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Metabolic engineering of *Saccharomyces cerevisiae* for bioconversion of D-xylose to D-xylonate

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ABSTRACT

An NAD^+-dependent D-xylose dehydrogenase, XylB, from *Caulobacter crescentus* was expressed in *Saccharomyces cerevisiae*, resulting in production of 17 ± 2 g D-xylonate l^{-1} at 0.23 g l^{-1} h^{-1} from 23 g D-xylose l^{-1} (with glucose and ethanol as co-substrates). D-Xylonate titre and production rate were increased and xylitol production decreased, compared to strains expressing genes encoding *T. reesei* or pig liver $NADP^+$-dependent D-xylose dehydrogenases. D-Xylonate accumulated intracellularly to ~70 mg g^{-1}; xylitol to ~18 mg g^{-1}. The aldose reductase encoding gene *GRE3* was deleted to reduce xylitol production. Cells expressing D-xylonolactone lactonase *xylC* from *C. crescentus* with *xylB* initially produced more extracellular D-xylonate than cells lacking *xylC* at both pH 5.5 and pH 3, and sustained higher production at pH 3. Cell vitality and viability decreased during D-xylonate production at pH 3.0. An industrial *S. cerevisiae* strain expressing *xylB* efficiently produced 43 g D-xylonate l^{-1} from 49 g D-xylose l^{-1}.

1. Introduction

D-Xylose is an abundant pentose sugar present in lignocellulosic plant material, which is currently considered primarily as a potential feed-stock for ethanol or xylitol production (Akinterinwa and Cirino, 2009; Nair and Zhao, 2010; Skorupa Parachin et al., 2011). However, its oxidation product D-xylonic acid or its conjugate base D-xylonate has potential applications as chelator, dispersant, clarifying agent, antibiotic, health enhancer, polyamide or hydrogel modifier or 1,2,4-butanetriol precursor (Millner et al., 1994; Chun et al., 2006; Markham, 1991; Tomoda et al., 2004; Pujos, 2006; Niu et al., 2003; Zamora et al., 2000). D-Xylonate could also serve as a non-food derived replacement of D-gluconic acid.

Microbial production of D-xylonate with bacteria e.g., *Pseudomonas sp.* or *Gluconobacter oxydans* has been well described (Buchert et al., 1986, 1988; Buchert, 1990). High D-xylonate yields and relatively high production rates from D-xylose are obtainable with bacteria, but when birch wood hydrolyzates were used as substrate, the conversion of D-xylose to D-xylonate decreased (Buchert et al., 1989, 1990). *Gluconobacter* species have periplasmic, membrane bound PQQ-dependent and intracellular $NAD(P)^+$-dependent dehydrogenases which oxidise D-xylose to D-xylonate. These enzymes are responsible for the oxidation of a variety of sugars and sugar alcohols, and the lack of specificity results in a mixture of acids when complex substrates such as lignocellulosic hydrolysate are provided (Buchert, 1991; Rauch et al., 2010; Hölscher et al., 2009). Resently, an *Escherichia coli* strain was engineered to produce D-xylonate from D-xylose (Liu et al., in press). However, for an industrial production process, an inhibitor tolerant organism such as the yeast *Saccharomyces cerevisiae* expressing a D-xylose specific D-xylose dehydrogenase would be advantageous. We recently described D-xylonate production with *S. cerevisiae* (Toivari et al., 2010) and *Kluyveromyces lactis* (Nygård et al., 2011) using D-xylose preferring D-xylose dehydrogenase from *T. reesei* (Berghäll et al., 2007). The activity of the *T. reesei* D-xylose dehydrogenase was relatively low in both hosts, even though the encoding gene was expressed in multiple copies. In addition, this enzyme is $NADP^+$-dependent, and reoxidation of the NADPH formed is not necessarily efficient. A D-xylose dehydrogenase with high activity, using NAD^+ as a cofactor, may provide improved D-xylonate production.

A NAD^+-dependent D-xylose dehydrogenase has been described in the oxidative catabolic D-xylose pathway (Dahms, 1974; Weimberg, 1961) of the fresh water bacterium *Caulobacter*

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crescentus (Stephens et al., 2007). In addition, NAD^+-dependent D-xylose dehydrogenase activity has been observed in *Trichoderma viridea* (Kanauchi and Bamforth, 2003) and an NAD^+-dependent D-xylose dehydrogenase was purified from *Arthrobacter sp.* (Yamanaka et al., 1977), but no gene has been annotated to these activities. Other enzymes, e.g., from mammalian tissues (dimeric dihydrodiol dehydrogenase e.g., from pig liver) (Zepeda et al., 1990; Aoki et al., 2001), Archaea *Haloarcula marismortui* (Johnsen and Schönheit, 2004), *Haloferax volcanii* (Johnsen et al., 2009), and *Pichia querquum* (Suzuki and Onishi, 1973), with D-xylose dehydrogenase activity use $NADP^+$ as cofactor. Overall, relatively little is known about the substrate specificities and kinetic properties of D-xylose dehydrogenases.

D-Xylose dehydrogenases convert D-xylose to D-xylonolactone, which is subsequently hydrolyzed either spontaneously or by a lactonase enzyme to yield D-xylonate. Only a few lactonases have been described and these have not been characterised. Spontaneous hydrolysis is relatively slow, and accumulation of D-xylonolactone inhibits growth of both *Pseudomonas fragi* (Buchert and Viikari, 1988) and recombinant *Pseudomonas putida* S12 (Meijnen et al., 2009), indicating an important role for the lactonase. In non-engineered bacteria, D-xylonolactone hydrolyzing lactonase enhances D-xylonate production, but its role in yeast has not been defined.

Here we describe the production of D-xylonate by *S. cerevisiae* using the NAD^+-dependent D-xylose dehydrogenase XylB from *C. crescentus*, and compare it with D-xylonate production using the *T. reesei* Xyd1 and another $NADP^+$-dependent enzyme, *SUS2DD* from pig liver. The recombinant XylB enzyme was purified to determine its substrate specificity and kinetic properties when expressed in yeast. This D-xylose dehydrogenase was also expressed in a strain deficient in the aldose reductase Gre3p for reduced xylitol production and in an industrial *S. cerevisiae* strain for high D-xylonate production. A strain expressing the D-xylonolactone lactonase encoding gene *xylC* of *C. crescentus* together with the D-xylose dehydrogenase encoding gene *xylB* was studied at pH 3 and pH 5. Physiological effects, such as intracellular pH, cell vitality and viability, of D-xylonolactone and D-xylonate production in *S. cerevisiae* were also assessed.

2. Materials and Methods

2.1. Strains and strain construction

Saccharomyces cerevisiae CEN.PK 113-17A (H2802; MATα, *ura3-52 HIS3 leu2-3/112 TRP1 MAL2-8^c SUC2*; Entian and Kötter, 1998) and B67002 (VTT Culture Collection) were used as parental strains. *S. cerevisiae* strain FY834 (Winston et al., 1995) was used for recombination cloning. The strains used in the study are listed in Table 1.

The D-xylose dehydrogenase encoding genes from *C. crescentus* (*xylB*, Stephens et al., 2007, CC_0821, Gene ID: 941308, NCBI) and pig liver (*SUS2DD*, Gene ID: 397337, NCBI) were obtained as synthetic genes, codon optimized for *S. cerevisiae* (Gene Art, Germany). The genes were ligated into the *Bgl*II site between the *PGK1* promoter and terminator of B1181 (derived from the multicopy plasmid YEplac195+*PGK1*PT containing *URA3*; Toivari et al., 2010), generating plasmids B3441 and B3443, respectively. Plasmids were introduced to *Saccharomyces cerevisiae* CEN.PK 113-17A strain H2802 to generate strains H3698 and H3700, respectively (Table 1). A control strain was created by introducing plasmid B1181 to *S. cerevisiae* CEN.PK 113-17A (H2802) to generate strain H3680 (Table 1). Plasmid B3441 was also introduced into the Gre3p-deficient strain H3613 (Toivari et al., 2010), resulting in strain H3722.

