Low pH \(\delta\)-xylonate production with *Pichia kudriavzevii*

Mervi Toivari *, Maija-Leena Vehkomäki, Yvonne Nygård, Merja Penttilä, Laura Ruohonen, Marilyn G. Wiebe

VTT, Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Espoo, Finland

**HIGHLIGHTS**

- *Pichia kudriavzevii* expressing *Caulobacter crescentus* xyIB produced 146 g \(\delta\)-xylonate 1\(^{-1}\) at pH 3.
- This *P. kudriavzevii* strain also produced 171 g \(\delta\)-xylonate 1\(^{-1}\) at pH 5.5.
- \(\delta\)-Xylonate production was less toxic to *P. kudriavzevii* than to *Saccharomyces cerevisiae*.
- *P. kudriavzevii* is thus an excellent production organism for \(\delta\)-xylonic acid.

**ABSTRACT**

\(\delta\)-Xylonic acid is one of the top 30 most desirable chemicals to be derived from biomass sugars identified by the US Department of Energy, being applicable as a non-food substitute for \(\delta\)-glucinic acid and as a platform chemical. We engineered the non-conventional yeast *Pichia kudriavzevii* VTT C-79090T to express a \(\delta\)-xylose dehydrogenase coding gene from *Caulobacter crescentus*. With this single modification the recombinant *P. kudriavzevii* strain produced up to 171 g L\(^{-1}\) of \(\delta\)-xylonate from 171 g L\(^{-1}\) \(\delta\)-xylose at a rate of 1.4 g L\(^{-1}\) h\(^{-1}\) and yield of 1.0 g [g substrate consumed] \(^{-1}\), which was comparable with \(\delta\)-xylonate production by *Glucosobacter oxydans* or *Pseudomonas* sp. The productivity of the strain was also remarkable at low pH, producing 146 g L\(^{-1}\) \(\delta\)-xylonate at 1.2 g L\(^{-1}\) h\(^{-1}\) at pH 3.0. This is the best low pH production reported for \(\delta\)-xylonate. These results encourage further development towards industrial scale production.

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1. Introduction

The current threats of increasing population, global warming and limited fossil resources have triggered a vision for a bioeconomy, i.e. sustainable production and conversion of biomass to products and energy, as exemplified in the EU white paper “The European Bioeconomy in 2030” and the “National Bioeconomy Blueprint” from the USA White House in 2012. Development of biorefineries to convert existing biomass, preferably non-utilized waste material, to usable products is a key element of the bioeconomy. Biorefineries will need to be economically viable, setting strict targets for the whole value chain. Microbes, when used as biocatalysts in the processes should be robust, tolerant to various inhibitors while maintaining high productivity, as well as being safe and economical to produce and use.

Several organic acids, such as gluconic acid (80 kton year\(^{-1}\)), acetic acid (150 kton year\(^{-1}\)) and citric acid (1600 kton year\(^{-1}\)) are already produced by microbes as bulk chemicals with wide application ranges (Sauer et al., 2008). Organic acids have traditionally been used as chelators, buffers and preservatives. They can also be important platform chemicals: around half of the current list of desirable sugar-derived platform chemicals could be produced using biotechnology are organic or amino acids (Bozell and Petersen, 2010; OECD, 2011). The feasibility of production has been demonstrated for e.g. glucaric acid (Lee et al., 2011), \(\delta\)-xylonic acid (Toivari et al., 2012a) and galactaric acid (Mojzita et al., 2010). Some organic acids like citric acid are produced in low pH processes, but most are produced at pH values closer to neutral because of physiological constraints, even though low pH processes would reduce costs during both production (less use of neutralizing base) and product separation (less acid addition and less salt produced as byproduct, Sauer et al., 2008).

* Corresponding author. Tel.: +358 40 708 1039; fax: +358 20 722 7071. E-mail address: mervi.toivari@vtt.fi (M. Toivari).

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Low pH
The 5-carbon sugar D-xylose is a major component of hemicellulose in lignocellulosic materials. It is thus abundant, but currently much less used than lignocellulosic D-glucose. Major efforts have been made to ferment the pentose sugars D-xylose and D-arabinose to ethanol (Van Vleet and Jeffries, 2009), however, the efficiency of C5 conversion is still low compared with D-glucose conversion. D-Xylose can also be reduced to xylitol or oxidized to D-xylonic acid, compounds with a variety of applications. D-Xylic acid can be used as a complexing agent and chelator in dispersal of concrete (Chun et al., 2006). It has also been used in co-polyamides, polyes- ters and hydrogels, and as a precursor for compounds such as 1,2,4-butanetriol or ethylene glycol (Liu et al., 2012b; Niu et al., 2003; reviewed in Toivari et al., 2012a). With increasing D-glucose prices, D-xyloxylic acid may provide a cheaper, non-food-derived alternative for gluconic acid which is widely used in pharmaceuticals, food products, solvents, adhesives, dyes, paints and polishes.

