined as gram of ethanol per liter per hour, was calculated from the final ethanol concentration. Ethanol yield and percent of theoretical yield were calculated using the following Eqs. (1), (2), respectively:

\[
Y_{P/S} = \frac{(\text{EtOH})_{\text{max}}}{(\text{Sugar})_{\text{ini}}}
\]

(1)

\[
T.Y.(\%) = \frac{(\text{EtOH})_{\text{max}}}{(\text{Sugar})_{\text{ini}}} 	imes 100
\]

(2)

where \((\text{EtOH})_{\text{max}} = \text{maximum ethanol concentration during fermentation (g L}^{-1}\)), \((\text{Sugar})_{\text{ini}} = \text{initial sugar concentration at onset of fermentation (g L}^{-1}\)), T.Y. (\%) = \% of theoretical yield (\%), and 0.511 = \% theoretical maximum ethanol yield per unit of hexose sugar from glycolytic fermentation (g g\(^{-1}\))

Specific growth rate \((\mu)\) was determined as a slope of straight line between ln\((X/X_0)\) and time, where \(X\) expresses cell concentration (g L\(^{-1}\)). For expressing quantitatively the effect of temperature, ethanol and lignocellulosic inhibitory compounds on cell growth, the following assumptions were adopted: (a) specific growth rate depends on temperature and is assumed to obey the Arrhenius relation and (b) ethanol and lignocellulosic inhibitory compounds non-competitively inhibit cell growth. Based on the assumptions above, the kinetic equations on cell growth rate at different environmental conditions were expressed as follows (Eqs. (3)–(6)):

\[
dX/dt = \mu_X X - \mu_D X
\]

(3)

\[
\mu_X = A_1 \exp(-E_X/RT)
\]

(4)

\[
\mu_D = A_2 \exp(-E_D/RT)
\]

(5)

\[
\mu = \mu_m \left( \frac{S}{K_S + S} \right) (1 - P/P_m)(1 - 1/I_m)
\]

(6)

where \(X\), \(S\) and \(P\) are the concentrations of biomass, substrate, and ethanol, \(\mu\), \(\mu_X\), and \(\mu_D\) are the overall specific growth rate, true specific growth rate and specific death rate. \(\mu_m\) represents the maximum specific growth rate, while \(E_X\) and \(E_D\) represent the activation and inactivation energies for cell growth and cell death. \(K_S\) is saturation constant for cell growth, and \(P_m\) and \(I_m\) are the inhibition terms of ethanol and/or lignocellulosic inhibitory compounds for cell growth, respectively. Biomass, glucose and ethanol concentrations were measured at 2 h intervals during log phase (which generally covered the time range of 2–18 h) for determination of specific growth rate. The initial parameters, \(\mu_m\) and \(K_S\) were tentatively estimated from the experimental data during the log phase by Lineweaver–Burk plot based on Monod rate equation (Muendler et al., 2006).

2.7. Analyses

Glucose, xylose, cellobiose, furfural, 5-HMF and vanillin from cornstalk hydrolysate and fermentation broth were analyzed by HPLC (HPLC-1100, Agilent Technologies, USA) with a refractive index detector using column HPX-87H (300 mm × 78 mm) (Bio, Richmond, CA, USA) maintained at 55°C in a column oven (Ehrman and Himmel, 1994). Sulfuric acid (0.01 N) in HPLC grade water was used as a mobile phase at a flow rate of 0.6 mL min\(^{-1}\).

Growth of cultures was estimated by measuring optical density at 660 nm in a spectrophotometer (UV-2100, Unico Instruments, Shanghai, China) after washing the cells twice and re-suspending them in 0.85% NaCl. Dry cell weight was determined for 10 mL sample washed twice with 0.85% NaCl solution and once with distilled water and dried at 90°C for 12 h. A calibration curve between absorbance and dry cell weight was established.
3. Results and discussion

3.1. Isolation and identification of thermotolerant yeast strain

Screening of the yeast isolates identified one strain, originating from cornstalk, that exhibited a growth rate similar to other isolates, but produced over 1.5 times more ethanol with a theoretical yield of ethanol of about 85% at 42 °C and a yield of 47% at 45 °C. Like other isolates tested, this strain was unable to grow or produce ethanol at 50 °C. This strain, designated as IPE100, produced 65.5 g L⁻¹ of ethanol from 150 g L⁻¹ of glucose, corresponding to 85.54% of theoretical yield at 42 °C. Strain IPE100 cells are ovoidal to elongate in shape and single or in pairs (Supplementary material, Fig. 1). Budding cells were present, and pseudomycelium were developed. This strain produced butyrous, light cream colored colonies on agar medium. The strain exhibited limited fermentative capability and could not utilize nitrate. Since ascosporulation was observed, the new isolate appeared to be a member of the genus Issatchenka (Van der Walt and Yarrow, 1984), and the ability to grow at 40 °C and to grow in vitamin-free medium and assimilate D-glucosamine suggested that the isolate is a strain of Issatchenka orientalis. The 18s rDNA sequence of strain IPE100 has 99.5% of similarity with the corresponding sequence of Issatchenka orientalis presented in GeneBank (Supplementary material, Fig. 2).

