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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}.

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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).

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