Bioresource Technology 133 (2013) 555–562

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Low pH D-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 xylB* produced 146 g D-xylonate l^{-1} at pH 3.
- This *P. kudriavzevii* strain also produced 171 g D-xylonate l^{-1} at pH 5.5.
- D-Xylonate production was less toxic to *P. kudriavzevii* than to *Saccharomyces cerevisiae*.
- *P. kudriavzevii* is thus an excellent production organism for D-xylonic acid.

ARTICLE INFO

Article history:
Received 14 November 2012
Received in revised form 23 January 2013
Accepted 29 January 2013
Available online 7 February 2013

Keywords:
Pichia kudriavzevii
Issatchenkia orientalis
D-Xylonate
D-Xylose
Low pH

ABSTRACT

D-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 D-gluconic acid and as a platform chemical. We engineered the non-conventional yeast *Pichia kudriavzevii* VTT C-79090T to express a D-xylose dehydrogenase coding gene from *Caulobacter crescentus*. With this single modification the recombinant *P. kudriavzevii* strain produced up to 171 g L^{-1} of D-xylonate from 171 g L^{-1} D-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 D-xylonate production by *Gluconobacter oxydans* or *Pseudomonas* sp. The productivity of the strain was also remarkable at low pH, producing 146 g L^{-1} D-xylonate at 1.2 g L^{-1} h^{-1} at pH 3.0. This is the best low pH production reported for D-xylonate. These results encourage further development towards industrial scale production.

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 lists of desirable sugar-derived platform chemicals which could be produced using biotechnology are organic or amino acids (Bozell and Petersen, 2010; OECD, 2011 http://dx.doi.org/10.1787/9789264126633-en; Werpy and Petersen, 2004). The number of organic acids produced from biomass sugars with microbes is increasing. Production of lactic acid, malic acid, succinic acid, and itaconic acid has been considerably improved during the last years (Lee et al., 2011; Erickson et al., 2012; Sauer et al., 2010) and the feasibility of production has been demonstrated for e.g. glucaric acid (Lee et al., 2011), D-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).

0960-8524 © 2013 Elsevier Ltd. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.biortech.2013.01.157

The 5-carbon sugar D-xylose is a major component of hemicellulose in lignocellosic 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-Xylonic acid can be used as complexing agent and chelator in dispersal of concrete (Chun et al., 2006). It has also been used in co-polyamides, polyesters 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-xylonic 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.

D-Xylonic 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 bacteria such as *Gluconobacter oxydans* or *Pseudomonas* sp. (reviewed in Toivari et al., 2012a). Recently D-xylonate has also been produced by genetically modified organisms, including the bacterium *Escherichia coli* (Liu et al., 2012a) and yeast *Saccharomyces cerevisiae* (Toivari et al., 2010, 2012b) and *Kluyveromyces lactis* (Nygård et al., 2011), to provide alternative, more robust production hosts which could be suitable for large scale production. D-Xylonate 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-xylonate 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 L-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-xylonic acid at low pH.

2. Methods

2.1. Strains, plasmids and strain construction

Pichia kudriavzevii (formerly *Issatchenkia orientalis* (Kurtzman et al., 2008), also referred to as *Candida krusei*) strain VTT C-79090T (ATCC 32196, isolated from cabbage waste, Kofu, Japan), VTT C-05705 (ATCC 60585, isolated from rye 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 IopPGKfrw2 5′TCCCCCGGGCGGATCCTTG CTGCAACGGCAACATCA ATG3' and IopPGKrev2 5′CCCAAGCTTGGAAGATCTTGTTGTTGTT GTTGTCGTTG TTTTTG3′. The 833 bp 5′ and 746 bp 3′ flanking regions of the *P. kudriavzevii PDC1*(*IoPDC1*) gene were amplified with primer pairs IoPDC 5′flank frw 5′ATAAGAATGCGGCCGCACTGCAG AGTATATGGAATTGACG GCTCATC3′/IoPDC 5′flank rev 5′ACTGAC GCGTCGACGGATCCGTATCATTTGTAGCCCACGC CACC3′ and IoPDC 3′flank frw 5′GGA ATTGATATCGACTAGTCTTGGCTACCCACTTACCAA GAGAT3′/IoPDC 3′flank rev 5′ATAAGAATGCGGCCGCAATAGAGA GTGACCTATCCAAGCT3′.

