Bioconversion of D-xylose to D-xylonate with *Kluyveromyces lactis*

D-Xylose is converted to D-xylonic acid, as well as cytoplasmic, dehydrogenases (Buchert et al., 1990) produced by bacteria such as Pseudomonas. Several bacteria are able to convert D-xylose to D-xylonate with high yields and production rates. However, commercial production is not established. D-Xylonate can be produced by fungi such as Fusarium, but the best process for production of D-xylonate is to use the yeast K. lactis. K. lactis can naturally use D-xylose as a carbon source and may have more efficient uptake of D-xylose than other sugars, which would be present in lignocellulosic hydrolysates, into other acids, such as D-gluconate and L-arabinoate. Other species produce a wide range of oxidising enzymes, which have periplasmic, membrane bound, PQQ-dependent D-xylose dehydrogenase from Trichoderma reesei.

Fungal species have high potential for production of organic acids (Porro et al., 1999; Magnuson and Lasure, 2004; Ilmén et al., 2008). With the current wide interest in using plant biomass derived Gordon et al., 2007). Several fungi have the added benefits of being well known industrial production organisms, with generally regarded as safe (GRAS) status. Media requirements are generally simple and the availability of various sequenced genomes has further improved the ability of micro-organisms to convert the pentose sugar D-xylose, abundant in lignocellulosic biomass, into D-xylonic acid is worth noting. With the availability of different genetic tools, it has been possible to generate fungal strains that can produce D-xylonate with high yields. Several fungal species have been engineered to produce D-xylonate by overexpression of genes coding for enzymes involved in the metabolism of D-xylose to D-xylonate. For example, the yeast K. lactis has been engineered to produce D-xylonate with high yields by overexpressing the D-xylose dehydrogenase (XYD) from Trichoderma reesei.

The metabolism of D-xylose in K. lactis has been studied extensively. D-Xylose uptake was not affected by deletion of either the D-xylose reductase (KmXYL1) or the xylitol dehydrogenase encoding gene (XYL2). However, when the endogenous xyd1 gene encoding D-xylose dehydrogenase (XYD) was deleted, production of D-xylonate was increased. When 12 mmol O2 l−1 were provided, D-xylonate production was more efficient when 12 mmol O2 l−1 were provided. D-Xylose uptake was not affected by deletion of either the D-xylose reductase (KmXYL1) or the xylitol dehydrogenase encoding gene (XYL2). However, when the endogenous xyd1 gene encoding D-xylose dehydrogenase (XYD) was deleted, production of D-xylonate was increased. When 12 mmol O2 l−1 were provided, D-xylonate production was more efficient when 12 mmol O2 l−1 were provided.

D-xylonate production with the yeast K. lactis xyd1 was produced when K. lactis xyd1 was grown on 10.5 g D-galactose l−1. K. lactis was produced when D-xylonate was produced from D-xylose using Gluconobacter. Other species produce a wide range of oxidising enzymes, which have periplasmic, membrane bound, PQQ-dependent D-xylose dehydrogenase from Trichoderma reesei. Several bacteria are able to convert D-xylose to D-xylonate with high yields and production rates. However, commercial production is not established.

In contrast, K. lactis carbon needs to be provided for biomass and energy generation. Further, the xyd1 strain containing the XYL1 gene or a putative XYL1 gene produced high concentrations of xylitol and significantly less D-xylonate. Several fungi have the added benefits of being well known industrial production organisms, with generally regarded as safe (GRAS) status. Media requirements are generally simple and the availability of various sequenced genomes has further improved the ability of micro-organisms to convert the pentose sugar D-xylose, abundant in lignocellulosic biomass, into D-xylonic acid is worth noting. With the availability of different genetic tools, it has been possible to generate fungal strains that can produce D-xylonate with high yields. Several fungal species have been engineered to produce D-xylonate by overexpression of genes coding for enzymes involved in the metabolism of D-xylose to D-xylonate. For example, the yeast K. lactis has been engineered to produce D-xylonate with high yields by overexpressing the D-xylose dehydrogenase (XYD) from Trichoderma reesei.
Bioconversion of d-xylose to d-xylonate with Kluyveromyces lactis

Yvonne Nygård *, Mervi H. Toivari, Merja Penttilä, Laura Ruohonen, Marilyn G. Wiebe

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

A R T I C L E   I N F O

Article history:
Received 21 December 2010
Received in revised form
18 March 2011
Accepted 5 April 2011
Available online 22 April 2011

Keywords:
• d-xylonic acid
• d-xylose
• Kluyveromyces lactis
• Xylitol dehydrogenase
• Xyitol dehydrogenase

A B S T R A C T

D-Xylonate was produced from d-xylose using Kluyveromyces lactis strains which expressed the gene for NADP⁺-dependent d-xylose dehydrogenase from Trichoderma reesei (xyd1). Up to 19 ± 2 g d-xylonate l⁻¹ was produced when K. lactis expressing xyd1 was grown on 10.5 g d-galactose l⁻¹ and 40 g d-xylose l⁻¹. Intracellular accumulation of d-xylonate (up to ∼70 mg [g biomass]⁻¹) was observed.

D-Xylose was metabolised to d-xylose, xylitol and biomass. Oxygen could be reduced to 6 mmol O₂ l⁻¹ h⁻¹ without loss in titre or production rate, but metabolism of d-xylose and xylitol were more efficient when 12 mmol O₂ l⁻¹ h⁻¹ were provided.