- Xyloxylic acid is formed in the oxidative metabolism of D-xylose by some archaea and bacteria and may accumulate in high extracellular concentrations when sufficient D-xylose is provided to bac- teria such as Gluconobacter oxydans or Pseudomonas sp. (reviewed in Toivari et al., 2012a). Recently D-xyloxylic acid has also been produced by genetically modified organisms, including the bacterium Escherichia coli (Liu et al., 2012a) and yeast Saccharomyces cerevisi- ae (Toivari et al., 2010, 2012b) and Klyveromycetes lactis (Nygård et al., 2011), to provide alternative, more robust production hosts which would be suitable for large scale production. D-Xyloxylic acid titres up to 43 g L\(^{-1}\) have been obtained, but the production rates were low compared to those observed with G. oxydans (Toivari et al., 2012a, b) and these strains have not been useful for D-xylo- xylinate production at low pH (Liu et al., 2012a; Toivari et al., 2012b), with its associated benefits in reducing contamination risk, reducing use of neutralisation agent, generating new options for down stream purification, and the corresponding reductions in costs.

While S. cerevisiae and E. coli are well known and extensively used production organisms, other production organisms may be superior in production of organic acids. The yeast Pichia kudriavzevii (previously named as Issatchenkia orientalis) has recently been described as a multi-stress-tolerant, robust organism with tolerance to low pH, high salt concentrations, and temperatures as high as 42 °C (Gallardo et al., 2011; Isono et al., 2012; Kitagawa et al., 2010; Kwon et al., 2011). The potential of P. kudriavzevii for the production of bioethanol at 40 °C has already been demonstrated (Dhaliwal et al., 2011). Examples of genetic modifications of P. kudriavzevii (I. orientalis) are still scarce: it has been engineered for production of β-glucosidase and examples of its engineering for γ-lactic acid production can be found in patents (Suominen et al., 2009; Kitagawa et al., 2010).

The purpose of this study was to assess the possibility of using P. kudriavzevii to produce D-xyloxylic acid at low pH.

2. Methods

2.1. Strains, plasmids and strain construction

Pichia kudriavzevii (formerly Issatchenka orientalis (Kurtzman et al., 2008), also referred to as Candida kruzei) strain VTT C-79090T (ATCC 32196, isolated from cabbage waste, Kofu, Japan), VTT C-05705 (ATCC 60585, isolated from rice sour dough starter, Germany) and VTT C-75010 (isolated from a sample of commercial baker's yeast, Finland), and S. cerevisiae VTT B-67002 were obtained from the VTT Culture Collection (http://culturecollection.vtt.fi/). An industrial K. lactis strain (GG799) was obtained from New England Biolab (MA). All strains were maintained as streaks on YPD agar or in 15% v/v glycerol at -80 °C.

A synthetic gene for the D-xylose dehydrogenase from Caulobacter crescentus (xyLB, CC_0821, Gene ID: 941308, NCBI) codon optimized for S. cerevisiae was obtained from Gene Art (Germany). The gene was cloned under the P. kudriavzevii PGK1 promoter (IoPGK1) and was introduced as a single copy into the P. kudriavzevii genome with targeted integration into the PDC1 locus. The primers used for the PCR amplification of sequences from the genomic DNA of the diploid strain P.kudriavzevii VTT C-79090T were previously described in Suominen et al. (2009). The IoPGK1 promoter, 624 bp 5' of ATG, was amplified with primers lopPGKfrw2 5'TCCCCCGGCGGCATCCTTG CTTGAAACCAGAAATC TAATG3' and lopPGKrev2 5'CCGAACTTTGAAGATTTGTGTGTT GATTGCTGTGTTT TTTTTG3'. The 833 bp 5' and 746 bp 3' flanking regions of the P. kudriavzevii PDC1 (IoPDC1) gene were amplified with primers flanked with its associated benefits in reducing contamination risk, reducing use of neutralisation agent, generating new options for down stream purification, and the corresponding reductions in costs.

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2.2. Media and culture conditions

YPD plates contained 10 g yeast extract L\(^{-1}\), 20 g bacto-peptone L\(^{-1}\), 20 g d-glucose L\(^{-1}\) and 15 g agar L\(^{-1}\), and were supplemented with 40 μg mL\(^{-1}\) X-α-Gal for selection of transformants. Medium for flask and bioreactor cultures contained yeast extract and peptone (YP, 10 g yeast extract L\(^{-1}\), 20 g bacto-peptone L\(^{-1}\)) with D-glucose (12.5–20 g L\(^{-1}\)) and/or D-xylose (20–171 g L\(^{-1}\)) for production of D-xylonate. Concentrations of added carbon source (D-glucose, D-xylose) are indicated in the text.