The D-xylonolactone lactonase encoding gene *xylC* ((Stephens et al., 2007) CC_0820, Gene ID: 941305, NCBI) was obtained as a synthetic gene, codon optimized for *S. cerevisiae* (Gene Art, Germany). The *xylC* gene was cloned using recombination. The gene was amplified with oligonucleotides 5′-TGCTTAAATCTA-TAACTACAAAAAACACATACAGGAATTCACAATGACTGCTCAAGTTAC-3′ and 5′-CTTATTCAGTTAGCTAGCTGAGCTCGACTCTAGAGGATCCC-AGATCTTTAAACCAATC-3′, and introduced into strain FY834 together with an *Eco*RI and *Bam*HI linearised plasmid B2158, modified from pYX242 (R&D systems, UK) as previously described for B2159 (Toivari et al., 2010). The resulting plasmid was named B3574. The lactonase containing plasmid was introduced into strain H2802 along with the *xylB* gene (on B3441) to create strain H3938 (Table 1).

The *xylB* expression cassette *pPGK-xylB-tPGK* was released as a *Hind*III-fragment from plasmid B3441 and the ends were made blunt with T4-polymerase. The fragment was ligated into bacterial plasmids pMLV23 and pMLV39, which had been cut with *Bam*HI and the ends modified to blunt ends. Plasmids pMLV23 and pMLV39 contain *loxP- S. cerevisiae MEL5* (α-*galactosidase*)-*loxP* and *loxP- pTEF*(*A. gossypii*)- *kan^r–tTEF*(*A. gossypii*) -*loxP* marker cassettes, respectively, with 60 bp flanking regions for targeting to the *GRE3* locus in *S. cerevisiae*. The *GRE3* flanking regions were from nucleotide −250 to −193 and from nucleotide 981 to +1040, where numbers are relative to nucleotide A in the *GRE3* ATG start codon. The *Bam*HI cloning site was included in one of the *GRE3* flanking sequences. The resulting plasmids, containing *xylB*, were named pMLV81B and pMLV82C.

Table 1
Strains of *S. cerevisiae* used or referred to in this study. Plasmids are described in the text.

Strain	H-number	Genotype or parent strain+plasmid	Dehydrogenase gene, source of gene
CEN.PK113-17A	H2802	MATα, *ura3-52 HIS3 leu2-3/112 TRP1 MAL2-8^c SUC2*	
Control	H3680	H2802+B1181	
xylB	H3698	H2802+B3441	*xylB, C. crescentus*
SUS2DD	H3700	H2802+B3443	*SUS2DD,* pig liver
xyd1[a]	H3725	H2802+B2871	*xyd1, T. reesei*
Gre3p deficient[a]	H3613	MATα, *ura3-52 HIS3 leu2-3/112 TRP1 MAL2-8^c SUC2 gre3Δ::kanMX*	
Gre3p deficient	H3720	H3613+B1181	
Gre3p deficient		H3613+B3441	*xylB, C. crescentus*
xylB	H3722		
xylB xylC	H3938	H2802+B3441+B3574	*xylB, C. crescentus*
pHluorin control	H3909	H3720+pMV118	
pHluorin *xylB*	H3910	H3722+pMV118	*xylB, C. crescentus*
N-Strep-tag *xylB*	H3779	H2802+B3694	*xylB, C. crescentus*
B67002 control		Isolated from spent sulfite liquor	
B67002 *xylB*	H3935	Two copies of *xylB*	*xylB, C. crescentus*

[a] Described in Toivari et al. (2010).

The expression cassette for XylB, together with the *loxP-kan*^{r}-*loxP* marker, was released from pMLV82C with *Not*I, introduced into B67002 cells by transformation and G418 resistant yeast colonies were collected. A second copy of *xylB* was introduced into the yeast chromosome of the B67002/pMLV82C transformant with the XylB expression cassette and *loxP-MEL5-loxP* marker, released from pMLV81B with *Not*I. Blue coloured, *MEL5* (α-galactosidase) expressing, G418 resistant yeast colonies were collected from agar-solidified YP containing 2% w/v D-galactose, supplemented with 5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside (*X-α-Gal*, 40 μg/ml) and G418 (200 μg/ml). To remove the marker cassettes from the yeast chromosome, the double transformant was retransformed with plasmid pKlNatCre (Steensma and Ter Linde, 2001), expressing the Cre recombinase. Integration of the expression cassette *pPGK-xylB-tPGK* into two *GRE3* loci in the B67002 genome was verified with PCR. The strain generated was named H3935.

Plasmid pYES2-PACT1-pHluorin (Orij et al., 2009), containing the pH responsive pHluorin gene (Miesenböck et al., 1998), was a kind gift from Dr. Smits (University of Amsterdam, The Netherlands). The pHluorin gene was cloned into vector B2158 with the constitutive *TPI1* promoter and terminator, resulting in plasmid pMV118. pMV118 was introduced into the Gre3p-deficient strain H3722, which also contains the *C. crescentus* xylose dehydrogenase *xylB*, resulting in strain H3910 (Table 1) and into the Gre3p-deficient control strain H3720, resulting in strain H3909 (Table 1).

For protein characterisation work, the *C. crescentus xylB* was expressed as an N-terminally Strep-tagged construct with an additional tobacco etch virus (TEV) cleavage site downstream of the Strep-tag. The Strep-tag was added by amplifying the *xylB* gene from plasmid B3441 by PCR using oligonucleotides 5′-GGTTCAGATCTACAATGTGGTCTCATCCACAATTCGAGAAGGAGA-ATTTGTATTTCCAATCATCTGCAATTTACCC-3′ and 5′-GGTTCAGATC-TTCATCTCCAACCAGC-3′. *Bgl*II sites were introduced to the 5′ and 3′ ends, respectively. Amplified fragments were digested with *Bgl*II and ligated into the *Bgl*II site of vector B1181, resulting in plasmid B3694. Plasmid B3694 was introduced to H2802 to generate strain H3779 (Table 1).

2.2. Media and culture conditions

Yeast-Peptone (YP) medium contained 10 g yeast extract l^{-1} and 20 g bacto-peptone l^{-1}, with D-glucose and D-xylose concentrations as indicated in the text. YP medium with 20 g D-glucose l^{-1} (YPD) was sometimes solidified with addition of 15 g agar l^{-1}.

For small scale cultures, yeast were cultured in 20 or 50 ml YP or modified synthetic complete medium (YSC, (Sherman et al., 1983)) in 100 or 250 ml Erlenmeyer flasks, respectively, at 250 rpm, 30 °C. Concentrations of added carbon source (D-glucose, D-xylose or ethanol) are indicated in the text. For larger scale cultures, yeast were grown in 500 ml medium (YP, YSC-ura, or YSC-ura-leu) in Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland) at pH 5.5 (unless otherwise indicated), 30 °C, 1 volume air [volume culture]$^{-1}$ min^{-1} (vvm) and 500 rpm agitation with two marine impellors, as previously described (Toivari et al., 2010) or in 2 l YP medium in B. Braun Biotech International (Sartorius AG, Germany) Biostat® CT (2.5 l working volume) bioreactors at pH 5.5, 30 °C, 500 rpm and 0.5 vvm aeration. 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}) was added to prevent foam formation.

2.3. Measurement of biomass

Biomass was measured as optical density (OD) at 600 nm (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.

