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Low pH D-xylonate production with *Pichia kudriavzevii*

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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.

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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.

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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 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).

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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

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