The defined medium described by Verdun et al. (1992) was used to assess growth in the Bioscreen analyser (Bioscreen C MBR automated turbidometric analyser, Growth Curves Ltd., Finland). To determine specific growth rates at different pH values, the medium was buffered with potassium hydrogen phthalate (KH₂HPO₄, pH 2.2–5.5) or monopotassium phosphate (KH₂PO₄, pH 6.2–8). Bioscreen microtiter plates (100-Well Honeycomb plate) containing 270 μL medium were inoculated with 30 μL cell suspension to an initial OD of 0.05. Growth at 30 °C with continuous, extra intensive shaking was measured as optical density at 600 nm (OD₆₀₀) at 30 min intervals for up to 24 h. The pH of the medium was measured at the end of the exponential growth phase. The buffering capacity of the buffers used was adequate to maintain a specific pH during the logarithmic growth phase and thus for determination of the relationship of specific growth rate and pH. Specific growth rates were determined from the exponential phase after the OD₆₀₀ was 0.2 or higher. Each condition as assessed in the Bioscreen was performed in at least four replicates. Growth at pH 6.2 was also assayed in bioreactors, using 200 mL defined medium (Verdun et al., 1992), inoculated to an OD of
0.05. The experiments were done without pH control in medium buffered with potassium hydrogen phthalate for direct comparison with the Bioscreen measurement, and also in medium lacking potassium hydrogen phthalate at constant pH, maintained by addition of 2 M NaOH or 1 M H₂PO₄. Logarithmic growth was monitored by hourly measurement of OD₆₀₀ and by measurement of CO₂ production. The gas concentration (CO₂, ¹³CO₂, O₂, N₂ and Ar) was analysed in an Omnistar quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO₂ in Ar.

Flask cultures were carried out in 50 mL YP medium in 250 mL Erlenmeyer flasks, at 250 rpm, 30 °C. Multifors bioreactors (max. working volume 500 mL, Infors HT, Switzerland) were used to obtain pH controlled cultivations. Yeast were grown in 500 mL YP medium at pH 3.0 or 5.5, 30 °C, 1–1.8 volume air [volume culture]⁻¹ min⁻¹ (vvm) and 800–1000 rpm agitation with 2 Rushton turbine impellers, as previously described (Toivari et al., 2010). The pH was maintained constant by addition of 2 M NaOH or 1 M H₂PO₄. Clerol antifoaming agent (Cognis, France, 0.08–0.10 μL L⁻¹) or mixed molecular weight polypropylene glycol was added to prevent foam formation.

2.3. Analytical methods

2.3.1. Measurement of biomass

Biomass was measured as OD₆₀₀ or as dry weight. For dry weight, samples were collected in 2 mL pre-dried, pre-weighed microcentrifuge tubes, washed twice with equal volume distilled water and dried at 100 °C.

2.3.2. Chemical analyses

To determine intracellular D-xylonate and xylitol amounts, cells were collected from 10 mL culture, washed and extracted as described by (Nygård et al., 2011). The intracellular concentration is given as mg per g dry biomass. A conservative estimate of intracellular concentrations can be derived by assuming that 1 g dry cell weight corresponds to 2 mL cell volume, as with S. cerevisiae (Nygård et al., 2011).

D-Xyloonic acid and/or D-xylulonolactone, ethanol, glycerol, pyruvate and acetate, D-glucose and D-xylulose concentrations from intra and extracellular samples were analysed by HPLC, as described previously (Toivari et al., 2010). Extracellular D-xylonate acid concentrations were also measured as the lactone using the hydroxyxamate method (Toivari et al., 2010).

Xylose dehydrogenase activity was measured as described in Toivari et al. (2012b).

2.3.3. Determination of vitality and viability of D-xylonate producing cells

The number of metabolically active (vital) cells was determined microscopically by methylene blue (0.25 g L⁻¹ in 0.04 M Na citrate buffer pH 8.3) staining. Viability was determined by comparing the number of viable colony forming units (CFU) on YPD to the total cell number determined microscopically. Viability determined as CFU correlated well with the per cent of metabolically active cells determined microscopically, although microscopic observation generally indicated slightly higher vitality/viability than CFU determination (data not shown).