α-Galactosidase activity derived from melibiase coded by *S. cerevisiae MEL5* (Gene ID: 547463, NCBI) was used for selection of transformants. The double expression cassette for expression of *xylB* and *MEL5* was constructed in the plasmid pSP72 (Promega) and the final construct, pMLV100A, contained the *IoPDC1* flank (5′) – [*IoPGK1* promoter – *S. cerevisiae MEL5-S. cerevisiae MEL5* terminator] – [*IoPGK1* promoter – *xylB* – *S. cerevisie ADH1* terminator] – *IoPDC1* flank (3′). The double expression cassette with *PDC1* regions was released from pMLV100A with *NotI* and introduced into *P. kudriavzevii* cells using the lithium acetate transformation protocol (Gietz et al., 1992). The transformants were selected based on blue colour formation on YPD plates containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (*X-α-Gal*). A transformant containing a functional copy of the *xylB* was deposited as VTT-C-12903 in the VTT Culture Collection.

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 Verduyn 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 ($KHC_8H_4O_4$, pH 2.2–5.5) or monopotassium phosphate (KH_2PO_4, 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 (OD_{600}) 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 OD_{600} was 0.2 or higher. Each condition assessed in the Bioscreen was performed in at least four replicates. Growth at pH 6.2 was also assessed in bioreactors, using 200 mL defined medium (Verduyn 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 H_2PO_4. Logarithmic growth was monitored by hourly measurement of OD_{600} and by measurement of CO_2 production. The gas concentration (CO_2, $^{13}CO_2$, O_2, N_2 and Ar) was analysed in an Omnistar quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO_2 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 impellors, as previously described (Toivari et al., 2010). The pH was maintained constant by addition of 2 M NaOH or 1 M H_2PO_4. 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 OD_{600} 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-Xylonic acid and/or D-xylonolactone, ethanol, glycerol, pyruvate and acetate, D-glucose and D-xylose concentrations from intra and extracellular samples were analysed by HPLC, as described previously (Toivari et al., 2010). Extracellular D-xylonic acid concentrations were also measured as the lactone using the hydroxymate 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}), VTT C-05705 (μ = 0.56 ± 0.02 h^{-1}) and VTT-C-75010 (μ = 0.50 ± 0.00 h^{-1}) all had maximal specific growth rates at pH 6 when grown in defined medium in Bioscreen micro titre 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. krusei*) 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.

3.2. Construction and characterization of D-xylonate producing P. kudriavzevii

P. kudriavzevii has been described as unable to utilise D-xylose, based on API-ID32C screening (Gallardo et. al., 2011). However, *P. kudriavzevii* VTT-C-75010 consumed D-xylose 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-xylose L^{-1} on YP media, which was higher than the D-xylose consumption rate of the D-xylose utilising yeast *K. lactis* (0.22 g L^{-1} h^{-1}, Nygård et al., 2011). The yield of xylitol on D-xylose was only 0.22 ± 0.01 g xylitol [g D-xylose]$^{-1}$, whereas the yield of biomass on D-xylose 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 metabolize D-xylose. The genes encoding the three enzymes needed for D-xylose utilization, D-xylose 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

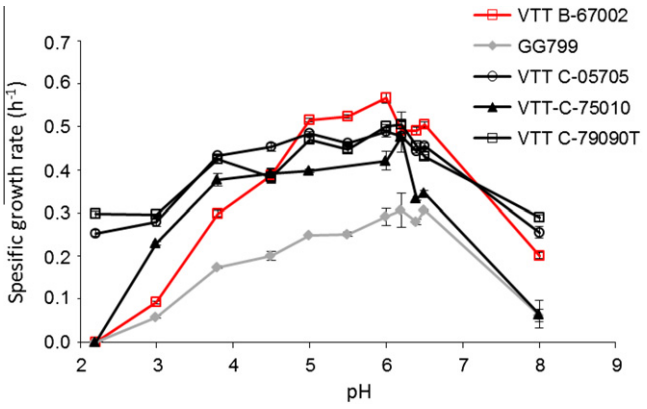

Fig. 1. Comparison of specific growth rates of *S. cerevisiae* VTT B-67002 (red square), *K. lactis* GG799 (grey diamond) and *P. kudriavzevii* VTT-C-75010 (black triangle), VTT C-79090T (open square), and VTT C-05705 (open circle) at pH 2–8 in defined medium with D-glucose as carbon source, 30 °C. Cells were grown in 300 μL microtitre wells and OD_{600} 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 CO_2 production in a bioreactor at pH 6.2. Error bars represent ± SEM (n = 4–10).

incubation (data not shown). Externally added D-xylonate (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 vitality (<5% non-vital cells in cultures with and without D-xylonate added).

Although *P. kudriavzevii* (*I. orientalis*) is increasingly being recognised as a robust production organism, e.g. for ethanol production (Dhaliwal 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-xylose dehydrogenase expression cassette was integrated into the genome, with the native *PGK* promoter driving expression of both the *MEL5* marker gene and the D-xylose dehydrogenase encoding gene *xylB*. Transformation frequencies of 1–3 colonies per μg DNA were obtained for both *P. kudriavzevii* VTT C-79090T and VTT-C-75010.