2.4. Chemical analyses

For determination of intracellular D-xylonate, D-xylose and xylitol, cells were collected from 10 ml culture. Cell pellets were washed with 1.8 ml deionised H_2O or NaCl (9 g l^{-1}), followed with 1.8 ml deionised water and frozen at −20 °C to disrupt membranes. The frozen pellets were freeze-dried using a Christ Alpha 2–4 lyophiliser (Biotech international, Belgium), removing all excess moisture. Intracellular D-xylonate, D-xylose and xylitol were extracted from the lyophilized pellets (~60 mg biomass ml^{-1}) in 5 mM H_2SO_4, as described by Nygård et al. (2011). Cell debris was removed by centrifugation and the supernatant analysed by HPLC. Intracellular concentrations are given as mg per g dry biomass, but a conservative estimate of intracellular concentrations can be derived by assuming that 1 g dry cell weight corresponds to 2 ml cell volume (Gancedo and Serrano, 1989; de Koning and van Dam, 1992). This estimate is conservative since it does not take into account the volume of intracellular organelles, variation in cell wall thickness, or the contribution of dead cells to the dry biomass.

Extracellular and intracellular compounds (D-xylonic acid and/or D-xylonolactone, ethanol, glycerol, pyruvate and acetate, D-glucose and D-xylose) were analysed by HPLC using a Fast Acid Analysis Column (100 mm × 7.8 mm, BioRad Laboratories, Hercules, CA) linked to an Aminex HPX-87 H column (BioRad Labs, USA) with 2.5 mM H_2SO_4 as eluent and a flow rate of 0.5 ml min^{-1}. 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. When D-xylonic acid was present, D-xylose concentrations were estimated by subtraction of the D-xylonic acid (detected by UV) from the combined D-xylose and D-xylonic acid/D-xylonolactone peak detected by RI.

Extracellular D-xylonic acid concentrations were also measured as the lactone using the hydroxymate method (Lien, 1959) as described by Toivari et al. (2010).

2.5. 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 NaCitrate buffer pH 8.3 or in 0.1 M phosphate buffer pH 4.6) staining. Viability was determined by comparing the number of viable colony forming units (CFU) on YPD to the total cell number determined microscopically or to the number of viable CFU of a control strain.

2.6. Enzyme purification

The *S. cerevisiae* strain expressing the Strep-tagged XylB (Strep-TEV-XylB) was grown in YCS-ura for 16 h and the cells were harvested by centrifugation at 3000 rpm (1600 × *g*) for 10 min, +4 °C. The cells were washed with Milli-Q water and disrupted with acid-washed glass beads (Ø 425–600 μm, Sigma–Aldrich Corporation, USA) in 50 mM sodium phosphate buffer pH 8.0, containing protease inhibitor (cOmplete Mini EDTA-free, Roche). The cell lysate was centrifuged at 4000 × *g* for 30 min at +4 °C. Crude protein extract was loaded on an affinity column (Strep-Tactin Superflow Plus (1 ml, QIAGEN) and the protein purified using an ÄKTAPurifier FPLC system (GE Healthcare). The affinity column was equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8 and washed with 10 column volumes

after sample loading. The same buffer containing 2.5 mM of desthiobiotin (Sigma) was used to elute the protein from the column. Fractions of 800 μl were collected and analyzed by SDS-PAGE 4–20% gradient gel (Criterion SF, Bio-Rad Laboratories) and using an NADH absorbance-based activity assay (see below). Fractions containing active enzyme were pooled and concentrated to a total volume of 2.5 ml with a VIVASPIN 20 concentrator tube ((Sartorius Vivascience) with a molecular weight cutoff of 10 kDa. The resulting concentrated solution was desalted using a PD 10 desalting column (GE Healthcare, Sephadex™ G-25 M) to a final volume of 3.5 ml of 50 mM Tris-Cl buffer pH 8. All purification steps were carried out at +4 °C.

The protein concentration of the purified Strep-TEV-XylB enzyme was determined by measuring absorbance at 280 nm using a theoretical molar extinction coefficient, ε=43,680 M^{-1} cm^{-1}, which was calculated from the primary amino acid sequence.

2.7. Enzyme activity

D-xylose dehydrogenase activity from crude cell extracts was measured according to Berghäll et al. (2007) in 100 mM Tris/HCl, pH 8.1 in the presence of 2 mM $MgCl_2$ using either 1 mM NAD^+ or $NADP^+$ as cofactor and 100 mM D-xylose (L-arabinose, D-glucose, or D-ribose) as substrate. The measurements were performed at 30 °C using a Konelab Arena photometric analyzer (Thermo Electron Oy, Finland). Protein concentration from crude cell extracts was determined using a Bio-Rad protein kit, based on the assay developed by Bradford (1976).

The pH optimum of the purified Strep-tagged XylB dehydrogenase (Strep-TEV-XylB) was determined in the pH range from 2.4 to 10.1 using 10 mM D-xylose and 5 mM NAD^+ with McIlvaine (50 mM, pH 2.4–7.3), Tris-HCl (100 mM, pH 8.0–9.2) and Glycine (100 mM, pH 10.1) buffers. Substrate specificity of the purified Strep-TEV-XylB was measured in 50 mM bis-Tris propane (BTP) buffer, pH 9.0 using 1, 10 and 100 mM substrate, 5 mM NAD^+ and 19 nM enzyme. Pentose sugars D-xylose, L-arabinose, D-lyxose and D-ribose, and hexose sugars D-glucose, D-galactose and D-mannose were used as substrates. The kinetic constants (k_{cat} and K_m values) of the purified Strep-TEV-XylB on D-xylose, L-arabinose and NAD^+ were determined in 50 mM BTP buffer pH 9.0, 50 mM BTP buffer pH 7.0 and in vivo like (IVL) buffer pH 6.8, described by van Eunen et al. (2010). Ten different substrate or cofactor concentrations (0–10 mM, 0–800 mM and 0—5 mM, for D-xylose, L-arabinose and NAD^+, respectively), were used. D-Xylose dehydrogenase activity was measured at 25 °C as the formation of NADH, detected at 340 nm using a Varioskan kinetic plate reader (Thermo Electron Corporation, USA). The kinetic parameters were obtained by curve fitting analysis using GraphPad Prism software 5.03 (GraphPadPrism Software Inc., USA). The effect of $MgCl_2$ up to 10 mM on XylB dehydrogenase activity was also tested, but was found to be negligible and thus omitted.

2.8. NMR analyses

NMR analysis of culture supernatant and cell extracts for D-xylonolactone lactonase activity measurements were carried out at 22 °C on a 500 MHz Bruker Avance III. The D_2O needed for the spectrometer lock was confined in a coaxially located glass capillary, so the samples were in 100% H_2O environment. ^1H NMR spectra were recorded with a standard 1 s presaturation of the water signal.

The hydrolysis of chemically produced xylono-1,4-lactone was monitored in vitro by ^1H NMR spectroscopy. The D-xylono-1,4-lactone was obtained from pure Ca-D-xylonate by incubating it in 0.1 M HCl at 95 °C for 3 h. Approximately 65% of the D-xylonate was converted to the 1,4-lactone form under these conditions and a small amount of another product, putatively 1,5-lactone, was detected. The pH of the D-xylonic acid – lactone mixture was adjusted by adding 50 mM Na-phosphate D_2O buffers of different pH and the hydrolysis was monitored by ^1H NMR. NMR spectra were recorded at 22 °C on a 500 MHz Varian Inova NMR spectrometer with 1 s presaturation of the water signal. The pH of the solutions was measured after the analysis.

2.9. In situ pHluorin calibration and measurement

Ratiometric pHluorin was used to determine intracellular pH as described by Miesenböck et al. (1998). For calibration of the probe, yeast strains expressing pHluorin were grown in flasks in YSC-ura-leu medium with 50 g D-glucose l^{-1} to an OD_{600} of approximately 1.0. Calibration buffers contained 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 200 mM ammonium acetate, 10 mM NaN_3, 10 μM nigericin (Sigma) and the pH was adjusted with NaOH or HCl (Brett et al., 2005). For imaging, the cells were grown in flasks in YSC-ura-leu medium (pH5.5) with 10 g D-glucose l^{-1}, 20 g D-xylose l^{-1}, and 10 g $CaCO_3$ l^{-1} to buffer the cultures.