3.2. Growth and fermentation characteristics

3.2.1. Effect of temperature and ethanol

The influence of temperature on cell growth and ethanol production is shown in Fig. 1A and Table 1. The temperature dependency of the apparent maximum specific growth rate expressed by Arrhenius relationships (Eqs. (4) and (5)) fit well with the experimental results. From the Arrhenius relationships, the estimated values of the activation or inactivation energy for cell growth (E_a) and death (E_d) were 4.00 × 10^3 kJ Kmol⁻¹ and 3.22 × 10^3 kJ Kmol⁻¹, respectively.

These values are lower for cell growth and higher for cell death than the corresponding values observed for Saccharomyces cerevisiae and Pichia tannophilus (Muenduen et al., 2006).

Fig. 1B shows that exogenously added ethanol decreased the maximum specific growth rate. The cell yield was decreased with the increase of ethanol concentration added, and the cell growth was strongly repressed at the concentration of about 9% (w/v). The inhibition term of ethanol for cell growth was determined as 5.56 g L⁻¹ from the plot of 1 – μ/μ_0 vs. ethanol concentration which is acceptable for ethanol fermentation processes.

3.2.2. Batch fermentation performance in a 5-L bioreactor

In the 5-L bioreactor, the concentration of glucose was gradually decreased while the cell density and ethanol production increased during 36–42 h (Supplementary material, Fig. 3). The cell growth curve was a typical sigmoidal shape (S-shape) and the maximum cell concentration in batch fermentation was 8.89 g L⁻¹. The highest ethanol concentration reached 65.5 g L⁻¹ (equivalent to 85.54% of theoretical yield and 1.56 g L⁻¹ h⁻¹ of productivity) at 48 h. There was no significant difference in ethanol yield in 250-ml shaking flasks and 5-L bioreactor, but the productivity of ethanol in the 5-L bioreactor (1.56 g L⁻¹ h⁻¹) was higher than (1.37 g L⁻¹ h⁻¹) that in the 250-ml shaking flasks. Strain IPE100 showed higher ethanol production efficiency at 42 °C than other yeasts (Table 1).

3.3. Effect of lignocellulosic inhibitory compounds on cell growth and ethanol production

3.3.1. Effect of inhibitors on cell growth

Fig. 2 shows the cell growth over time with varying initial concentration of furfural, 5-HMF or vanillin in YPG media. All lignocellulosic inhibitory compounds not only prolonged the lag phase of cell growth but also decreased the cell yield with increasing the concentration. In case of furfural, the lag phase lasted 4–6 h, but the cell yield was not significantly affected at concentrations of 1 and 2 g L⁻¹ furfural, whereas a remarkable decrease of cell yield and long lag phase was observed at concentrations above 3 g L⁻¹ furfural (Fig. 2A). 5-HMF affected cell growth less than furfural did (Fig. 2B), but vanillin depressed cell growth even at a concentration of 2 g L⁻¹ (Fig. 2C).

Plots of relative specific growth rate vs. concentration of inhibitors (Supplementary material Fig. 4), suggest that the maximum inhibitory levels of inhibitors for cell growth were 5.56 g L⁻¹ furfural, 7.81 g L⁻¹ 5-HMF and 3.17 g L⁻¹ vanillin, respectively. From l-
The inhibitory effects on cell growth are dose-dependent but the sensitivities are different with microorganisms and the kinds of inhibitors. These inhibitory concentrations for the strain IPE 100 are higher than those for the strains *Pichia stipitis* and *S. cerevisiae* (Mussatto and Roberto, 2004), and similar to those for the strain *S. cerevisiae* Tembec T1 adapted in spent sulfite liquor (Keatig et al., 2006).