3. Results and discussion

3.1. Effect of pH on specific growth rate for S. cerevisiae, K. lactis and P. kudriavzevii growing on D-glucose

D-Xylonate production has previously been studied with yeast S. cerevisiae (Toivari et al., 2012b) and K. lactis (Nygård et al., 2011). The specific growth rates of these strains growing on D-glucose were compared with those of P. kudriavzevii strains for pH values from 2 to 8. S. cerevisiae VTT B-67002 (μ = 0.57 ± 0.01 h⁻¹), K. lactis GG799 (μ = 0.29 ± 0.02 h⁻¹), and P. kudriavzevii VTT C-79090T (μ = 0.48 ± 0.01 h⁻¹), and VTT-C-05705 (μ = 0.56 ± 0.02 h⁻¹) and VTT-C-79010 (μ = 0.50 ± 0.00 h⁻¹) all had maximal specific growth rates at pH 6 when grown in defined medium in Bioscreen microtitre wells (Fig. 1). K. lactis GG799 was the least tolerant of these species to low pH (Fig. 1). Both P. kudriavzevii and S. cerevisiae strains grew at rates greater than 0.30 h⁻¹ at pH values as low as 3.8, and with slower growth at pH 3.0 (Fig. 1). At pH 3 the specific growth rates of the P. kudriavzevii strains were about threefold higher than those of S. cerevisiae and K. lactis strains, and only P. kudriavzevii VTT C-79090T and VTT-C-05705 grew at pH 2.2 (μ = 0.30 ± 0.01; Fig. 1). P. kudriavzevii (C. kruzei) has previously been shown to have greater tolerance to lactic acid than S. cerevisiae (Halm et al., 2004) and the relatively good growth of P. kudriavzevii VTT C-79090T, VTT-C-05705 and VTT-C-75010 (pH 3 only) at pH 2.2–3, suggested that P. kudriavzevii is a potential host for D-xylonate production.

P. kudriavzevii has been described as unable to utilise D-xylulose, based on API-ID32C screening (Gallardo et al., 2011). However, P. kudriavzevii VTT-C-75010 consumed D-xylulose at a rate of 0.28 ± 0.02 g L⁻¹ h⁻¹ in flasks with 7 g D-glucose L⁻¹ and 20 g D-xylulose L⁻¹ on YP media, which was higher than the D-xylulose consumption rate of the D-xylulose utilising yeast K. lactis (0.22 g L⁻¹ h⁻¹, Nygård et al., 2011). The yield of xylitol on D-xylulose was only 0.22 ± 0.01 g xylitol [g D-xylose]⁻¹, whereas the yield of biomass on D-xylulose was initially about 0.5 g g⁻¹, decreasing in older cultures (∼0.14 g [g biomass]⁻¹), confirming that P. kudriavzevii VTT-C-75010 was able to metabolise D-xylulose. The genes encoding the three enzymes needed for D-xylulose utilization, D-xylulose reductase, xylitol dehydrogenase and xylulokinase, are present in the genome of P. kudriavzevii (Chan et al., 2012) and we demonstrate that they are active in at least some P. kudriavzevii strains. In contrast, P. kudriavzevii VTT-C-75010 did not utilise D-xylonate as a carbon source at pH 5 in either defined or complex medium, with or without D-glucose present, even after 138 h.

Fig. 1. Comparison of specific growth rates of S. cerevisiae VTT B-67002 (red square), K. lactis GG799 (grey diamond) and P. kudriavzevii VTT-C-75010 (black triangle), VTT-C-79090T (open square), and VTT-C-05705 (open circle) at pH 2–8 in defined medium with D-glucose as carbon source, 30 °C. Cells were grown in 200 μL microtitre wells and OD₆₀₀ measured in a Bioscreen C MBR automated analyser. The specific growth rate of K. lactis VTT B-67002 and VTT-C-75010 was also determined from biomass and CO₂ production in a bioreactor at pH 6.2. Error bars represent ± SEM (n = 4–10).
incubation (data not shown). Externally added D-xylulose (100 g L\(^{-1}\) at pH 3.5 or pH 4.5) did not inhibit growth of VTT-C-75010, nor cause an increase in loss of cell viability (<5% non-vital cells in cultures with and without D-xylulose added).

Although *P. kudriavzevii* (*I. orientalis*) is increasingly being recognised as a robust production organism, e.g. for ethanol production (Dhalwal et al., 2011; Isono et al., 2012; Kitagawa et al., 2010; Kwon et al., 2011), reports on genetic manipulation of this organism are scarce (Kitagawa et al., 2010). No episomal vectors are known for *P. kudriavzevii*. Kitagawa et al. (2010) noticed integration of the *S. cerevisiae* 2µ episomal plasmid into the *P. kudriavzevii* (*I. orientalis*) genome. Our D-xylulose dehydrogenase expression cassette was integrated into the genome, with the native PGK promoter driving expression of both the MEL5 marker gene and the D-xylulose dehydrogenase encoding gene *xylB*. Transformation frequencies of 1–3 colonies per µg DNA were obtained for both *P. kudriavzevii* VTT-C79090T and VTT-C75010.