Several *xylB* transformants of VTT C-79090T and VTT-C-75010 were assessed for production of D-xylonate in medium containing D-glucose and D-xylose. No variation between transformants of the same strain was seen. VTT C-79090T *xylB* produced 50 g D-xylonate L^{-1} in 48 h when grown in unbuffered YP medium (final pH 2.96) in flasks with 20 g D-glucose L^{-1} and 50 g D-xylose L^{-1}, whereas VTT-C-75010 *xylB* produced only 42 ± 1 g D-xylonate L^{-1} (final pH 3.05) in the same conditions. Thus, the better growth of VTT C-79090T, compared with VTT-C-75010, at low pH (Fig. 1) was reflected in production of more D-xylonic acid by its transformants. A representative *xylB* transformant of VTT C-79090T was deposited in the VTT Culture Collection as strain VTT-C-12903 and used in bioreactor cultivations. D-xylose dehydrogenase activity in crude cell extract of this strain after ~20 h growth in YPD was 2.0 ± 0.2 nkat [mg protein]$^{-1}$, which was much lower than has been reported for the *S. cerevisiae* CEN.PK *xylB* transformant (45 nkat [mg protein]$^{-1}$, Toivari et al., 2012b), which may reflect the difference in copy number (a single copy of the gene in *P. kudriavzevii*, multiple copies on plasmids in *S. cerevisiae*), differences in promoter strength or suitability of *S. cerevisiae* codon optimisation for expression in *P. kudriavzevii*. The activity was none-the-less adequate for good D-xylonate production.

3.3. Production of D-xylonate by P. kudriavzevii VTT-C-12903 at pH 5.5

VTT-C-12903, expressing the D-xylose dehydrogenase encoding gene *xylB*, was grown in YP medium with D-glucose and D-xylose under conditions similar to those used to produce D-xylonate with *S. cerevisiae* VTT B-67002 *xylB* (Toivari et al., 2012b), but with higher concentrations of D-xylose. D-Glucose was provided to obtain rapid biomass production during the initial stage of the cultures, as in *S. cerevisiae* cultures (Toivari et al., 2010, 2012b), and was consumed at a rate of 3.6 ± 0.02 g L^{-1} h^{-1}. Ethanol, produced from D-glucose, was consumed at 0.34 ± 0.02 g L^{-1} h^{-1}. In some cultures additional D-glucose was provided after 28.4 h, since addition of ethanol or D-glucose helped sustain D-xylonate production in *S. cerevisiae* (Toivari et al., 2010, 2012b). Cells grown at pH 5.5, produced 88 ± 0.4 g D-xylonate L^{-1} from 98 ± 1 g D-xylose L^{-1}, at an initial rate of 1.4 ± 0.02 g L^{-1} h^{-1} (Table 1). Up to 8.2 ± 0.6 g xylitol L^{-1} were produced, but xylitol was consumed after 74 h at a rate ⩾0.36 ± 0.02 g L^{-1} h^{-1} when D-xylose was no longer present in the supernatant. Further cultures were provided a total of 171 ± 5 g D-xylose L^{-1}, in pulses approximately every 24 h (Fig. 2), and 171 ± 5 g D-xylonate L^{-1} were produced (Table 1). Only 17.1 ± 1.5 g xylitol L^{-1} were produced, which was being consumed after 146 h (Fig. 2) Production of D-xylonate at the rate of 1.4 ± 0.03 g L^{-1} h^{-1} was sustained for the first 100 h, after which the amounts of D-xylose provided limited the D-xylonate production rate and should be optimised. The initial rate was comparable to that of *Pseudomonas fragi* (1.4 g L^{-1} h^{-1}) and *Enterobacter cloacea* (1.6 g L^{-1} h^{-1}) producing similar concentrations of D-xylonate (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. cloacea*, which produced these concentrations of D-xylonate from initial D-xylose concentrations of 150–200 g L^{-1}, *P. kudriavzevii* produced only 103 ± 1 g D-xylonate L^{-1} (yield 0.48 g D-xylonate [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-xylonate 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-xylonate production, while its high stress tolerance (Kitagawa et al., 2010) makes it a good choice for D-xylonate production from complex plant biomass hydrolysates.

The approximate yield of D-xylonate 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-xylonate with *P. kudriavzevii* VTT-C-12903 and *S. cerevisiae* VTT B-67002 *xylB* at pH 5.5 or pH 3.0, with data from *P. fragi* and *E. cloacea* (pH 6.5), and *G. oxydans* (pH 3.5) for comparison. D-Glucose was used as a co-substrate.