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>$S_0$ (g L⁻¹)</th>
<th>Sugar source</th>
<th>$P$ (g L⁻¹)</th>
<th>$T$ (°C)</th>
<th>$Q_p$ (g L⁻¹ h⁻¹)</th>
<th>T.Y. (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Kluyveromyces marxianus</em> IBM3</td>
<td>160</td>
<td>Glucose</td>
<td>55</td>
<td>37</td>
<td>0.83</td>
<td>67.3</td>
<td>Banat et al. (1992)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> VS3</td>
<td>150</td>
<td>Glucose</td>
<td>75</td>
<td>35</td>
<td>1.56</td>
<td>97.9</td>
<td>Sridhar et al. (2002)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> F111</td>
<td>180</td>
<td>Sucrose</td>
<td>84</td>
<td>43</td>
<td>1.19</td>
<td>93.4</td>
<td>Abdel-Fattah et al. (2000)</td>
</tr>
<tr>
<td><em>Kluyveromyces marxianus</em> WR 12</td>
<td>180</td>
<td>Sucrose</td>
<td>80</td>
<td>43</td>
<td>2.86</td>
<td>91.3</td>
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<tr>
<td><em>Kluyveromyces marxianus</em> DMKU 3-1042</td>
<td>220</td>
<td>Sucrose</td>
<td>87</td>
<td>37</td>
<td>1.45</td>
<td>77.5</td>
<td>Limtong et al. (2007)</td>
</tr>
<tr>
<td><em>Issatchenkia orientalis</em> IPE 100</td>
<td>150</td>
<td>Glucose</td>
<td>64.3</td>
<td>37</td>
<td>1.07</td>
<td>83.9</td>
<td>This work</td>
</tr>
<tr>
<td><em>Issatchenkia orientalis</em> IPE 100</td>
<td>95.7</td>
<td>Cornstalk hydrolysate</td>
<td>45.9</td>
<td>42</td>
<td>0.75</td>
<td>47.0</td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>103.3</td>
<td>Cornstalk hydrolysate</td>
<td>49.5</td>
<td>30</td>
<td>1.03</td>
<td>94</td>
<td>Lu et al. (2010)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>56.3</td>
<td>Cornstalk hydrolysate</td>
<td>27.8</td>
<td>42</td>
<td>0.52</td>
<td>90.2</td>
<td></td>
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<tr>
<td><em>Issatchenkia orientalis</em> IPE 100</td>
<td>42.4</td>
<td>Cornstalk hydrolysate</td>
<td>19.4</td>
<td>35</td>
<td>0.81</td>
<td>89.5</td>
<td>Öhgren et al. (2007)</td>
</tr>
<tr>
<td><em>Issatchenkia orientalis</em> IPE 100</td>
<td>36.7</td>
<td>Cornstalk hydrolysate</td>
<td>16.8</td>
<td>35</td>
<td>0.73</td>
<td>89.6</td>
<td></td>
</tr>
</tbody>
</table>

$S_0$: initial sugar concentration (g L⁻¹), $P$: concentration of ethanol produced (g L⁻¹), $Q_p$: productivity of ethanol (g L⁻¹ h⁻¹), T.Y.: theoretical yield of ethanol (%).

* Enzymatic hydrolysate of water-washed steam-exploded cornstalk.

* Enzymatic hydrolysate of unwashed steam-exploded cornstalk.

**Fig. 2.** Effect of lignocellulosic inhibitory compounds on cell growth of *I. orientalis* IPE 100 and ethanol production. (A): growth vs. furfural, (B): growth vs. 5-HMF, (C): growth vs. vanillin, (D): ethanol production vs. furfural, (E): ethanol production vs. 5-HMF, (F): ethanol production vs. vanillin.

3.3.2. *Effect of inhibitors on ethanol production*

Fig. 2D–F shows ethanol production profiles in inhibitors-supplemented YPG media. All of the inhibitors affected ethanol yield. There was little difference in ethanol yield at furfural concentrations of 0.5–2.0 g L⁻¹, while significant decrease was observed above 3.0 g L⁻¹ (Fig. 2D). Ethanol production was influenced less by 5-HMF than by furfural, and a significant yield was obtained.
even at 4 g L\(^{-1}\) 5-HMF. The ethanol production decreased to 65% in the presence of 0.25–2.0 g L\(^{-1}\) vanillin at 54 h (Fig. 2F). Glucose utilization decreased with increasing inhibitor concentrations (Fig. 3). The ethanol yield of the strain IPE 100 was higher than that of \textit{S. cerevisiae} and \textit{Kluyveromyces maxianus} at 1.5 g L\(^{-1}\) furfural (Palmqvist and Hahn-Hägerdal, 2000; Keatig et al., 2006). In addition, the IPE 100 cultures grown with furfural were successfully transferred to subsequent fermentations containing the same concentrations of furfural without inhibition of cell growth and ethanol yield (Fig. 4).

### 3.4. Ethanol fermentation from enzymatic hydrolysate of steam-exploded cornstalk

During batch fermentation from enzymatic hydrolysate of the washed steam-exploded cornstalk, glucose was gradually consumed while the biomass and ethanol increased. The highest concentrations of biomass (8.12 g L\(^{-1}\)) and ethanol (45.92 g L\(^{-1}\), equivalent to 93.8% of theoretical yield corresponding to initial glucose and 0.91 g L\(^{-1}\) h\(^{-1}\) of productivity) were reached at 48 h (Fig. 5). There was no significant difference in ethanol efficiency from enzymatic hydrolysate between washed and unwashed steam-exploded cornstalk, but fermentation rate in the unwashed substrate was lower than that in the washed substrate (Table 1). Similar results were also observed in ethanol fermentation of hydrolysate from washed and unwashed steam-exploded cornstalk (Öhgren et al., 2007; Lu et al., 2010).

### 4. Conclusion

A new thermotolerant yeast strain of \textit{I. orientalis} IPE 100 was isolated and identified. This strain has good tolerances to temperature, ethanol, and lignocellulosic inhibitors and presents high efficiency of ethanol productivity at elevated temperature of 42 °C. The current results provide the prerequisite basis for industrial application of the isolated strain for efficient simultaneous saccharification and fermentation of lignocellulosic biomass due to both improvement of enzymatic hydrolysis at elevated temperatures and reduction of the pretreatment cost for detoxification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.06.035.

References