A Leica TCS SP5 laser scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Germany) with an HCX plan apo 63 × (NA 1.4) oil immersion objectives was used for imaging. For pH probe recording, a method described by Bagar et al. (2009) was adopted. For sequential excitation, a 50 mW 405 nm diode laser and the 476 nm line of a 25 mW argon laser were used. Laser powers 6% for the diode laser and 12% for the argon laser were employed. Successive images excited at 405 and 476 nm were captured within 1.2 s of each other.

Images were analysed using ImageJ (Rasband, 1997–2011; Abramoff et al., 2004) and ratio calculations and conversion to pH values were determined as in Bagar et al. (2009)

3. Results

3.1. D-xylose dehydrogenases and their activity in recombinant S. cerevisiae strains

Crude cell extract containing XylB had activity of 45.4 ± 3.0 nkat [mg protein]$^{-1}$ with D -xylose as substrate and was strictly NAD^+-dependent. The cell extract containing *SUS2DD* had activity of 1.32 ± 0.11 nkat [mg protein]$^{-1}$ and required $NADP^+$ as a cofactor. No D-xylose dehydrogenase activity was detectable in crude cell extracts from the control strain (H3680), lacking a D-xylose dehydrogenase gene.

The XylB was purified from *S. cerevisiae* using an *N*-terminal Strep-tag, and its kinetic properties and pH optimum determined (Table 2). XylB was most active at pH 9 but remained active over a wide pH range from pH 7 to 10, and showed 30% activity at pH 5 (data not shown). The purified enzyme had activity only with the pentose sugars D-xylose, L-arabinose and D-lyxose (measured at pH 9); D-xylose was clearly the best substrate and D-lyxose the worst (data not shown). There was no activity detected with D-ribose, D-glucose, D-galactose or D-mannose. The measured kinetic constants (K_m, k_{cat} and k_{cat}/K_m) for D-xylose and L-arabinose are shown in Table 2. XylB was very specific for D-xylose, having a specificity constant (k_{cat}/K_m) for D-xylose which is 400–600 fold higher than for L-arabinose at the physiological (pH 6.8) as well as in the optimum pH (pH 9).

3.2. Production of D-xylonate with the D-xylose dehydrogenase XylB

S. cerevisiae strains expressing *xylB* and *SUS2DD* genes coding for D-xylose dehydrogenases were grown in bioreactor cultures at

Table 2
Kinetic properties (k_{cat}, K_m for NAD^+, D-xylose or L-arabinose and specificity constant k_{cat}/K_m) of the N-terminally Strep tagged XylB produced in *S. cerevisiae* at pH 6.8 (IVL physiological buffer), pH 7 (50 mM BTP buffer) and pH 9 (50 mM BTP buffer). NAD^+ (5 mM) was used as cofactor to determine the properties with D-xylose and L-arabinose as substrate.

Substrate	Parameter	pH 6.8	pH 7	pH 9
NAD^+	k_{cat} (min^{-1})	1550 ± 55	2140 ± 65	1860 ± 65
	K_m (mM)	0.26 ± 0.04	0.31 ± 0.03	0.46 ± 0.05
	k_{cat}/K_m (mM^{-1} min^{-1})	6100 ± 1200	6900 ± 1000	4000 ± 650
D-Xylose	k_{cat} (min^{-1})	1570 ± 60	1820 ± 50	1360 ± 50
	K_m (mM)	0.40 ± 0.06	0.24 ± 0.03	0.08 ± 0.02
	k_{cat}/K_m (mM^{-1} min^{-1})	4000 ± 800	7500 ± 1100	16800 ± 4700
L-Arabinose	k_{cat} (min^{-1})	1335 ± 45	1755 ± 50	1455 ± 40
	K_m (mM)	180 ± 15	100 ± 10	40 ± 5
	k_{cat}/K_m (mM^{-1} min^{-1})	7 ± 1	17 ± 3	36 ± 6

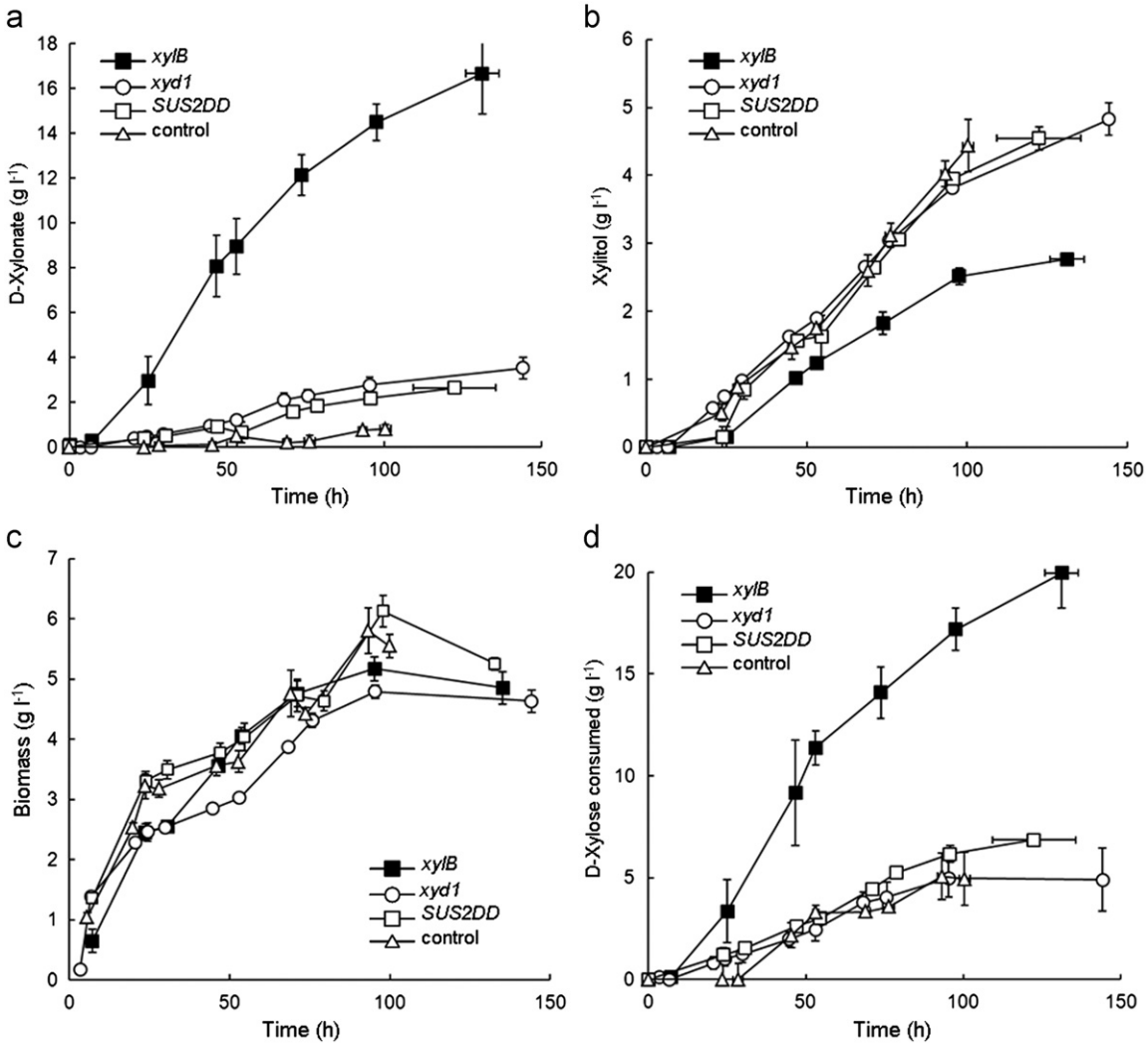

Fig. 1. D-xylonate (a), xylitol (b) and biomass (c) production and D-xylose consumed (d) by *S. cerevisiae* expressing *xylB* (solid square), *xyd1* (open circle, Toivari et al., 2010), or *SUS2DD* (open square), or containing an empty vector (open triangle). Cells were grown in bioreactors with 23 ± 1 g D-xylose l^{-1} and 9 ± 1 g D-glucose l^{-1}, pH 5.5, 30 °C, supplemented with 5 ± 1 g ethanol l^{-1} after ~54 h. Cultures were agitated at 500 rpm with 1 vvm aeration. Error bars show SEM (n=2 or 3).