D-Xylose uptake was not affected by deletion of either the d-xylose reductase (XYL1) or a putative xylitol dehydrogenase encoding gene (XYL2) in xyd1 expressing strains. K. lactis xyd1ΔXYL1 did not produce extracellular xylitol and produced more d-xylonate than the xyd1 strain containing the endogenous XYL1. K. lactis xyd1ΔXYL2 produced high concentrations of xylitol and significantly less d-xylonate than the xyd1 strain with the endogenous XYL2.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

With the current wide interest in using plant biomass derived products to replace fossil fuel derived materials, the ability of micro-organisms to convert the pentose sugar d-xylose, abundant in lignocellulosic biomass, into d-xylonic acid is worth noting. d-Xylose is an organic acid similar to d-gluconic acid, which is widely used in the food, chemical, construction and pharmaceutical industries (Singh and Kumar, 2007). d-Xylonate can be produced by bacteria such as Pseudomonas and Gluconobacter species, which have periplasmic, membrane bound, PQQ-dependent, as well as cytoplasmatic, dehydrogenases (Buchert et al., 1986, 1988; Buchert, 1990). d-Xylose is converted to d-xylono-γ-lactone by d-xylose dehydrogenase and the d-xylo-γ-lactone is subsequently hydrolysed either spontaneously or enzymatically by a lactonase to yield d-xylonate (Buchert and Viikari, 1988). Several bacteria are able to convert d-xylose to d-xylonate with high yields and production rates. However, commercial production of d-xylonate has not been established. Pseudomonas and Gluconobacter species produce a wide range of oxidising enzymes, with various degrees of specificity, resulting not only in the conversion of d-xylose to d-xylonate, but also in conversion of other sugars, which would be present in lignocellulosic hydrolysates, into other acids, such as d-glucanate and L-arabinonate.

Fungal species have high potential for production of organic acids (Porro et al., 1999; Magnuson and Lasure, 2004; Ilimén et al., 2007). Several fungi have the added benefits of being well known industrial production organisms, with generally regarded as safe (GRAS) status. Media requirements are generally simple and the availability of various sequenced genomes has further improved the molecular biology tools available. We recently described d-xylonate production with the yeast Saccharomyces cerevisiae using the d-xylonate dehydrogenase (XYD) from T. reesei (Toivari et al., 2010), but production rates (25–36 mg d-xylonate l⁻¹ h⁻¹) and yields (∼0.4 g d-xylonate [g d-xylose consumed]⁻¹) were low.

S. cerevisiae does not naturally utilise d-xylose as a carbon source and it does not have specific uptake for d-xylose. d-Xylose is transported by hexose transporters, which have much lower Km values for d-xylose compared to d-glucose (Kötter and Ciriacy, 1993; Hamacher et al., 2002), so that d-xylose transport is inhibited in the presence of excess d-glucose (Saloheimo et al., 2007) and alternative carbon needs to be provided for biomass and energy generation during d-xylonate production in batch culture (Toivari et al., 2010). In contrast, Kluyveromyces lactis can naturally use d-xylose as a carbon source and may have more efficient uptake of d-xylose than S. cerevisiae. Since d-xylose can be metabolised, it will also serve as a carbon, redox balance and energy source for K. lactis. Further, the T. reesei YXD requires NADP⁺ as a co-factor (Berghäll et al., 2007) and K. lactis has several routes, in addition to the pentose phosphate pathway, for regeneration of intracellular NADP⁺, such as a NAD(P)⁺-accepting external dehydrogenase (Tarrio et al., 2006), NAD(P)⁺-accepting glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Verho et al., 2002) and glutathione reductase (NIGR1, González-Siso et al., 2009) and would not require redox engineering to regenerate NADP⁺ (Verho et al., 2002, 2003; Martínez et al., 2008). Although redox balancing was not able to improve d-xylonate
production by *S. cerevisiae* (Toivari et al., 2010), these inherent routes for NADP⁺ regeneration may still be beneficial in *K. lactis*. None-the-less, the activity of the native pathway for D-xylose conversion may need to be reduced in order to redirect D-xylose to n-xylose rather than to biomass and/or xylitol. This can be achieved by deleting either the n-xylose reductase (XR) or the xylitol dehydrogenase into the encoding gene of the native pathway or by controlling oxygen provision.

*K. lactis* is used in the industrial production of proteins. Although it is not particularly tolerant to hydrolysate inhibitors (M. Wiebe, unpublished results), with a sequenced genome and well defined tools for genetic modification it can serve as a model source, G418 (200 mg l⁻¹), and solidified by the addition of agar (1.5%). Yeast peptone (YP) medium, containing yeast extract (10 g l⁻¹), and solidified by the addition of agar (1.5%) was used to select strains in which the *K. lactis* XYL1 gene basically as described (Y. Nygård et al., 2013) medium containing 20 g D-xylose l⁻¹ as sole carbon source. Previous experiments demonstrated that a strong history of D-xylose utilisation was needed for *K. lactis* strains to have efficient D-xylose utilisation. Pre-growth on D-xylose increased the D-xylose consumption rate of the control strain H3632 by 77% and of *xyd1*-expressing strains by almost 12-fold (unpublished data). The deletion strains grew weakly on n-xylose, but sufficient to maintain the strains and generate inoculum with induced n-xylose uptake.