Species	pH	D-Xylose provided (g L^{-1})	Co-substrate provided (g L^{-1})	D-Xylonate (g L^{-1})	$Yield_{P/S}$ (approx., g g^{-1})	Volumetric production rate (initial) (g L^{-1} h^{-1})	Biomass (g L^{-1})	Time (h)	Process
P. kudriavzevii VTT-C-12903	5.5	96 ± 1	12.5	88 ± 1	0.9	1.4 ± 0.1	12.1 ± 0.2	97	Fed-batch [c]
P. kudriavzevii VTT-C-12903	5.5	99 ± 1	20.5	87 ± 0	0.9	1.4 ± 0.0	15.3 ± 0.2	97	Fed-batch [c]
P. kudriavzevii VTT-C-12903	5.5	171 ± 5	24.7	171 ± 5	1.0	1.4 ± 0.0	12.6 ± 0.3	170	Fed-batch [c]
S. cerevisiae VTT B-67002 *xylB* [a]	5.5	49	12	43	0.8	0.44	7	120	Fed-batch [c]
P. fragi [b]	6.5	150	0	162	1.1	1.4	6.9	120	Batch
E. cloacea [b]	6.5	200	<2	190	1.0	1.6	nd	120	Batch
P. kudriavzevii VTT-C-12903	3.0	153 ± 1	24.1	146 ± 5	1.0	1.2 ± 0.0	10.7 ± 0.1	170	Fed-batch [c]
P. kudriavzevii VTT-C-12903	3.0	55 ± 1	12.5	57 ± 0	1.0	1.2 ± 0.0	9.1 ± 0.1	74	Batch
S. cerevisiae VTT B-67002 xylB	3.0	60	12.6	13 ± 0.3	0.7	0.2 ± 0.0	5.0 ± 0.2	117	Fed-batch [c]
G. oxydans (ATCC621) [b]	3.5	40	2.3	41	1.0	1.0	0.2	52	Batch

nd, no data.
[a] Toivari et al. (2012b).
[b] See Toivari et al. (2012a).
[c] With discontinuous addition of substrate, see Figs. 2 and 4.

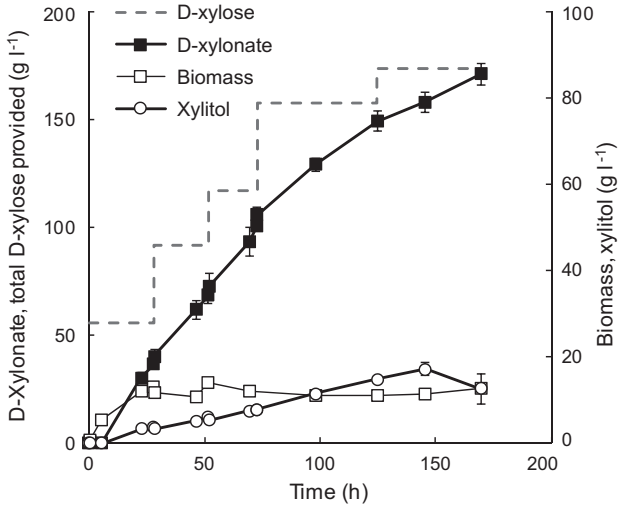

Fig. 2. D-Xylonate (solid square), xylitol (open circle) and biomass (open square) produced by *P. kudriavzevii* VTT-C-12903 at pH 5.5 in cultures provided a total 171 g D-xylose in pulses at ~24 h intervals (indicated by grey line). YP medium initially contained 15.4 g L^{-1} D-glucose and 54.0 g D-xylose 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.

L^{-1} (Table 1). Biomass, as well as D-xylonate and xylitol (yield prior to consumption 0.09 ± 0.01 mol mol^{-1}), was produced from D-xylose mainly during the first 50 h cultivation, resulting in lower yields of D-xylonate on D-xylose for shorter cultures. Yield was not affected ($p > 0.05$, Table 1) by the amount of D-glucose added and D-glucose is probably not needed as co-substrate, other than for the initial rapid production of biomass. Further, production of 171 g D-xylonate L^{-1} did not require the consumption of more D-xylose as an energy source than production of 88 g D-xylonate L^{-1}, probably because ATP was provided by the regeneration of NAD^+ through the respiratory chain.

Cell viability and/or vitality were monitored at intervals throughout the cultivations and was similar in all cultures with pulsed D-xylose feed (Fig. 3). Cell vitality was strongly correlated (R^2 0.86 to 0.96) with the extracellular D-xylonate concentration (Fig. 3b). Even after 146 h (extracellular D-xylonate = 158 ± 5 g L^{-1}), 61 ± 2% of the population remained metabolically active (52 ± 2% viable; Fig. 3a) in the cultures provided 171 g D-xylose L^{-1}. In contrast, only 23% of *S. cerevisiae* VTT B-67002 *xylB* 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-xylonate 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-xylonate could be produced if more D-xylose were provided.