pH 5.5 as described by Toivari et al. (2010). The strain expressing *SUS2DD* produced 2.7 ± 0.1 g D-xylonate l^{-1} and 4.5 ± 0.2 g xylitol l^{-1} when provided 23 ± 1 g D-xylose l^{-1} at pH 5.5 (Fig. 1), similar to that produced by the stain expressing *T. reesei xyd1* (Toivari et al., 2010). The strain expressing *xylB* produced 16.7 ± 1.8 g D-xylonate l^{-1} and 2.7 ± 0.1 g xylitol l^{-1} from 23 ± 1 g D-xylose l^{-1} (Fig. 1). The negative control produced no D-xylonate but similar amounts of xylitol (4.4 ± 0.4 g xylitol l^{-1}) as the strains expressing *SUS2DD* or *xyd1* (Toivari et al., 2010). *S. cerevisiae* expressing *xylB* produced D-xylonate at an approximately 10 times higher rate than observed in the strains expressing the $NADP^+$-dependent dehydrogenase encoding genes *xyd1* or *SUS2DD* (Table 3)

Table 3
Initial D-xylose consumption rate, D-xylonate and xylitol production rates, approximate yield of D-xylonate and xylitol on consumed D-xylose and specific D-xylonate production rate for recombinant *S. cerevisiae*. CEN.PK strains were grown in YSC medium with 9 ± 1 g D-glucose l^{-1} and 23 ± 1 g D-xylose l^{-1}, supplemented with 5 ± 1 g ethanol l^{-1} after ~50 h. B67002 cells were grown in YP with 8 g D-glucose l^{-1} and 21 g D-xylose l^{-1}, supplemented with 4 g D-glucose l^{-1} and 28 g D-xylose l^{-1} after 47.4 h. Cultures were maintained at 30 °C, 500 rpm, 1 vvm aeration and pH 5.5, except as indicated. Values are mean ± SEM from 2 to 4 cultivations.

Strain	D-xylose consumption rate ($g\,l^{-1}\,h^{-1}$)	D-xylonate production rate ($g\,l^{-1}\,h^{-1}$)	D-xylitol production rate ($g\,l^{-1}\,h^{-1}$)	Yield D-xylonate/ D-xylose ($g\,g^{-1}$)	Yield D-xylitol/ D-xylose ($g\,g^{-1}$)	Specific D-xylonate production rate ($mg\,g^{-1}\,h^{-1}$)
control	0.06 ± 0.01	0	0.04 ± 0.00	0	0.9	0
xylB	0.23 ± 0.01	0.23 ± 0.02	0.03 ± 0.01	0.8	0.1	58
SUS2DD	0.07 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	0.4	0.6	6
xyd1[a]	0.06 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.4	0.6	7
gre3 xylB	0.21 ± 0.01	0.20 ± 0.01	0.01 ± 0.00	0.8	0.03	58
xylB xylC	0.27 ± 0.01	0.24 ± 0.01	0.04 ± 0.00	0.8	0.1	61
xylB pH 3	0.12 ± 0.02	0.08 ± 0.01	0.03 ± 0.00	0.8	0.2	28
xylB xylC pH 3	0.15 ± 0.02	0.13 ± 0.01	0.02 ± 0.00	0.8	0.1	48
B67002 0.5 vvm	0.48 ± 0.02	0.44 ± 0.01	0.11 ± 0.01	0.8	0.2	64

[a] Data from Toivari et al. (2010).

and was able to consume D-xylose 3 to 4 times faster than these strains or the parent which did not produce D-xylonate. Expression of *xylB* in *S. cerevisiae* did not affect biomass production (Fig. 1).

Deletion of the aldose reductase encoding gene *GRE3* reduced xylitol production to only 0.5 g xylitol l^{-1}, without reducing the D-xylose consumption rate or D-xylonate production rate in *S. cerevisiae* expressing *xylB* (Table 3). D-Xylonate production (15.8 ± 0.6 g l^{-1}) was similar to *S. cerevisiae xylB*, but biomass production (4.3 ± 0.1 g l^{-1}, compared to 5.2 ± 0.2 g l^{-1} for *S. cerevisiae xylB*, Fig. 1) was slightly reduced when *GRE3* was deleted.

The *xylB* gene was also integrated in two of several *GRE3* loci present in the industrial, hydrolysate tolerant *S. cerevisiae* strain B67002, which is polyploid or aneuploid. The strain was grown in complex medium at pH 5.5 with 8 g D-glucose l^{-1} and 21 g D-xylose l^{-1}, supplemented with 4 g D-glucose l^{-1} and 28 g D-xylose l^{-1} at 47.4 h (Fig. 2, Table 3). From 49 g D-xylose l^{-1} this strain produced 43 ± 1 g D-xylonate l^{-1}, and 8 g xylitol l^{-1} in 120 h, with an initial D-xylonate production rate of 0.44 $g\,l^{-1}\,h^{-1}$ (Fig. 2, Table 3).

3.3. Effect of D-xylonolactone lactonase XylC on production of D-xylonate

The *xylB xylC* strain produced 13 ± 2 g D-xylonate l^{-1} at pH 5.5. It released more D-xylonate into the culture supernatant during the first 50 h cultivation than the *xylB* strain, although during the production phase the initial production rate (0.24 ± 0.01 $g\,l^{-1}\,h^{-1}$) was similar to that of *xylB* (0.23 ± 0.02 $g\,l^{-1}\,h^{-1}$; Fig. 3, Table 3). After ~50 h, D-xylonate production by *xylB xylC* essentially stopped, although D-xylose was still available. The *xylB xylC* strain produced less biomass than *S. cerevisiae xylB* (Fig. 3) and the specific D-xylonate production rate of the two strains was similar (Table 3). Since the lactone form is more stable at low pH, production of D-xylonic acid by *xylB* and *xylB xylC* was also compared at pH 3. Production of D-xylonic acid, xylitol and biomass were reduced for both strains at pH 3, compared to pH 5.5 (Fig. 3, Table 3). At pH 3, the *xylB xylC* strain produced more D-xylonic acid (6.7 ± 1.1 $g\,l^{-1}$) and less xylitol (1.5 ± 0.2 $g\,l^{-1}$) than the *xylB* strain (5.5 ± 0.9 and 2.2 ± 0.1 $g\,l^{-1}$, respectively) at higher initial volumetric and specific production rates (Table 3).

^{1}H NMR analysis demonstrated that in the culture supernatants of the *xylB*, *xylB xylC* and *xylB*-B67002 strains both linear D-xylonate and D-xylonolactone were present at pH 5.5 and pH 3. Spontaneous hydrolysis of D-xylonolactone in aqueous solution at pH 6.8 or 7.2 was relatively slow, with only 12% (pH 6.8) and 20% (pH 7.2) of the lactone hydrolysed spontaneously within 16 h. When gel filtered cell extract of the *xylB xylC* strain was used, the hydrolysis of D-xylonolactone was more rapid (100% in < 1 h) compared to similar cell extract from the *xylB* strain lacking *xylC* (40 to 90% hydrolysed in 16 to 24 h).