For small scale cultures, yeast were grown in either 20 or 50 ml modified yeast synthetic complete (YSC, Sherman et al., 1983) medium containing 20 g D-xylose l⁻¹ in 100 or 250 ml Erlenmeyer flasks, respectively, with 250 rpm shaking at 30 °C. For larger scale cultures, yeast were grown in 500 ml yeast nitrogen base (YNB, Becton, Dickinson and Company, USA) medium containing 20 or 40 g D-xylose l⁻¹ with 10 g n-galactose l⁻¹ to induce the *lac4* promoter, in Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland) with 2 marine impellers (4 blades) at pH 5.5, 30 °C, 0.3–1.4 volume air [volume culture]⁻¹ min⁻¹ (vvm) and 150–700 rpm agitation, to provide estimated oxygen transfer rates (OTRs) between 3 and 22 mmol l⁻¹ h⁻¹ (Table 1). Cultures provided with 40 g D-xylose l⁻¹ were given an additional 0.5 g n-galactose l⁻¹ after 53 h to sustain expression of *xyd1*. The pH was maintained constant by addition of 2 M NaOH or 1 M H₃PO₄. Clerol antifoaming agent (Cognis, France, 0.08–0.10 ml l⁻¹) was added to prevent foam accumulation. Gas concentration (CO₂, 13CO₂, O₂, N₂, and Ar) was measured continuously in an Omnistor quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO₂ in Ar.

### 2.2. Media and culture conditions

Yeast peptone (YP) medium, containing yeast extract (10 g l⁻¹) and bacter peptone (20 g l⁻¹), with n-glucose (20 g l⁻¹) as a carbon source, G418 (200 mg l⁻¹), and solidified by the addition of 15 g agar l⁻¹ was used to select strains in which the *XYL1* and *XYL2* genes had been deleted. All strains were stored on agar-solidified YP medium containing 20 g D-xylose l⁻¹ as sole carbon source. Previous experiments demonstrated that a strong history of D-xylose utilisation was needed for *K. lactis* strains to have efficient D-xylose utilisation. Pre-growth on D-xylose increased the D-xylose consumption rate of the control strain H3632 by 77% and of *xyd1*-expressing strains by almost 12-fold (unpublished data). The deletion strains grew weakly on n-xylose, but sufficient to maintain the strains and generate inoculum with induced n-xylose uptake.

For small scale cultures, yeast were grown in either 20 or 50 ml modified yeast synthetic complete (YSC, Sherman et al., 1983) medium containing 20 g D-xylose l⁻¹ in 100 or 250 ml Erlenmeyer flasks, respectively, with 250 rpm shaking at 30 °C. For larger scale cultures, yeast were grown in 500 ml yeast nitrogen base (YNB, Becton, Dickinson and Company, USA) medium containing 20 or 40 g D-xylose l⁻¹ with 10 g n-galactose l⁻¹ to induce the *lac4* promoter, in Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland) with 2 marine impellers (4 blades) at pH 5.5, 30 °C, 0.3–1.4 volume air [volume culture]⁻¹ min⁻¹ (vvm) and 150–700 rpm agitation, to provide estimated oxygen transfer rates (OTRs) between 3 and 22 mmol l⁻¹ h⁻¹ (Table 1). Cultures provided with 40 g D-xylose l⁻¹ were given an additional 0.5 g n-galactose l⁻¹ after 53 h to sustain expression of *xyd1*. The pH was maintained constant by addition of 2 M NaOH or 1 M H₃PO₄. Clerol antifoaming agent (Cognis, France, 0.08–0.10 ml l⁻¹) was added to prevent foam accumulation. Gas concentration (CO₂, 13CO₂, O₂, N₂, and Ar) was measured continuously in an Omnistor quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO₂ in Ar.

### 2.3. Measurements of biomass

Biomass was measured as optical density at 600 nm (OD₆₀₀) or as dry mass of the cells. For dry mass measurement, samples were
collected in 2 ml pre-dried, pre-weighed microcentrifuge tubes, washed twice with equal volume distilled water and dried at 100 °C. One OD<sub>600</sub> unit was equal to 0.22 g l<sup>-1</sup> cell dry mass.

### 2.4. Chemical analyses

Culture supernatant was stored at −20 °C for substrate and product analyses. To determine the intracellular concentrations of D-xylose, D-xylonate and xylitol, cells from 10 ml of culture were collected by centrifugation. Pellets were washed with 1.8 ml deionised water to remove extracellular compounds and frozen at −20 °C to disrupt membranes. The frozen pellets were freeze-dried using a Christ Alpha 2-4 lyophiliser (Biotech international, Belgium) to remove the interstitial liquid enabling the concentrations to be measured in a known volume. Intracellular D-xylose, D-xylonate and xylitol were extracted from the lyophilised pellets (−60 mg biomass ml<sup>−1</sup>) in 5 mM H<sub>2</sub>SO<sub>4</sub>. The resuspended pellets were incubated for approximately 1 h. Samples could be refrozen at this stage if desired. Thawed samples were centrifuged and the supernatant collected for analyses. Cells could also be disrupted with glass beads, resulting in release of the same amounts of D-xylose, D-xylonate and xylitol as slow freezing with mild acid extraction. Results are presented as mg substrate or product per g dry biomass.

Intracellular and extracellular substrates and products (D-xylonic acid, ethanol, glycerol, pyruvate and acetate, D-glucose and D-xylose) were analysed by HPLC on a Fast Acid Analysis Column linked to an Aminex HPX-87H column (BioRad Labs, USA) with 2.5 mM H<sub>2</sub>SO<sub>4</sub> as eluent and a flow rate of 0.5 ml min<sup>−1</sup>. The column was maintained at 55 °C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector. D-Xylose could not be accurately determined using this HPLC method when D-xylonic acid was present in the sample.