In *G. oxydans*, *P. fragi* and other natural D-xylonate producers, D-xylonate 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-xylonate may be more toxic than the linear form (Buchert and Viikari, 1988; Meijnen et al., 2009). In D-xylonate producing yeast, D-xylonate 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-xylonate concentration was high (115–143 mg D-xylonate [g biomass]$^{-1}$) within 6–9 h of providing D-xylose to the culture (Fig. 4), even though the extracellular D-xylonate concentration was very low (0.1–7 g L^{-1}). High initial D-xylonate 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-xylonate 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-xylonate concentrations in *P. kudriavzevii* were much lower than those observed in *S. cerevisiae* VTT B-67002 *xylB* (~170 mg [g biomass]$^{-1}$, Toivari et al., 2012b). *P. kudriavzevii* was exporting D-xylonate (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-Xylonate is not naturally produced by these yeast and nothing is known about its export from the cells, but it is interesting to note that the yeast which can naturally

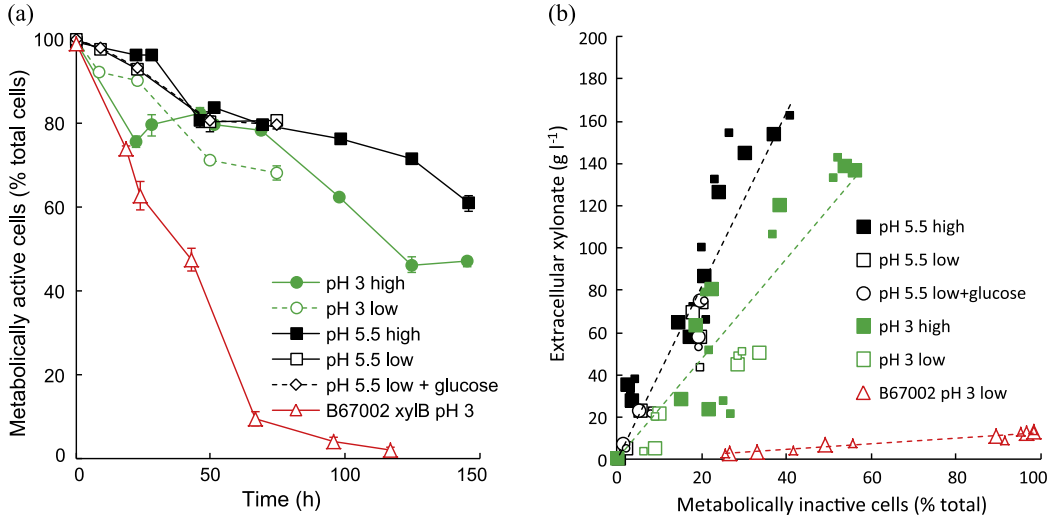

Fig. 3. (a) Metabolically active cells (as % total cells) in cultures of *P. kudriavzevii* VTT-C-12903 at pH 5.5 (squares, diamond) or 3.0 (circles) and *S. cerevisiae* VTT B-67002 *xylB* at pH 3.0 (triangle) in YP medium. (b) The relationship between the amount of extracellular D-xylonate in the culture 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 *xylB* 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 5), 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 *xylB* received 60.5 g D-xylose L^{-1} and 12.6 g D-glucose L^{-1}.