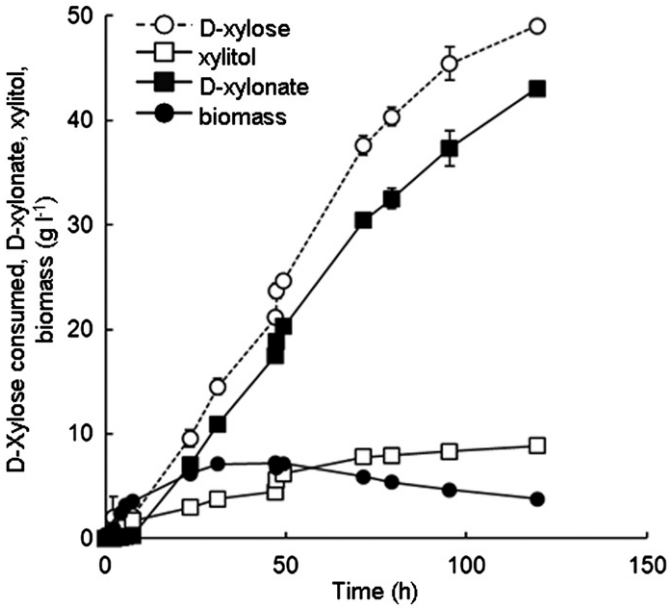

Fig. 2. D-xylonate (solid square), xylitol (open square) and biomass (solid circle) produced and D-xylose consumed (open circle) by *S. cerevisiae* B67002 *xylB* grown on YP with 8 g D-glucose l^{-1} and 21 g D-xylose l^{-1}, supplemented with 4 g D-glucose l^{-1} and 28 g D-xylose l^{-1} after 47.4 h. Cells were maintained at pH 5.5, 30 °C, with 500 rpm and 0.5 vvm aeration. Error bars show SEM for three cultivations and where not seen are smaller than the symbol.

3.4. Effect of D-xylonate production on cellular physiology

D-xylonate accumulated intracellularly to 40 to 80 $mg\,g^{-1}$ (i.e., at least 20 to 40 $g\,l^{-1}$; Fig. 4(a)) in *S. cerevisiae* CEN.PK expressing D-xylose dehydrogenase (Fig. 1). Intracellular accumulation of D-xylonate did not reflect extracellular D-xylonate concentration. Xylitol also accumulated intracellularly (10 to 50 $mg\,g^{-1}$, Fig. 4(b) and (d)), and was generally correlated to the extracellular concentration ($r^2 = 0.66$ to 0.91). The intracellular xylitol concentration was lower in the *gre3 xylB* strain than in the strain with a functional Gre3p (Fig. 4(b)). Industrial strain B67002 had higher intracellular D-xylonate concentrations than the CEN.PK strains, maintaining ~170 mg D-xylonate [g dry biomass]$^{-1}$ in the cytoplasm after 48 h incubation in D-xylose containing medium (Fig. 4(a)). Intracellular D-xylonate was initially present in higher concentrations

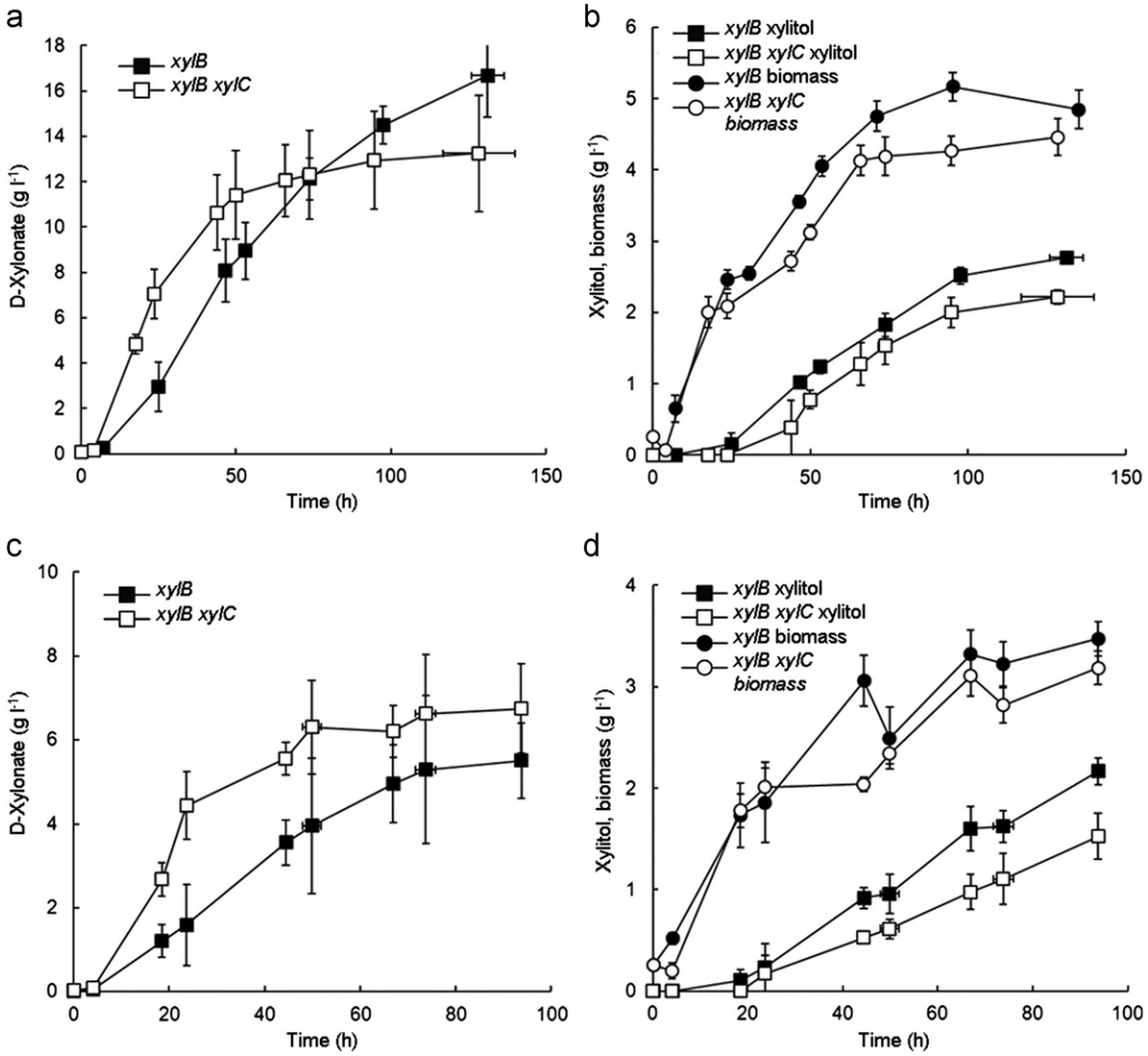

Fig. 3. Production of D-xylonate (a), (c), xylitol and biomass (b), (d) at pH 5.5 (a), (b) or at pH 3.0 (c), (d) by *S. cerevisiae xylB* (solid symbols) and *xylB xylC* (open symbols) strains in YSC medium with 9 ± 1 g D-glucose l^{-1} and 23 ± 1 g D-xylose l^{-1}, supplemented with 5 ± 1 g ethanol l^{-1} after ~50 h. Cells were maintained at pH 5.5 or at pH 3.0, 30 °C, with 500 rpm and 1.0 vvm aeration. Error bars show SEM for (n=2 to 4).

in the *xylB xylC* strain than in the *xylB* strain at both pH 5.5 and pH 3.0, but after ~50 h concentrations in *xylB xylC* were lower than in *xylB* (Fig. 4(c)). At pH 3, the intracellular D-xylonate concentration of both *xylB* and *xylB xylC* strains was lower compared to at pH 5.5 (Fig. 4(c)).