Several xyI transformants of VTT-C79090T and VTT-C75010 were assessed for production of D-xylulose in medium containing D-glucose and D-xylose. No variation between transformants of the same strain was seen. VTT-C79090T xyI produced 50 g D-xylulose L\(^{-1}\) in 48 h when grown in unbuffered VP medium (final pH 2.96) in flasks with 20 g D-glucose L\(^{-1}\) and 50 g D-xylose L\(^{-1}\), whereas VTT-C75010 xyI produced only 42 ± 1 g D-xylulose L\(^{-1}\) (final pH 3.05) in the same conditions. Thus, the better growth of VTT-C79090T, compared with VTT-C75010, at low pH (Fig. 1) was reflected in production of more D-xylose by its transformant, whereas VTT-C75010 xyI produced these concentrations of D-xylulose from initial D-xylose concentrations of 150–200 g L\(^{-1}\), pH 6.5; Table 1; Toivari et al., 2012a) at similar yield (Table 1). In contrast with *P. fragi* and *E. cloacae*, which produced these concentrations of D-xylulose from initial D-xylose concentrations of 150–200 g L\(^{-1}\), *P. kudriavzevii* produced only 103 ± 1 g D-xylulose L\(^{-1}\) (yield 0.48 g D-xylulose [g D-xylose consumed\(^{-1}\)] in 213 h when the initial concentration of D-xylose was high (251 g L\(^{-1}\), data not shown), even though this concentration of D-xylose would not normally be inhibitory for *P. kudriavzevii* (unpublished data), most of the D-xylose (215 ± 12 g L\(^{-1}\)) was consumed, and only 40 ± 0.4 g xylitol L\(^{-1}\) (yield 0.19 g [g D-xylose\(^{-1}\)]) were produced. Thus, pulsed feeding, as used here, or a fed-batch or continuous culture system are more appropriate for production of D-xylulose with *P. kudriavzevii* than the batch cultures used with bacteria. Although different production systems were used, the results demonstrate that *P. kudriavzevii* can perform as well (in titre, yield and volumetric rate) as the best reported bacteria for D-xylulose production, while its high stress tolerance (Kitagawa et al., 2010) makes it a good choice for D-xylulose production from complex plant biomass hydrolysates.

The approximate yield of D-xylulose on D-xylose was 0.92 ± 0.04 g g\(^{-1}\) (0.83 mol mol\(^{-1}\)) in the cultures fed 98 g D-xylose L\(^{-1}\), and 1.0 ± 0.0 g g\(^{-1}\) (0.90 mol mol\(^{-1}\)) in cultures fed 171 g D-xylose

### Table 1

Production of D-xylulose with *P. kudriavzevii* VTT-C12903 and *S. cerevisiae* VTT B-67002 xyI at pH 5.5 or pH 3.0, with data from *P. fragi* and *E. cloacae* (pH 6.5), and *G. oxydans* (pH 12.5) for comparison. D-Glucose was used as a co-substrate.