Extracellular D-xylonic acid concentrations were also measured using the hydroxamate method described by Lien (1959). Samples were diluted in 1.3 M HCl and heated at 100 °C to convert D-xylonic acid to D-xyloono-γ-lactone before adding 50 μl of the diluted sample to 100 μl hydroxylamine reagent (2 M hydroxylamine HCl in 2 M NaOH). HCl (65 μl, 3.2 M) was added, followed by addition of 50 μl FeCl<sub>3</sub> (100 g l<sup>−1</sup> in 0.1 M HCl). Absorbance was measured immediately at 550 nm and D-xyloono-γ-lactone concentration was determined by comparison with a standard curve. This assay was sensitive to <0.1 g D-xyloono-γ-lactone l<sup>−1</sup> and correlated well with HPLC measurements of D-xylonic acid, since D-xylonic acid was the only reacting compound present in the supernatant. The hydroxamate method was not used for intracellular D-xyloono-γ-lactone measurement, since it was not necessarily the only lactone present in the cell extract.

### 2.5. Enzyme activity

D-Xylose dehydrogenase activity was measured, according to Berghäll et al. (2007) from crude cell extracts. The specific D-xylose dehydrogenase activity was calculated as enzyme activity in nanokatal per mg protein. The protein concentration was determined using the Bio-Rad protein kit, based on the assay developed by Bradford (1976). Assays were performed at 30 °C using a Konelab Arena photometric analyzer (Thermo Electron Oy, Finland). The sample volume from which cells were harvested for the enzyme assays was 7–50 ml, depending on the OD<sub>600</sub> and expected activity.

### 3. Results

#### 3.1. Production of α-xylonate from α-xylose in K. lactis expressing the T. reesei α-xylose dehydrogenase encoding gene xyd1

The xyd1 expressing strain H3677 was grown with 20 or 40 g D-xylose l<sup>−1</sup> and 10 g D-galactose l<sup>−1</sup> which induced the LAC4 promoter as well as providing for biomass generation and maintenance of the energy and redox state of the cells. With 20 g D-xylose l<sup>−1</sup>, 6.3 ± 0.1 g α-xylonate l<sup>−1</sup> was produced at an initial rate of 80 ± 1 mg α-xylonate l<sup>−1</sup> h<sup>−1</sup>, whereas no α-xylonate was produced by the control strain, H3632 (Fig. 1). D-Galactose was consumed during the first 10–15 h, after which α-xylose consumption started.

---

**Table 1**

<table>
<thead>
<tr>
<th>Agitation (rpm)</th>
<th>Aeration (l min&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;a&lt;/sub&gt; (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Oxygen transfer rate (mmol l&lt;sup&gt;−1&lt;/sup&gt; h&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Maximum oxygen uptake rate (mmol l&lt;sup&gt;−1&lt;/sup&gt; h&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>0.14–0.15</td>
<td>0.003</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>300</td>
<td>0.3</td>
<td>0.007</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>500</td>
<td>0.5</td>
<td>0.014</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>700</td>
<td>0.6–0.67</td>
<td>0.024</td>
<td>22</td>
<td>13</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Biomass, D-xylose, D-xylonate, xylitol and ethanol concentrations of K. lactis H3677 (xyd1, solid symbols) and H3632 (parent, open symbols) during growth on 10 g D-galactose l<sup>−1</sup> and 20 g D-xylose l<sup>−1</sup> at pH 5.5, 30 °C, OTR = 12 mmol O<sub>2</sub> l<sup>−1</sup> h<sup>−1</sup>. Error bars show ± SEM for duplicate cultures.
A maximum of 2.9 ± 0.2 g ethanol l⁻¹ was produced from n-galactose and was consumed simultaneously with n-xylene. The D-xylene consumption rate of xyl1 expressing H3677 (0.27 ± 0.02 g n-xylene l⁻¹ h⁻¹) was significantly (p < 0.05) higher than that of the control strain, H3632 (0.20 ± 0.02 g n-xylene l⁻¹ h⁻¹). Both strains produced 6.1 ± 0.1 g biomass l⁻¹, but H3677 produced more (p < 0.05) xyitol (6.4 ± 0.1 g l⁻¹) than the parent strain (5.8 ± 0.3 g xyitol l⁻¹). Biomass was produced primarily during n-galactose and ethanol consumption, but at least 1.8 g biomass l⁻¹ was produced by the control and 2.2 g biomass l⁻¹ by H3677 when n-xylene and xyitol were the only carbon-sources being consumed. The yield of n-xylonate and xyitol on n-xylene and of biomass on total carbohydrate consumed are given in Table 2, along with production and consumption rates.

All n-xylene was consumed within 75 h when only 20 g n-xylene l⁻¹ was provided. When the n-xylene concentration was increased to 40 g l⁻¹ and an additional 0.5 g n-galactose l⁻¹ was added at 53 h to induce the lac promoter, 18.9 ± 2.3 g n-xylonate l⁻¹ was produced at an initial rate of 158 ± 11 mg D-xylonate l⁻¹ h⁻¹. Biomass (6.2 ± 0.2 g l⁻¹), xyitol (6.2 ± 0.2 g l⁻¹) and ethanol (2.5 ± 0.4 g l⁻¹) concentrations did not differ significantly (p > 0.05) from that produced with 20 g n-xylene l⁻¹. Yields and rates of n-xylonate production are presented in Table 2.

H3677 had approximately 2 nkat XYD activity [mg protein]⁻¹ after 24 h cultivation in n-galactose and n-xylene containing medium. From 48 to 140 h XYD activity was constant at 1.5 ± 0.0 nkat [mg protein]⁻¹. The control strain H3632 showed no XYD activity.