Production of D-xylonate resulted in a strong loss of vitality ($16 \pm 2\%$) for CEN.PK strains during the first 25 h cultivation (Fig. 5(a)). Subsequently, little or no cell death appeared to occur. Co-expression of pHluorin with *xylB* did not result in greater cell death (data not shown). In contrast with the CEN.PK strain, B67002 *xylB* did not show significant loss in vitality during the first 31 h of D-xylonate production (Fig. 5(a)), when 11 ± 0.3 g D-xylonate l^{-1} had been produced and 102 ± 9 mg D-xylonate [g biomass]$^{-1}$ was extracted from the cytoplasm. As extracellular D-xylonate continued to increase, inactive and empty cells began to accumulate until $77 \pm 1\%$ of the cells were no longer active at 120 h.

More inactive cells occurred in lactonase expressing cells than in the strain without *xylC* (Fig. 5(a) and (b)). More loss of vitality occurred at pH 3 than at pH 5.5 for both *xylB* and *xylB xylC* strains (Fig. 5(b)), and even small differences in pH (± 0.1 pH unit) had a large effect on the vitality of cells in replicate cultures at low pH.

Viable counts with B67002 *xylB* and CEN.PK *xylB* and *xylB xylC* strains at pH 3 showed that the loss in vitality reflected loss in viability (Fig. 5(c)). For simplicity, viability will be used in the discussion to refer to results from either staining or colony counting.

When strains expressing the pH sensitive green fluorescent protein pHluorin were grown at pH 5.5, both D-xylonate producing and non-producing cells had an initial average cytoplasmic pH of 7.5. Within 18 h, the average intracellular pH of the *xylB* strain (3 g D-xylonate l^{-1} produced) had decreased to 7.2, whereas that of the control cells was 7.4. Fewer cells remained fluorescent in the *xylB* than in the control strain.

4. Discussion

The bacterial D-xylose dehydrogenase XylB from *C. crescentus* had >30 fold higher activity in crude cell extracts with D-xylose as substrate, compared to the D-xylose dehydrogenase Xyd1 of *T. reesei* (Toivari et al., 2010) or the *SUS2DD* dehydrogenase from pig liver, when expressed in *S. cerevisiae*. The *S. cerevisiae* strain expressing the *xylB* gene had a more than 5 fold higher D-xylonate titre (Fig. 1) and 10 fold higher volumetric and specific production rates (Table 3), compared with the D-xylose dehydrogenases from *T. reesei* or pig liver. Even higher levels of D-xylonate production

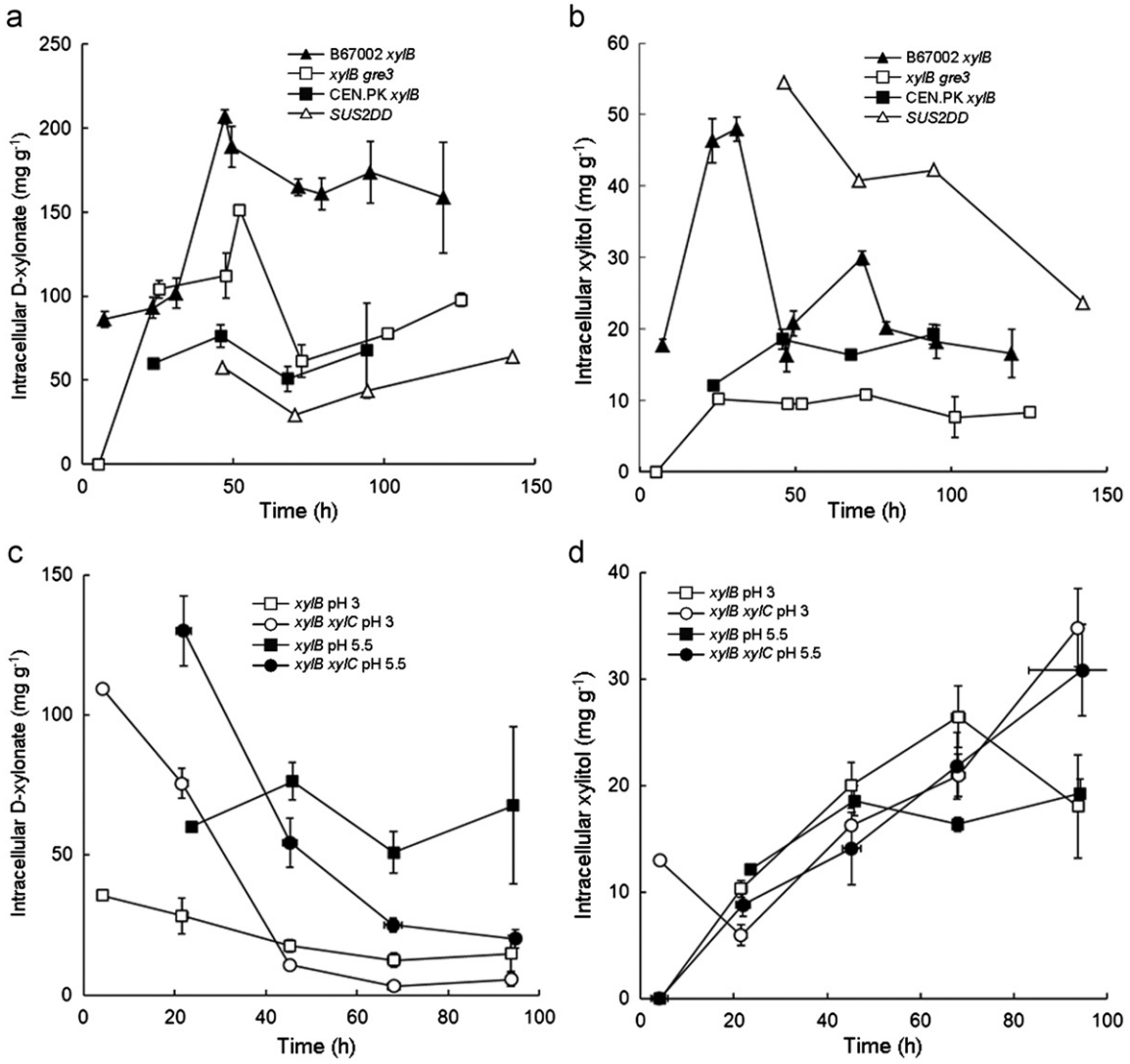

Fig. 4. Intracellular concentrations (expressed as mg per g dry biomass) of D-xylonate (a) and (c) and xylitol (b) and (d). Parts (a) and (b) show intracellular concentrations extracted from *S. cerevisiae xylB* (solid square), *SUS2DD* (open triangle), Gre3p-deficient *xylB* (open square) and B67002 *xylB* (solid triangle) grown on D-glucose and D-xylose as described in the Materials and Methods, and Figs. 2–4 at pH 5.5, 30 °C. Parts (c) and (d) show intracellular concentrations from *S. cerevisiae xylB* (squares) and *xylB xylC* (circles) grown at pH 3.0 (open symbols) or pH 5.5 (solid symbols). Error bars show SEM ($n=1$ to 3).