<table>
<thead>
<tr>
<th>Species</th>
<th>pH</th>
<th>D-Xylose provided (g L(^{-1}))</th>
<th>Co-substrate provided (g L(^{-1}))</th>
<th>D-Xylulose (g L(^{-1}))</th>
<th>Yield(_{xyl}) (approx., g g(^{-1}))</th>
<th>Volumetric production rate (initial) (g L(^{-1}) h(^{-1}))</th>
<th>Biomass (g L(^{-1}))</th>
<th>Time (h)</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. kudriavzevii</em> VTT-C12903</td>
<td>5.5</td>
<td>96 ± 1</td>
<td>12.5</td>
<td>88 ± 1</td>
<td>0.9</td>
<td>1.4 ± 0.1</td>
<td>12.1 ± 0.2</td>
<td>97</td>
<td>Fed-batch(^{c})</td>
</tr>
<tr>
<td><em>P. kudriavzevii</em> VTT-C12903</td>
<td>5.5</td>
<td>99 ± 1</td>
<td>20.5</td>
<td>87 ± 0</td>
<td>0.9</td>
<td>1.4 ± 0.0</td>
<td>15.3 ± 0.2</td>
<td>97</td>
<td>Fed-batch(^{c})</td>
</tr>
<tr>
<td><em>P. kudriavzevii</em> VTT-C12903</td>
<td>5.5</td>
<td>171 ± 5</td>
<td>24.7</td>
<td>171 ± 5</td>
<td>1.0</td>
<td>1.4 ± 0.0</td>
<td>126.3 ± 0.3</td>
<td>170</td>
<td>Fed-batch(^{c})</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> VTT B-67002 xyI</td>
<td>5.5</td>
<td>49</td>
<td>12</td>
<td>43</td>
<td>0.8</td>
<td>0.44</td>
<td>7</td>
<td>120</td>
<td>Fed-batch(^{c})</td>
</tr>
<tr>
<td><em>P. fragi</em></td>
<td>6.5</td>
<td>150</td>
<td>0</td>
<td>162</td>
<td>1.1</td>
<td>1.4</td>
<td>6.9</td>
<td>120</td>
<td>Batch</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>6.5</td>
<td>200</td>
<td>&lt;2</td>
<td>190</td>
<td>1.0</td>
<td>1.6</td>
<td>nd</td>
<td>120</td>
<td>Batch</td>
</tr>
<tr>
<td><em>P. kudriavzevii</em> VTT-C12903</td>
<td>3.0</td>
<td>153 ± 1</td>
<td>24.1</td>
<td>146 ± 5</td>
<td>1.0</td>
<td>1.2 ± 0.0</td>
<td>107.0 ± 0.1</td>
<td>170</td>
<td>Fed-batch(^{c})</td>
</tr>
<tr>
<td><em>P. kudriavzevii</em> VTT-C12903</td>
<td>3.0</td>
<td>55 ± 1</td>
<td>12.5</td>
<td>57 ± 0</td>
<td>1.0</td>
<td>1.2 ± 0.0</td>
<td>91.0 ± 0.1</td>
<td>74</td>
<td>Batch</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> VTT B-67002 xyI</td>
<td>3.0</td>
<td>60</td>
<td>12.6</td>
<td>13 ± 0.3</td>
<td>0.7</td>
<td>0.2 ± 0.0</td>
<td>5.0 ± 0.2</td>
<td>117</td>
<td>Fed-batch(^{c})</td>
</tr>
<tr>
<td><em>G. oxydans</em> (ATCC621)</td>
<td>3.5</td>
<td>40</td>
<td>2.3</td>
<td>41</td>
<td>1.0</td>
<td>1.0</td>
<td>0.2</td>
<td>52</td>
<td>Batch</td>
</tr>
</tbody>
</table>

\(^{a}\) Toivari et al. (2012b).
\(^{b}\) See Toivari et al. (2012a).
\(^{c}\) With discontinuous addition of substrate, see Figs. 2 and 4.
Cell vitality was strongly correlated throughout the cultivations and was similar in all cultures with metabolism of D-xylose as an energy source than production of 88 g D-xylonate L\(^{-1}\). In contrast, only 23% of S. cerevisiae VTT B-67002 xyB cultures which had produced 43 g D-xylose L\(^{-1}\) in similar production conditions remained active (Toivari et al. 2012b). The ability of P. kudriavzevii to utilise D-xylose/xylitol as an energy/carbon source, may contribute to its maintenance of viability while producing D-xylose even though only a small proportion of D-xylose (<10%) was consumed for biomass production. Because 60% of the cells were metabolically active after 146 h, we expect that much higher concentrations of D-xylose could be produced if more D-xylose were provided.

In G. oxydans, P. fragi and other natural D-xylose producers, D-xylose is typically produced in the periplasm and accumulation of high cytoplasmic concentrations has not been addressed beyond the observation that the lactone form of D-xylose may be more toxic than the linear form (Buchert and Viikari, 1988; Meijnen et al., 2009). In D-xylose producing yeast, D-xylose is produced in the cytoplasm, where it may accumulate, depending on the cell's capacity to export it. Thus the ability to tolerate intracellular acid concentrations is important and may be correlated with viability/vitality. In P. kudriavzevii VTT-C-12903 cultures at pH 5.5, intracellular D-xylose concentration was high (115–143 mg D-xylose [g biomass]\(^{-1}\)) within 6–9 h of providing D-xylose to the culture (Fig. 4), even though the extracellular D-xylose concentration was very low (0.1–7 g L\(^{-1}\)). High initial D-xylose concentrations (94 ± 4 mg [g biomass]\(^{-1}\)) were also observed in S. cerevisiae VTT B-67002 xylB (Toivari et al. 2012b), but unlike in B-67002, the intracellular concentration in P. kudriavzevii decreased during the first 40 h of D-xylose production to a concentration of 63 ± 2 mg [g biomass]\(^{-1}\), remaining more-or-less constant until D-xylose had been consumed (Fig. 4). Thus intracellular D-xylose concentrations in P. kudriavzevii were much lower than those observed in S. cerevisiae VTT B-67002 xylB (170 mg [g biomass]\(^{-1}\)). P. kudriavzevii was exporting D-xylose (either free or dissociated acid) against a concentration gradient from the intracellular to the extracellular environment after the first 50 h, whereas the opposite was generally the case for S. cerevisiae VTT B-67002 xylB. D-xylose is not naturally produced by these yeasts and nothing is known about its export from the cells, but it is interesting to note that the yeast which can naturally

![Fig. 2](image-url) **Fig. 2.** D-Xylonate (solid square), xylitol (open circle) and biomass (open square) produced by P. kudriavzevii VTT-C-12903 at pH 5.5 in cultures provided a total 171 g D-xylose in pulses at ~24 h intervals (indicated by grey line). YP medium initially contained 15.4 g L\(^{-1}\) D-glucose and 54.0 g D-xylose L\(^{-1}\). D-Glucose (9.4 g D-glucose L\(^{-1}\)) was also added at 28.4 h. Error bars represent SEM for two cultures and where not visible are smaller than the size of the symbol.