3.2 Effect of aeration on n-xylonate production by K. lactis

K. lactis H3677 was grown in bioreactors with 20 g n-xylene l⁻¹ at various OTRs, controlled by changing the agitation and aeration rates (Table 1). Oxygen uptake rate (OUR) was highest at the end of the exponential growth phase on n-galactose and remained below the estimated potential OTR throughout the cultivations in all conditions (Table 1).

Similar (p > 0.05) concentrations of n-xylonate were produced at OTRs of 6, 12 and 22 mmol O₂ l⁻¹ h⁻¹, but initial n-xylonate production rates were higher (p < 0.05) with oxygen provided at 6 mmol O₂ l⁻¹ h⁻¹ (77 ± 2 mg n-xylonate l⁻¹ h⁻¹) and 12 mmol O₂ l⁻¹ h⁻¹ (60 ± 1 mg n-xylonate l⁻¹ h⁻¹) than at 22 mmol O₂ l⁻¹ h⁻¹ (64 mg n-xylonate l⁻¹ h⁻¹, Fig. 2). In contrast, significantly less (p < 0.05) n-xylonate was produced at a lower volumetric rate when oxygen provision was reduced to 3 mmol O₂ l⁻¹ h⁻¹ (Fig. 2). However, initial specific production rates, ~33 mg [g biomass]⁻¹ h⁻¹, were similar in all conditions. As expected, less biomass and more ethanol were produced at lower OTRs (Fig. 2). Little or no apparent xyitol consumption occurred at OTRs of 3 or 6 mmol O₂ l⁻¹ h⁻¹, whereas xyitol was consumed at 12 mmol O₂ l⁻¹ h⁻¹ after n-xylene had been consumed from the supernatant. n-Xylonate metabolism at 22 mmol O₂ l⁻¹ h⁻¹ appeared inefficient, with less biomass, ethanol and xyitol being produced than at 12 mmol O₂ l⁻¹ h⁻¹ (Fig. 2). Although xyitol was produced at 22 mmol O₂ l⁻¹ h⁻¹, it was not completely consumed (data not shown).

3.3 Altering the n-xylene flux in n-xylene reductase and xylitol dehydrogenase-deficient K. lactis strains

Deletion of XYL1, encoding XR, disrupted xyitol production in K. lactis H3765, while deletion of the putative XYL2 gene, encoding XDH, disrupted metabolism of xyitol in K. lactis H3763. Deletion of XYL1 resulted in no accumulation of xyitol (<0.4 g xyitol l⁻¹) in the culture supernatant and production of 22% more n-xylonate (7.7 ± 0.4 g n-xylonate l⁻¹) than H3677 containing the endogenous XYL1 (6.3 ± 0.1 g n-xylonate l⁻¹; Fig. 3). K. lactis xyd1 ΔXYL1 (127 ± 2 mg n-xylonate l⁻¹ h⁻¹) had a significantly (p < 0.05) initial n-xylonate production rate than H3677 (80 mg n-xylonate l⁻¹ h⁻¹, Table 2). Deletion of the putative XYL2 resulted in accumulation of 14.1 ± 0.5 g xyitol l⁻¹ and production of 69% less n-xylose (2.0 ± 0.1 g n-xylose l⁻¹) than H3677 containing the endogenous XYL2 (Fig. 3). The initial xyitol production rate was not affected (Table 2). The n-xylonate consumption rate of H3765 (xyd1), H3675 (xyd1 ΔXYL1) and H3763 (xyd1 ΔXYL2) did not differ significantly (p > 0.05, Table 2).

Neither the XDH encoding gene nor the corresponding protein from K. lactis has been characterised, but the potential protein encoded by open reading frame KLA0D05511g in the genome of K. lactis NRRL Y-1140 shows 53% identity and 68% sequence similarity with the Scheffersomyces stipitis (formerly Pichia stipitis) XDH (BLASTP 2.2.6; http://www.genolevures.org/blast.html). Thus this open reading frame is a potential XDH encoding gene. Since deletion of the potential XYL2 gene resulted in significantly (p < 0.05) more xyitol being produced, with little apparent xyitol consumption, and a higher yield of xyitol on n-xylonate than in H3677 or the parent H362, we conclude that KLA0D05511g encodes an XDH. However, K. lactis xyd1 ΔXYL2 grew on rich medium with n-xylene as the sole carbon source to produce incula, and in n-xylonate production cultures approximately 3.4 g biomass l⁻¹ was produced when n-xylene and xyitol were