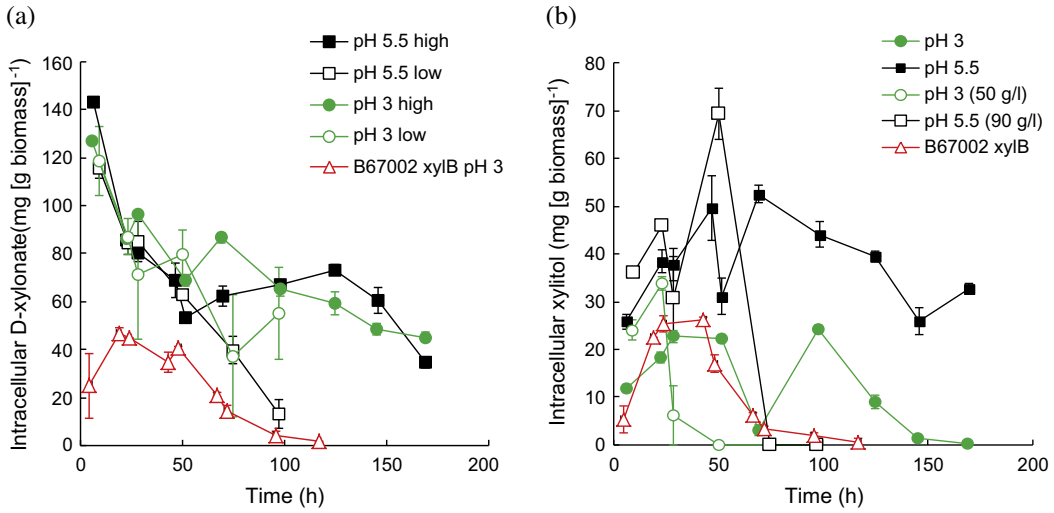

Fig. 4. Intracellular (a) D-xylonate and (b) xylitol in VTT-C-12903 at pH 5.5 (squares) or pH 3.0 (circles) and VTT B-67002 *xylB* at pH 3.0 (triangles) when provided with high (171 g L^{-1}) or low (98 g L^{-1} for VTT-C-12903; 55 g L^{-1} for VTT B-67002 *xylB*) amounts of D-xylose in YP medium with D-glucose as co-substrate, as described in the text. Error bars represent SEM.

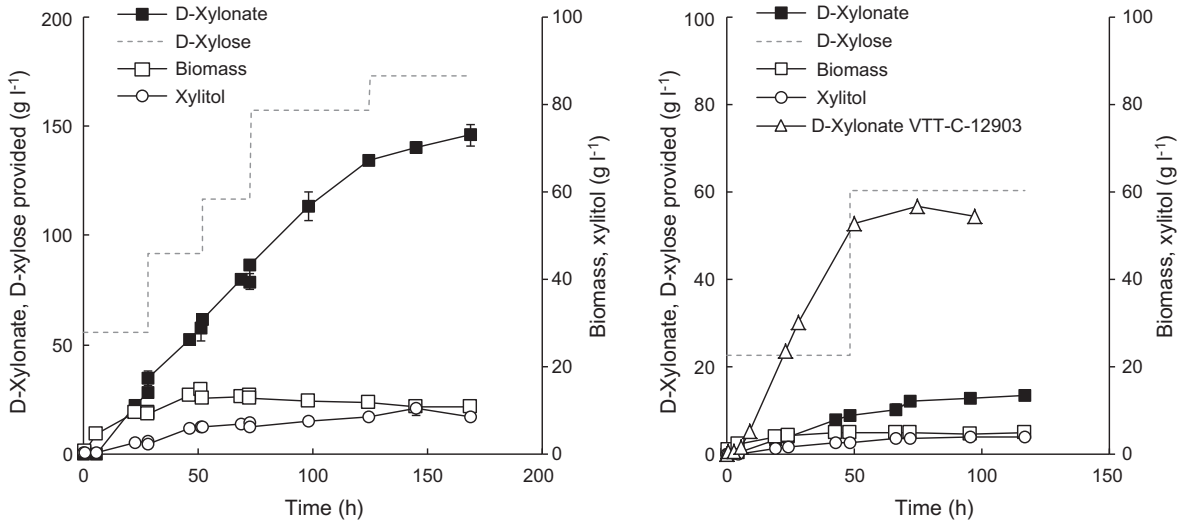

Fig. 5. D-Xylonate, biomass and xylitol production by *P. kudriavzevii* VTT C-12903 (left) and *S. cerevisiae* VTT B-67002 *xylB* (right)at pH 3.0 in YP medium from 60 (VTT B-67002 *xylB*) or 153 (VTT-C-12903) g D-xylose L^{-1}, provided in pulses as shown (dashed lines). VTT-C-12903 cultures initially contained 15 g L^{-1} D-glucose and 53.2 ± 0.8 g D-xylose L^{-1} and received 9.1 g D-glucose L^{-1} at 28.4 h. VTT B-67002 *xylB* cultures initially contained 8.2 g D-glucose L^{-1} and 22.7 g D-xylose L^{-1} and were provided 4.4 g D-glucose L^{-1} and 37.7 g D-xylose L^{-1} at 48 h. Data for D-xylonate production from VTT-C-12903 in batch culture (56 g D-xylose L^{-1} with 12.5 g D-glucose L^{-1}) are shown with the VTT B-67002 *xylB* data for comparison. Error bars represent SEM for two cultures and where not visible are smaller than the size of the symbol.

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 1.2 ± 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 (R^2 = 0.89, Fig. 3b). Intracellular accumulation of D-xylonate 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

(4 g L^{-1}) 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 *xylB* was negatively correlated to the extracellular D-xylonic acid concentration (R^2 = 0.94, Fig. 3b). D-Xylonate 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-xylonate (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-xylonate concentration in VTT-C-12903 at pH 3, indicating that cell lysis was not primarily responsible.