![Fig. 3](image-url) **Fig. 3.** (a) Metabolically active cells (% total cells) in cultures of P. kudriavzevii VTT-C-12903 at pH 5.5 (squares, diamond) or 3.0 (circles) and S. cerevisiae VTT B-67002 yxIB at pH 3.0 (triangle) in YP medium. (b) The relationship between the amount of extracellular D-xylonate in the culture supernatant and the percentage of cells which have lost metabolic activity, as determined by staining with methylene blue. Data from replicate cultures are indicated with large and small symbols in part b and the dashed straight lines represent the best fit linear regression for all cultures of VTT-C-12903 at pH 5.5 (\(R^2 = 0.86\)) or pH 3.0 (\(R^2 = 0.82\)) and of VTT B-67002 yxIB at pH 3 (\(R^2 = 0.93\)). Cultures of VTT-C-12903 received 98 (low) or 171 (high) g D-xylose L\(^{-1}\) at pH 5.5 and 55 (low) or 153 (high) g D-xylose L\(^{-1}\) at pH 3.0 (see Figs. 2 and 3), with 12.5 (pH 5.5 low, pH 3 low), 20.7 (pH 5.5 low + glucose) or 24 (pH 5.5 high) g D-glucose L\(^{-1}\). VTT B-67002 yxIB received 60.5 g D-xylose L\(^{-1}\) and 12.6 g D-glucose L\(^{-1}\).
 utilise D-xylose (P. kudriavzevii, as presented here, and K. lactis, Nygård et al., 2011) appear able to sustain lower intracellular concentrations than S. cerevisiae VTT B-67002.

The intracellular xylitol concentrations in P. kudriavzevii (25–55 mg [g biomass]⁻¹, Fig. 4) were similar to those of S. cerevisiae VTT B-67002 xylB in YP with D-glucose and D-xylose at pH 5.5 (20 to 50 mg [g biomass]⁻¹, Toivari et al. 2012b), but lower than those of K. lactis xyd1 strains (50–150 mg [g biomass]⁻¹) (Nygård et al. 2011). Intracellular xylitol concentrations decreased prior to measureable decreases in extracellular concentrations (cf. Figs. 2 and 4).

3.4. Production of D-xylonate by P. kudriavzevii VTT-C-12903 and S. cerevisiae VTT B-67002 xylB at pH 3

P. kudriavzevii VTT-C-12903 efficiently produced D-xylonic acid at pH 3 (Fig. 5) when D-xylose was provided in pulses, as with cultures at pH 5.5. P. kudriavzevii VTT-C-12903 produced 146 ± 5 g D-xylonic acid L⁻¹ from 153 ± 1 g D-xylose L⁻¹ at a rate of 12 ± 0.03 g L⁻¹ h⁻¹ and approximate yield of 0.95 ± 0.04 g [g D-xylose consumed]⁻¹ (Table 1, 0.86 mol mol⁻¹). There was more cell death at pH 3.0 than at pH 5.5, but 47 ± 1% of the population remained metabolically active (30% viable) after 145 h (Fig. 3). With nearly half of the population still metabolically active it is probable that higher concentrations of D-xylonic acid could have been produced if more D-xylose had been provided. As at pH 5.5, vitality was negatively correlated with extracellular D-xylonic acid concentration (R² = 0.89, Fig. 3b). Intracellular accumulation of D-xylonic acid was similar at pH 3.0 and pH 5.5 (Fig. 4). Less xylitol (10.5 ± 1.7 g L⁻¹, yield 0.06 ± 0.00 mol mol⁻¹, Fig. 5) was produced at pH 3.0 than at pH 5.5. As at pH 5.5, high initial D-xylose concentration was inhibitory (data not shown).