<table>
<thead>
<tr>
<th>Strain</th>
<th>n-xylene consumption rate (g l⁻¹ h⁻¹)</th>
<th>n-xylene production rate (mg l⁻¹ h⁻¹)</th>
<th>Xyitol production rate (mg l⁻¹ h⁻¹)</th>
<th>Biomass (g [g carbohydrate consumed]⁻¹)</th>
<th>n-xylonate (g [g n-xylene consumed]⁻¹)</th>
<th>Xyitol (g [g n-xylose consumed]⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3632</td>
<td>0.20 ± 0.02*</td>
<td>0</td>
<td>104 ± 6</td>
<td>0.26 ± 0.01</td>
<td>0</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>H3677 (xyd1)</td>
<td>0.27 ± 0.02*</td>
<td>80 ± 1*</td>
<td>119 ± 4*</td>
<td>0.27 ± 0.01*</td>
<td>0.29 ± 0.01*</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>H3677 (xyd1)</td>
<td>0.37 ± 0.01*</td>
<td>158 ± 11*</td>
<td>104 ± 4*</td>
<td>0.17 ± 0.01*</td>
<td>0.60 ± 0.03*</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>H3765 (ΔXYL1)</td>
<td>0.29 ± 0.03*</td>
<td>127 ± 2*</td>
<td>18 ± 5*</td>
<td>0.22 ± 0.02*</td>
<td>0.40 ± 0.04*</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>H3763 (ΔXYL2)</td>
<td>0.29 ± 0.00*</td>
<td>31 ± 5*</td>
<td>135 ± 5*</td>
<td>0.25 ± 0.01*</td>
<td>0.09 ± 0.01*</td>
<td>0.59 ± 0.03</td>
</tr>
</tbody>
</table>
the only available carbon sources, indicating that XYL2 is not the only gene encoding XDH present in *K. lactis*. Other putative xylitol dehydrogenase encoding sequences in the *K. lactis* genome have lower sequence similarity with *S. stipitis* XDH (28% or 27% identity and 45% or 46% sequence similarity for KLLA0B00451g and KLLA0D19929g, respectively).

Similarly, H3765 (xyd1DXYL1) grew on D-xylose as sole carbon source and produced 37.1 g biomass l\(^{-1}\) from D-xylose in the D-xylonate production cultures, indicating the presence of other D-xylose or aldose reductase(s) in the genome. The *K. lactis* XR shows 60% identity and 77% sequence similarity with the *S. stipitis* XR, but in addition, there are at least 5 hypothetical aldose reductases with app. 30% identity and 50% sequence similarity to the *S. stipitis* XR in the *K. lactis* genome. Deletion of XYL1 or XYL2 did not affect the yield of biomass on total carbohydrate consumed (Table 2).

Both *K. lactis* xyd1 DXYL1 and *K. lactis* xyd1 DXYL2 had similar levels of XYD activity as strain H3677.

### 3.4. Intracellular accumulation of D-xylonate, xylitol and D-xylose

D-Xylonate accumulated intracellularly in *K. lactis* H3677, expressing xyd1, when grown in the presence of D-xylose (Fig. 4). At 31 h, when only 0–3 g l\(^{-1}\) extracellular D-xylonate was detected, the intracellular concentration in *K. lactis* xyd1 was approximately 71 mg [g biomass\(^{-1}\)]. This represents an intracellular concentration of 35–47 g D-xylonate l\(^{-1}\), depending on the volume of cytoplasm per g dry biomass, which was not measured in this study but has been reported to be between 1.5 and 2 ml per g dry cell mass for *S. cerevisiae* (de Koning and van Dam, 1992; Gancedo and Serrano, 1989), and may vary depending on the stage of growth (Lagunas and Moreno, 1985; Austriaco, 1996) and differences in the volume of organelles, in particular the cell wall and the vacuole. Intracellular D-xylonate concentration then generally decreased as extracellular D-xylonate concentration increased, with a strong negative correlation (r=0.98). When either XYL1 or XYL2 were deleted, intracellular D-xylonate did not accumulate above 44 mg [g biomass\(^{-1}\)], and remained approximately constant at 26 (ΔXYL1) or 31 (ΔXYL2) mg D-xylonate [g biomass\(^{-1}\)] between 31 and 143 h. No (<3 mg [g biomass\(^{-1}\) background] D-xylonate was detected in the cell extracts of the parent strain H3632.

Xylitol also accumulated intracellularly in *K. lactis*. Concentrations over 135 mg xylitol [g biomass\(^{-1}\)] (65–90 g xylitol l\(^{-1}\)) were observed in both H3677 and the parent H3632 when cultivated in 20 g D-xylose l\(^{-1}\) with 12 mmol O\(_2\) l\(^{-1}\) h\(^{-1}\). High intracellular concentrations were maintained in H3677 and H3632 even when extracellular xylitol was being consumed. In *K. lactis* xyd1 DXYL1 ~19 mg intracellular xylitol [g biomass\(^{-1}\)] was observed, whereas in *K. lactis* xyd1 ΔXYL2, xylitol initially accumulated to ~100 mg [g biomass\(^{-1}\), similar to H3677 and H3632.
After 72 h the intracellular xylitol concentration in K. lactis xyd1 ΔXYL2 decreased to a concentration of only \( \sim 8 \) mg [g biomass] \(^{-1} \) at the end of the cultivation.

Along with \( \alpha \)-xylate and xylitol, \( \alpha \)-xylose was detected in the intracellular extracts and was positively correlated to extracellular concentrations. After 31 h cultivation, approximately 70 mg \( \alpha \)-xylose [g biomass] \(^{-1} \) (35–47 g \( \alpha \)-xylose l \(^{-1} \)) was present intracellularly in H3632 and H3677, when the extracellular \( \alpha \)-xylose concentration was \( \sim \) 16 g l \(^{-1} \). Less \( \alpha \)-xylose accumulated intracellularly in the \( \Delta XYL1 \) (max. 44 mg \( \alpha \)-xylose [g biomass] \(^{-1} \)) and \( \Delta XYL2 \) (max. 12 mg \( \alpha \)-xylose [g biomass] \(^{-1} \)) strains than in H3677, but as in H3677, intracellular \( \alpha \)-xylose was positively correlated with the extracellular concentration.