Production of D-xylonate at pH 3.0 has previously been reported for *S. cerevisiae* CEN.PK *xylB* expressing strains, but these produced only 5–7 g D-xylonate 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-xylonic acid could not be produced by recombinant *E. coli* if the medium pH was below 4.0. *P. fragi* did not produce D-xylonate at pH values below 5 (Buchert et al., 1986). *G. oxydans* can produce D-xylonate 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-xylonic acid at pH 3.0 than any other reported strain producing D-xylonic acid, 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, gluconic, 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 yeast 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-xylonate (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-xylonate 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-xylonate 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-xylonate in hemicellulose 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-xylonic acid. By introducing a D-xylose dehydrogenase gene we produced 171 g D-xylonate L^{-1} at pH 5.5 and 146 g D-xylonic acid L^{-1} at pH 3.0, at rates of 1.2–1.4 g L^{-1} h^{-1}. VTT-C-12903 produces D-xylonic acid as well or better than natural D-xylonate 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-xylonate production.

Acknowledgements

This work has been financially supported by the Academy of Finland through the Centre of Excellence in White Biotechnology – Green Chemistry (Grant 118573) and the European Commission through the Seventh Framework Programme (FP7/2007-2013) under Grant agreement N° FP7-241566 BIOCORE. Financial support from the VTT Graduate School is acknowledged (Yvonne Nygård).

References

Bozell, J.J., Petersen, G.R., 2010. Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energy's "Top 10" revisited. Green Chem. 12, 539–554.
Buchert, J., Viikari, L., 1988. Oxidative D-xylose metabolism of *Gluconobacter oxydans*. Appl. Microbiol. Biotechnol. 29, 375–379.
Buchert, J., Viikari, L., Linko, M., Markkanen, P., 1986. Production of xylonic acid by *Pseudomonas fragi*. Biotechnol. Lett. 8, 541–546.
Chan, G.F., Gan, H.M., Ling, H.L., Aini, N., Rashid, A., 2012. Genome sequence of *Pichia kudriavzevii* M12, a potential producer of bioethanol and phytase. Eukaryot. Cell 2012, 1300–1301.
Chun, B.W., Dair, B., Macuch, P.J., Wiebe, D., Porteneuve, C., Jeknavorian, A., 2006. The development of cement and concrete additive: based on xylonic acid derived via bioconversion of xylose. Appl. Biochem. Biotechnol. 129–132, 645–658.
Dhaliwal, S.S., Oberoi, H.S., Sandhu, S.K., Nanda, D., Kumar, D., Uppal, S.K., 2011. Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. Bioresour. Technol. 102, 5968–5975.
Erickson, B., Nelson, J.E., Winters, P., 2012. Perspective on opportunities in industrial biotechnology in renewable chemicals. Biotechnol. J. 7, 176–185.
Gallardo, J.C., Souza, C.S., Cicarelli, R.M., Oliveira, K.F., Morais, M.R., Laluce, C., 2011. Enrichment of a continuous culture of *Saccharomyces cerevisiae* with the yeast *Issatchenkia orientalis* in the production of ethanol at increasing temperatures. J. Ind. Microbiol. Biotechnol. 38, 405–414.
Gietz St., D., Jean, A., Woods, R.A., Schiestl, R.H., 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20, 1425.
Halm, M., Hornbäk, T., Arneborg, N., Sefa-Dedeh, S., Jespersen, L., 2004. Lactic acid tolerance determined by measurement of intracellular pH of single cells of *Candida krusei* and *Saccharomyces cerevisiae* isolated from fermented maize dough. Int. J. Food Microbiol. 94, 97–103.
Isono, N., Hayakawa, H., Usami, A., Mishima, T., Hisamatsu, M., 2012. A comparative study of ethanol production by *Issatchenkia orientalis* strains under stress conditions. J. Biosci. Bioeng. 113, 76–78.
Kitagawa, T., Tokuhiro, K., Sugiyama, H., Kohda, K., Isono, N., Hisamatsu, M., Takahashi, H., Imaeda, T., 2010. Construction of a beta-glucosidase expression system using the multistress-tolerant yeast *Issatchenkia orientalis*. Appl. Microbiol. Biotechnol. 87, 1841–1853.
Kurtzman, C.P., Robnett, C.J., Basehoar-Powers, E., 2008. Phylogenetic relationships among species of *Pichia*, *Issatchenkia* and *Williopsis* determined from multigene sequence analysis, and the proposal of *Barnettozyma* gen.nov., *Lindnera* gen.nov. and *Wickerhamomyces* gen.nov. FEMS Yeast Res. 8, 939–954.
Kwon, Y.J., Ma, A.Z., Li, Q., Wang, F., Zhuang, G.Q., Liu, C.Z., 2011. Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant *Issatchenkia orientalis*. Bioresour. Technol. 102, 8099–8104.
Lee, J.W., Kim, H.U., Choi, S., Yi, J., Lee, S.Y., 2011. Microbial production of building block chemicals and polymers. Curr. Opin. Biotechnol. 22, 758–767.