In contrast, the robust S. cerevisiae VTT B-67002 xylB produced only 13 ± 0.3 g D-xylonic acid L⁻¹ at pH 3.0 (Fig. 5, Table 1). Xylitol
was also produced. Cell vitality decreased rapidly, so that less than 10% of the population remained metabolically active after 67 h cultivation in the presence of D-xylose and D-xylonic acid (Fig. 3a). Vitality of S. cerevisiae VTT-B-67002 xy18 was negatively correlated to the extracellular D-xylonic acid concentration \((R^2 = 0.94, \text{Fig. 3b})\). D-xylonic acid accumulation within the cytoplasm (Fig. 4) was lower than at pH 5.5 (Toivari et al., 2012b) or in P. kudriavzevii VTT-C-12903 and decreased with time. A similar decrease in intracellular xylitol concentration was observed (Fig. 4) \((R^2 = 0.77)\), indicating that this release of D-xylurate (and xylitol) to the supernatant resulted from cell death and lysis. In contrast, only 7–45% of the changes in intracellular xylitol were reflected in changes in the intracellular D-xylurate concentration in VTT-C-12903 at pH 3, indicating that cell lysis was not primarily responsible.

Production of D-xylurate at pH 3.0 has previously been reported for S. cerevisiae CEN.PK xy18 expressing strains, but these produced only 5–7 g D-xylurate L\(^{-1}\) (Toivari et al., 2012b). The viability of CEN.PK strains was severely affected by production of D-xylonic acid at pH 3.0 (Toivari et al., 2012b). Liu et al. (2012a) reported that D-xylose could not be produced by recombinant E. coli if the medium pH was below 4.0. P. frugi did not produce D-xylurate at pH values below 4 (Buchert et al., 1986). G. oxydans can produce D-xylurate at pH 3.5 at a production rate of 1.0 g L\(^{-1}\) h\(^{-1}\) (Toivari et al., 2012a), but pH values below 3.5 have not been tested and concentrations above 40 g L\(^{-1}\) have not been produced (Toivari et al., 2012a).

Thus P. kudriavzevii is able to produce over 10-fold more D-xylurate at pH 3.0 than any other reported strain producing D-xylurate, at rates which are almost as high as those at pH 5.5. While some acids such as citric and itaconic acid have traditionally been produced at pH values of 2–3 (Magnuson and Lasure, 2004), it has been more common to produce acids at higher pH values, either because of enzymatic constraints, as in the case of gluconic acid production by A. niger, or because of physiological constraints, as in the production of acetic, lactic, glutamic, butyric and other acids with bacteria which are generally unable to grow at pH values below 4 or 4.5. Recent interest in developing processes for the production of lactic (Sauer et al., 2010; Suominen et al., 2009) and succinic (Yuzbashev et al., 2010) acid at low pH to reduce subsequent downstream processing steps has highlighted both the potential and the challenges of producing organic acids at low pH.

P. kudriavzevii is one of the yeasts which has been successfully engineered to produce lactic acid at low pH, producing 67–70 g L\(^{-1}\) at pH 3.0 (Suominen et al., 2009). The underlying mechanisms of tolerance of low pH are not well understood (Mira et al., 2010; Warnecke and Gill, 2005), nor are acid export mechanisms. However, it is clear that the ability to maintain intracellular pH at near-neutral level is important (Mira et al., 2010; Warnecke and Gill, 2005). Halm et al. (2004) measured intracellular pH in C. krusei (synonym of I. orientalis, and thus P. kudriavzevii) to demonstrate that P. kudriavzevii had better pH homeostasis than S. cerevisiae at pH 2.5 in the presence of lactic acid. This was measured in the presence of exogenous lactic acid. The intracellular pH of S. cerevisiae has also been shown to decrease in response to synthesising L-lactic acid (Valli et al., 2006), and possibly D-xylurate (Toivari et al., 2012b), but equivalent studies have not been carried out with P. kudriavzevii.

In addition to maintaining intracellular pH more effectively than S. cerevisiae, P. kudriavzevii may also have better methods of exporting organic acids than S. cerevisiae, since VTT-C-12903 maintained lower intracellular D-xylurate levels than S. cerevisiae VTT B-67002 (Toivari et al., 2012b) at pH 5.5. Thus P. kudriavzevii is an interesting subject for comparative genomic studies, while transcriptomic studies of the D-xylurate producing strain would also be of interest. With the recent publication of a P. kudriavzevii genome (Chan et al., 2012), such investigations have become possible. Meanwhile, the ability of P. kudriavzevii to produce D-xylurate in hemicellulosic hydrolysates also needs to be addressed.

4. Conclusions

P. kudriavzevii is an excellent production organism not only for traditional products such as ethanol, but also for novel products like D-xylurate acid. By introducing a D-xylose dehydrogenase gene we produced 171 g D-xylurate L\(^{-1}\) at pH 5.5 and 146 g D-xylurate acid L\(^{-1}\) at pH 3.0, at rates of 1.2–1.4 g L\(^{-1}\) h\(^{-1}\). VTT-C-12903 produces D-xylurate acid as well or better than natural D-xylurate producers, such as G. oxydans, and has the benefit of also producing it very efficiently at low pH. Choice of strain, gene and culture conditions have led to significant improvements in D-xylurate production.

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