4. Discussion

Expression of \( T. \) reesei xyd1 in K. lactis resulted in a 5-fold increase in titre, 4-fold increase in production rate and a 50% increase in the yield of \( \alpha \)-xylate from \( \alpha \)-xylose, compared to expression of the same gene in S. cerevisiae (Toivari et al., 2010). Activity levels of the XYL1 in K. lactis and S. cerevisiae were similar. K. lactis H3677 produced 19 ± 2 g l \(^{-1} \) \( \alpha \)-xylate from \( \alpha \)-xylose at a maximum rate of 158 ± 11 mg l \(^{-1} \) h \(^{-1} \) and yield of \( \sim 0.60 \pm 0.03 \) g \( \alpha \)-xylate [g \( \alpha \)-xylose consumed] \(^{-1} \) when grown on 10 g \( \alpha \)-galactose l \(^{-1} \) and 40 g \( \alpha \)-xylose l \(^{-1} \), with re-induction of the LAC4 promoter with 0.5 g \( \alpha \)-galactose l \(^{-1} \) after 53 h. \( \alpha \)-Galactose was provided to K. lactis instead of \( \alpha \)-glucose or ethanol (cf. Toivari et al., 2010), in order to induce the LAC4 promoter, but also provided carbon for biomass and as an energy source.

Various factors may contribute to the higher \( \alpha \)-xylate production by K. lactis than S. cerevisiae, such as more efficient uptake of \( \alpha \)-xylose, better tolerance to intracellular acid accumulation and/or better \( \alpha \)-xylate export capacity. In contrast to S. cerevisiae, K. lactis is able to use \( \alpha \)-xylose as a carbon source and produced biomass as well as \( \alpha \)-xylate and xylitol when grown on \( \alpha \)-xylose. The uptake of \( \alpha \)-xylose has not been studied in K. lactis. K. lactis has several uniporers for sugar transport (Palma et al., 2009), and also a putative active sugar transporter belonging to the putative \( \alpha \)-xylose proton symporter family (Palma et al., 2007). Although evolved strains of S. cerevisiae have been shown to take up \( \alpha \)-xylose at rates of 0.2–1.1 g \( \alpha \)-xylose l \(^{-1} \) h \(^{-1} \), non-evolved strains consume \( \alpha \)-xylose at rates between 0.05 and 0.1 g l \(^{-1} \) h \(^{-1} \) (Pitkänen et al., 2005; van Vleet and Jeffries, 2009; Liu and Hu, 2010). \( \alpha \)-Xylate-producing strains of S. cerevisiae consumed \( \alpha \)-xylose at 0.06 g \( \alpha \)-xylose l \(^{-1} \) h \(^{-1} \) (Toivari et al., 2010), which was three to six times slower than the rate of \( \alpha \)-xylose consumption observed for K. lactis strains (0.2–0.4 g \( \alpha \)-xylose l \(^{-1} \) h \(^{-1} \)). Further, since \( \alpha \)-xylose could be used as both carbon and energy source by K. lactis, it would not be necessary to add a co-substrate during the cultivation to sustain \( \alpha \)-xylate production, as was necessary with S. cerevisiae (Toivari et al., 2010).

Production of \( \alpha \)-xylate from \( \alpha \)-xylose is an oxidative reaction and production occurs under aerobic conditions (Buchert et al., 1986; Hardy et al., 1993; Toivari et al., 2010). Oxygen is also essential for the growth of K. lactis. However, since \( \alpha \)-xylose provides carbon for both \( \alpha \)-xylate and biomass production, it would be desirable to limit biomass production by limiting oxygen provision, while providing sufficient oxygen for \( \alpha \)-xylose oxidation. In oxygen limited conditions (e.g. \( \sim 12 \) mmol O \(_2\) l \(^{-1} \) h \(^{-1} \)), biomass production decreased as expected. However, \( \alpha \)-xylate production
was not affected until the oxygen supply was reduced to 3 mmol O$_2$ l$^{-1}$ h$^{-1}$. Thus, completely aerobic conditions were not necessary and *K. lactis* could produce D-xylonate with OTRs as low as 6 mmol O$_2$ l$^{-1}$ h$^{-1}$. None-the-less, D-xylose uptake rate was significantly ($p < 0.05$) slower with 6 mmol O$_2$ l$^{-1}$ h$^{-1}$ (0.19 g D-xylose l$^{-1}$ h$^{-1}$) than with 12 mmol O$_2$ l$^{-1}$ h$^{-1}$ (0.27 g D-xylose l$^{-1}$ h$^{-1}$) and D-xylose consumption may be incomplete, so 12 mmol O$_2$ l$^{-1}$ h$^{-1}$ was used as the standard condition for strain comparisons.

Deletion of the XR encoding gene improved the D-xylonate titre, yield and production rate in comparison with the strain with the intact XR encoding gene and completely eliminated extracellular xylitol production. In *S. cerevisiae* expressing *xyd1*, deletion of the aldose reductase encoding gene GRE3 did not improve the titre or rate of D-xylonate production (Toivari et al., 2010). This may indicate that xylitol production was not as important in *K. lactis* as in *S. cerevisiae* in regenerating NADP$^+$ for XYL.

Although extracellular xylitol was low or undetected, the *xyd1*AXYL1 derivative (H3765) of the industrial *K. lactis* strain GG799 was able to grow and produce biomass with D-xylose as the sole carbon source. Intracellular xylitol (~19 mg [g biomass]$^{-1}$) was also detected in this strain. Thus, although Billard et al. (1995) described XYL1 as the gene encoding XR in the genome of *K. lactis* MW270-7B, it appears that H3632 (GG799) has alternative aldose reductases which enable growth on D-xylose. Indeed, there are at least 5 hypothetical aldose reductases with approx. 30% identity and 50% sequence similarity to the *S. stipitis* XR in the *K. lactis* genome.