Liu, H., Valdehuesa, K.N., Nisola, G.M., Ramos, K.R., Chung, W.J., 2012a. High yield production of D-xylonic acid from D-xylose using engineered *Escherichia coli*. Bioresour. Technol. 115, 244–248.

Liu, H., Ramos, K.R., Valdehuesa, K.N., Nisola, G.M., Lee, W.K., Chung, W.J., 2012b. Biosynthesis of ethylene glycol in *Escherichia coli*. Appl. Microbiol. Biotechnol.. http://dx.doi.org/10.1007/s00253-012-4618-7.

Magnuson, J.K., Lasure, L.L., 2004. Organic Acid Production by Filamentous Fungi. In: Tracz, J.S., Lange, L. (Eds.), Advances in Fungal Biotechnology for Industry, Agriculture and Medicine. Kluwer/Plenum, New York, USA, pp. 307–340.

Meijnen, J.P., de Winde, J.H., Ruijssenaars, H.J., 2009. Establishment of oxidative D-xylose metabolism in *Pseudomonas putida S12*. Appl. Environ. Microbiol. 75, 2784–2791.

Mira, N.P., Teixeira, M.C., Sa-Correia, I., 2010. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. OMICS 14, 525–540.

Mojzita, D., Wiebe, M., Hilditch, S., Boer, H., Penttilä, M., Richard, P., 2010. Metabolic engineering of fungal strains for conversion of D-galacturonate to meso-galactarate. Appl. Environ. Microbiol. 76, 169–175.

Niu, W., Molefe, M.N., Frost, J.W., 2003. Microbial synthesis of the energetic material precursor 1,2,4-butanetriol. J. Am. Chem. Soc. 125, 12998–12999.

Nygård, Y., Toivari, M.H., Penttilä, M., Ruohonen, L., Wiebe, M.G., 2011. Bioconversion of D-xylose to D-xylonate with *Kluyveromyces lactis*. Metab. Eng. 13, 383–391.

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 2008. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 26, 100–108.

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 2010. 16 Years research on lactic acid production with yeast – ready for the market? Biotechnol. Genet. Eng. Rev. 27, 229–256.

Suominen, P., Aristidou, A., Penttilä, M., Ilmen, M., Ruohonen, L., Koivuranta, K., Roberg-Perez, K., et al., 2009. Genetically modified yeast of the species *Issatchenkia orientalis* and closely relates species, and fermentation processes using same, US 2009/0226989 A1.

Toivari, M.H., Ruohonen, L., Richard, P., Penttilä, M., Wiebe, M.G., 2010. *Saccharomyces cerevisiae*engineered to produce D-xylonate. Appl. Microbiol. Biotechnol. 88, 751–760.

Toivari, M.H., Nygård, Y., Penttilä, M., Ruohonen, L., Wiebe, M.G., 2012a. Microbial D-xylonate production. Appl. Microbiol. Biotechnol. 96, 1–8.

Toivari, M., Nygård, Y., Kumpula, E.P., Vehkomäki, M.L., Bencina, M., Valkonen, M., Maaheimo, H., Andberg, M., Koivula, A., Ruohonen, L., Penttilä, M., Wiebe, M.G., 2012b. Metabolic engineering of *Saccharomyces cerevisiae* for bioconversion of D-xylose to D-xylonate. Metab. Eng. 14, 427–436.

Valli, M., Sauer, M., Branduardi, P., Borth, N., Porro, D., Mattanovich, D., 2006. Improvement of lactic acid production in *Saccharomyces cerevisiae* by cell sorting for high intracellular pH. Appl. Environ. Microbiol. 72, 5492–5499.

Van Vleet, J.H., Jeffries, T.W., 2009. Yeast metabolic engineering for hemicellulosic ethanol production. Curr. Opin. Biotechnol. 20, 300–306.

Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501–517.

Warnecke, T., Gill, R.T., 2005. Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. Microb. Cell Fact. 4, 25.

Werpy, T., Petersen, G., 2004. Top value added chemicals from biomass: Volume I – Results of screening for potential candidates from sugars and synthesis gas (U.S. Department of Energy, Oak Ridge, TN, August 2004; available at www.eere.energy.gov/biomass/pdfs/35523.pdf).

Yuzbashev, T.V., Yuzbasheva, E.Y., Sobolevskaya, T.I., Laptev, I.A., Vybornaya, T.V., Larina, A.S., Matsui, K., Fukui, K., Sineoky, S.P., 2010. Production of succinic acid at low pH by a recombinant strain of the aerobic yeast *Yarrowia lipolytica*. Biotechnol. Bioeng. 107, 673–682.