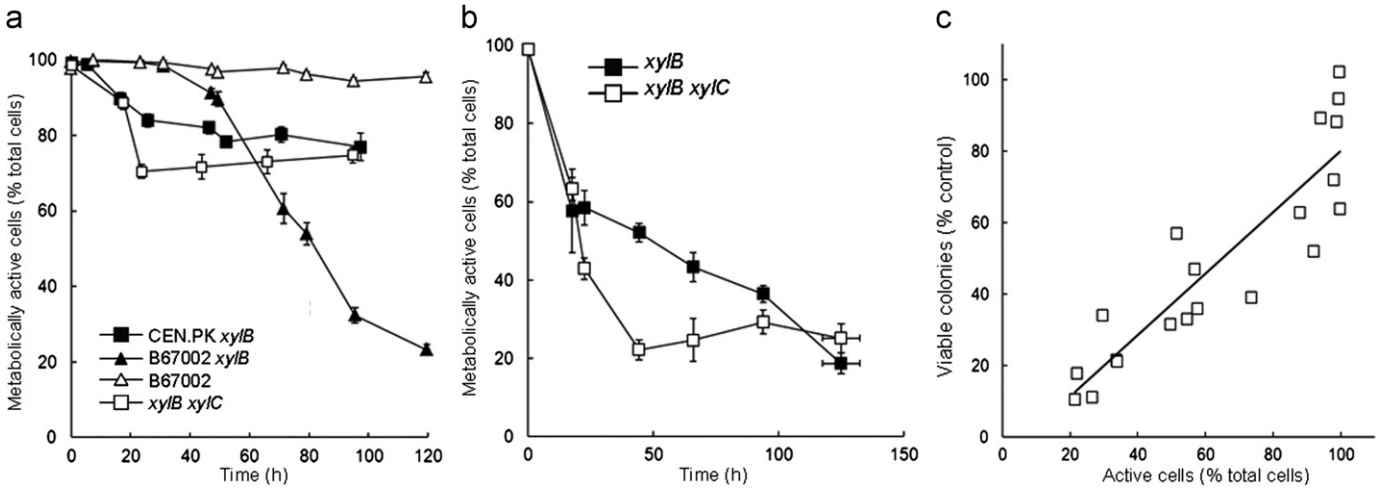

Fig. 5. Percentage of metabolically active cells, as determined by methylene blue staining, in populations of (a) *S. cerevisiae xylB* (solid squares), B67002 *xylB* (solid triangle), *xylB xylC* (open square) and B67002 (open triangle) grown in D-glucose and D-xylose containing medium at pH 5.5, as described in the Materials and Methods, and (b) *S. cerevisiae xylB* (solid squares) and *xylB xylC* (open squares) grown in D-glucose and D-xylose containing medium at pH 3.0. Extracellular D-xylonate concentrations are shown in Figs. 2 and 3 and intracellular D-xylonate concentrations in Fig. 4. The correlation between metabolically active cells and cell viability (expressed as the % viable colony forming units, CFU, relative to the non-D-xylonate-producing control strain, B67002) for B67002 *xylB* at pH 5.5 is presented in panel c.

(43 g D-xylonate l^{-1}) were obtained with an industrial, hydrolysate-tolerant strain expressing *xylB*.

NAD^+-dependency, kinetic properties and/or enzyme concentration presumably all contributed to enhanced D-xylonate production with *xylB* expressing *S. cerevisiae* strains. NADH generated by XylB activity can be efficiently reduced via oxidative phosphorylation in aerobic conditions, replenishing the NAD^+ supply and providing additional ATP for energy, whereas availability of $NADP^+$ would be mostly dependent on anabolic metabolic reactions.

Previously the best D-xylonate production levels with yeast have been reported for *K. lactis* expressing the *T. reesei xyd1* gene (Nygård et al., 2011). Compared to the *K. lactis xyd1* strain the *S. cerevisiae* CEN.PK *xylB* strain produced >2 fold more D-xylonate at a higher volumetric production rate, while producing less xylitol. Yield of D-xylonate on D-xylose consumed was thus also substantially improved with XylB in *S. cerevisiae*, compared to Xyd1 in *K. lactis*. Expression of *xylB* in *S. cerevisiae* CEN.PK resulted in a D-xylose consumption rate which was only slightly lower (0.23 g l^{-1} h^{-1}) than that of *K. lactis xyd1* (0.27 g l^{-1} h^{-1}; Nygård et al., 2011), demonstrating that the low natural D-xylose transport rates observed during ethanol production (typically less than 0.1 g l^{-1} h^{-1}; Pitkänen et al., 2005; van Vleet et al., 2008; Liu and Hu, 2010) can be increased with D-xylonate production.

The high specificity of XylB for D-xylose was confirmed (Table 2) (Stephens et al., 2007). The binding affinity for D-xylose (K_m=0.08–0.40 mM) is clearly higher than that of the *T. reesei* Xyd1 (K_m=43 mM when purified from *S. cerevisiae*; Berghäll et al., 2007) or the native aldose reductase Gre3p (K_m=14–28 mM; Jeong et al., 2002; Kuhn et al., 1995). The k_{cat} of XylB (1360–1820 min^{-1}) is also higher than that of Gre3p (k_{cat}=202–864 min^{-1}; Jeong et al., 2002; Kuhn et al., 1995). This facilitates D-xylose flux to D-xylonate rather than xylitol and strains expressing *xylB* produced less xylitol (Table 3), compared to the control strain or strains expressing *SUS2DD* or *xyd1* (Toivari et al., 2010). Deletion of *GRE3* in the *xylB* strain reduced xylitol production by ~80% compared to the *xylB* strain with *GRE3*, although its deletion generally reduces xylitol production by ~67% (Toivari et al., 2010; Träff et al., 2001).

D-Xylose dehydrogenases convert D-xylose to D-xylonolactone, which is subsequently hydrolysed either spontaneously or by a lactonase to yield D-xylonate. *S. cerevisiae* expressing the *C. crescentus* D-xylonolactone lactonase encoding gene *xylC* along with *xylB* excreted more D-xylonate earlier during the cultivation, both at pH 5.5 and at pH 3, than the strains lacking the lactonase. The greater initial reduction in viability of the *xylB xylC* strain, compared to the *xylB* strain, and the earlier stop in production at pH 5.5, suggested that in *S. cerevisiae* the linear form may be more toxic than the lactone form and that a more gradual hydrolysis of the lactone form may be advantageous to the cells.

The greatest loss in viability in CEN.PK *xylB* strains occurred during the first 24 h of cultivation when D-xylonate was accumulating within the cytoplasm, but extracellular concentrations remained low. This may suggest that intracellular accumulation of D-xylonate contributes to cell death, but that the cells which survive are able to adapt physiologically to the high intracellular D-xylonate concentrations. B67002 *xylB* cells were more viable than CEN.PK *xylB* cells with the same concentration of extracellular D-xylonate, but did not appear to adapt to the presence of D-xylonate. This probably reflects the continued D-xylonate production and/or the high intracellular D-xylonate concentration (Fig. 4). In vivo imaging of intracellular pH using the pH responsive protein pHluorin showed that the intracellular pH decreased more rapidly in D-xylonate producing CEN.PK strains than in control cells, as also observed for lactic acid producing *S. cerevisiae* using the pH responsive colour cSNARF-4f (Valli et al., 2006).

As has previously been observed with lactic acid production at low pH (Porro et al., 1999; van Maris et al., 2004a, b), less D-xylonic acid was produced at a lower rate at low compared to high pH. At low pH, maintenance of pH homeostasis may be more energy demanding than at pH values closer to the cytoplasmic pH. A larger supply of D-glucose/ethanol might improve low pH production by providing energy for cell maintenance.

Expression of the NAD^+ -specific *C. crescentus* XylB D-xylose dehydrogenase encoding gene in an industrial *S. cerevisiae* strain improved D-xylonate production to more than 40 g l^{-1}, and higher titres would be expected with provision of more D-xylose and optimisation of production conditions. However, the production rate is still 4 fold lower than observed with some bacteria (~1.7 g l^{-1} h^{-1}, Buchert, 1990) and a higher production rate would be needed for industrial production. Physiological studies indicate that export, maintenance energy and maintenance of cell viability are key issues for efficient production. Transcriptome and/or metabolome analysis of the industrial D-xylonic acid producing *S. cerevisiae* strain may provide information on how the cells adapt to D-xylonate production, which pathways are upregulated and whether specific factors are controlling cell death.

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