The *K. lactis* XDH has not been characterised. We suggest that the open reading frame KLAB0005511g of *K. lactis* strain Y-1140, which has 53% identity to the *S. stipitis* XDH encoded by *XYL1*, codes for xylitol dehydrogenase, since deletion of the corresponding gene in strain H3632 diminished the ability to use D-xylose and xylitol as carbon sources. However, the *xyd1*AXYL1 strain (H3763) was still able to grow on D-xylose as the sole carbon source, suggesting that there are other enzymes present which perform the same reaction. There are other putative xylitol dehydrogenase encoding sequences in the *K. lactis* genome which have lower sequence similarity with *S. stipitis* XDH than that encoded by KLAB0005511g.

Deletion of the XDH encoding gene resulted in significantly lower D-xylose and increased xylitol production compared to H3677. Extracellular xylitol accumulated, as with H3632 and H3677 which contain the intact XDH encoding gene, during the first ca. 70 h of growth, but unlike strains with an intact endogenous D-xylose utilisation pathway, there was no apparent xylitol consumption. Intracellular xylitol concentrations decreased after 79 h, indicating the activity of an alternative xylitol dehydrogenase, increased export of xylitol or increased cell lysis.

D-xylose (0–70 mg [g biomass]$^{-1}$) and xylitol (10–150 mg [g biomass]$^{-1}$) both accumulated within the cytoplasm. The mechanism of D-xylose excretion across the cell membrane is not known and no eukaryotic D-xylose transporters have been described. Organic acids, in the charged dissociated form at intracellular pH, may be transported by specific ABC-type transporters or by facilitated or passive diffusion across the membrane (Nicolau et al., 2010), but are generally thought to not freely diffuse through the membrane as the free acid is thought to do (Casal et al., 2008; Abbott et al., 2009). Maintenance of a high extracellular pH is thus useful in reducing the re-import of acid to the cell, but would contribute less to the initial export of the acid.

Concentrations of intracellular xylitol similar to those observed here have been observed in the D-xylose utilising yeast
The high concentration of intracellular α-xylene (\(\sim 70\) mg [g biomass] \(^{-1}\), i.e. \(>35\) g l \(^{-1}\)) supports the hypothesis that \(K.\) lactis has an active α-xylene transporter. α-Xylene did not accumulate in \(P.\) tannophilus (Xu and Taylor, 1993). Intraplantar α-xylene concentrations around 15 g l \(^{-1}\) have been observed in \(S.\) cerevisiae and \(D.\) hansenii, while higher concentrations (\(>30\) g l \(^{-1}\)) were observed in hybrid (\(S.\) cerevisiae with \(D.\) hansenii) yeast (Loray et al., 1997; Gârdonyi et al., 2003). In the case of \(S.\) cerevisiae and \(D.\) hansenii intracellular α-xylene concentration was also highly correlated with the extracellular concentration, as observed here for \(K.\) lactis. The high intracellular concentrations suggest that transport does not limit α-xylene metabolism in \(K.\) lactis.

In this study we have significantly increased the production of α-xylene over that achieved earlier with \(S.\) cerevisiae, demonstrating the benefit of using a strain with good α-xylene uptake. Using a more robust α-xylene utilising yeast, such as \(K.\) marxianus and introducing xylinases for a consolidated process, as already demonstrated for ethanol production in other yeast (Katahira and introducing xylanases for a consolidated process, as already demonstrated for ethanol production in other yeast (Katahira 2002; van de Vondervoort et al., 2006).

**Acknowledgments**

The high concentration of intracellular α-xylene (\(\sim 70\) mg [g biomass] \(^{-1}\), i.e. \(>35\) g l \(^{-1}\)) supports the hypothesis that \(K.\) lactis has an active α-xylene transporter. α-Xylene did not accumulate in \(P.\) tannophilus (Xu and Taylor, 1993). Intraplantar α-xylene concentrations around 15 g l \(^{-1}\) have been observed in \(S.\) cerevisiae and \(D.\) hansenii, while higher concentrations (\(>30\) g l \(^{-1}\)) were observed in hybrid (\(S.\) cerevisiae with \(D.\) hansenii) yeast (Loray et al., 1997; Gârdonyi et al., 2003). In the case of \(S.\) cerevisiae and \(D.\) hansenii intracellular α-xylene concentration was also highly correlated with the extracellular concentration, as observed here for \(K.\) lactis. The high intracellular concentrations suggest that transport does not limit α-xylene metabolism in \(K.\) lactis.

In this study we have significantly increased the production of α-xylene over that achieved earlier with \(S.\) cerevisiae, demonstrating the benefit of using a strain with good α-xylene uptake. Using a more robust α-xylene utilising yeast, such as \(K.\) marxianus and introducing xylinases for a consolidated process, as already demonstrated for ethanol production in other yeast (Katahira et al., 2004; den Haan et al., 2007; Voronovysh et al., 2009), would further improve the process. In \(K.\) lactis, the deletion of \(XYI\) was beneficial for α-xylene production. However, the accumulation of α-xylene in the cytoplasm may indicate difficulties with export.

**References**


Bergdoll, S., Hilditch, S., Penttilä, M., Richard, P., 2007. Identification in the mould demonstrated for ethanol production in other yeast (Katahira and introducing xylanases for a consolidated process, as already demonstrated for ethanol production in other yeast (Katahira and introducing xylanases for a consolidated process, as already demonstrated for ethanol production in other yeast (Katahira 2002; van de Vondervoort et al., 2006).


