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The glucose/xylose facilitator Gxf1 from *Candida intermedia* expressed in a xylose-fermenting industrial strain of *Saccharomyces cerevisiae* increases xylose uptake in SSCF of wheat straw

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ABSTRACT

Ethanolic fermentation of lignocellulose raw materials requires industrial xylose-fermenting strains capable of complete and efficient D-xylose consumption. A central question in xylose fermentation by *Saccharomyces cerevisiae* engineered for xylose fermentation is to improve the xylose uptake. In the current study, the glucose/xylose facilitator Gxf1 from *Candida intermedia*, was expressed in three different xylose-fermenting *S. cerevisiae* strains of industrial origin. The *in vivo* effect on aerobic xylose growth and the initial xylose uptake rate were assessed. The expression of Gxf1 resulted in enhanced aerobic xylose growth only for the TMB3400 based strain. It displayed more than a 2-fold higher affinity for D-xylose than the parental strain and approximately 2-fold higher initial specific growth rate at 4 g/L D-xylose. Enhanced xylose consumption was furthermore observed when the GXF1-strain was assessed in simultaneous saccharification and co-fermentation (SSCF) of pretreated wheat straw. However, the ethanol yield remained unchanged due to increased by-product formation. Metabolic flux analysis suggested that the expression of the Gxf1 transporter had shifted the control of xylose catabolism from transport to the NAD^+ dependent oxidation of xylitol to xylulose.

1. Introduction

Although significant progress has been made on the development of xylose fermenting *Saccharomyces cerevisiae* strains using inserted heterologous pathways [1,2], the rate of xylose conversion is still an order of magnitude lower than that of glucose. Since many potential feedstocks for biomass based ethanol production, e.g. agricultural residues, hardwood and several short rotation crops, contain a large fraction of xylose [3,4], there would be a significant benefit from improving the conversion rate of xylose. The transport of xylose into the cell was early recognized as a potential limitation for xylose catabolism in *S. cerevisiae* [5]. *S. cerevisiae* is not known to have specific transport systems for D-xylose. Instead xylose enters the cell via hexose transporters, which have several orders of magnitude lower affinity for D-xylose (overall K_m > 150 mM) than for D-glucose. One option to improve the transport is therefore to express xylose transporters from other organisms.

The glucose/xylose facilitator (Gxf1) from *Candida intermedia* was found to be the most efficient transporter when compared with other known heterologous xylose transporters; Sut1 and At5g59250 from *Pichia stipitis* and *Arabidopsis thaliana*, respectively, in an isogenic strain background [6]. Expression of the *GXF1* gene encoding the glucose/xylose facilitator in a xylose-fermenting laboratory strain of *S. cerevisiae* increased the rate of xylose utilization and ethanol production at low xylose concentration [7]. However, at high xylose concentration the Gxf1 transporter had no effect, suggesting that xylose catabolism is controlled by other factors than transport under these conditions [6–8].

Industrial *S. cerevisiae* strains are in general more robust and well-adapted to harsh environments than haploid laboratory strains [1], and an industrial lignocellulose-based ethanol process will with necessity be based on such strains. The objective of the current study was to investigate the effect of Gxf1 in xylose-fermenting yeasts strains of industrial origin, and assess their performance in undetoxified xylose-rich hydrolyzate. Three

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xylose-fermenting *S. cerevisiae* strains with industrial background: TMB3400 [9], BH42 [10] and BH46 [11] harbouring the xylose reductase (XR)/xylitol dehydrogenase (XDH)/xylulokinase (XK) pathway were used. The specific growth rate of the Gxf1 expressing strains was assessed in defined xylose medium and xylose transport was determined by zero trans-influx measurements. The industrial strain background – combined with stable chromosomal integration of the *GXF1* gene – enabled investigation of improved transport capacity in an undetoxified hydrolyzate medium. Simultaneous saccharification and co-fermentation (SSCF), which is a promising process option for industrial ethanol production [12], was chosen for this purpose.

The largest effect of Gxf1 expression on the xylose uptake rate was found for the strain TMB3400, and this strain also showed increased aerobic specific growth rate at low xylose concentration, which was not found for the other two strains. In SSCF of pretreated wheat straw the xylose uptake increased from 76 to 88% when Gxf1 was expressed in TMB3400. However, this was not accompanied by an increased ethanol yield; instead the increased xylose uptake generated other reduced by-products.

2. Material and methods

2.1. Strains and media

Plasmids and yeast strains used in this study are summarized in Table 1. *Escherichia coli* strain MOSblue (GE Healthcare) was used for cloning and grown in liquid or solid LB-medium supplemented with 15 mg L^{-1} tetracyclin and 100 mg L^{-1} ampicillin. Additionally, kanamycin (50 mg L^{-1}) was used for selection during subcloning of the *KanMX* cassette (see Section 2.2.2). *S. cerevisiae* strains were maintained on YPD-agar plates (20 g L^{-1} glucose, 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} agar) supplemented, when required, with 200 mg L^{-1} geneticin (G-418, Apollo Scientific Ltd., UK).

During cultivation in defined media *S. cerevisiae* strains were grown in Yeast Nitrogen Base (YNB) (6.7 g L^{-1} Difco Yeast Nitrogen Base without amino acids), buffered with 50 mM potassium hydrogen phthalate, pH 5.5 [13] and supplemented with a carbon source (glucose and/or xylose). When the concentration of the sugar exceeded 20 g L^{-1}, the concentration of YNB was increased proportionally. In anaerobic batch cultivation the medium was supplemented with 0.4 g L^{-1} Tween 80 and 0.01 g L^{-1} ergosterol.

2.2. Molecular biology

2.2.1. Techniques and material

Standard molecular biology protocols were used [14]. Restriction endonucleases, T4 DNA Ligase and Alkaline Phosphatase (Roche) were used according to the manufacturer specifications. Polymerase chain reaction (PCR) was performed using DNA polymerases (Dream™ *Taq* and *Pfu*) from Fermentas and primers from MWG. DNA fragments were purified with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Blunt-ended PCR Cloning Kit (GE Healthcare) was used for *KanMX* sub-cloning. *E. coli* competent cells were transformed by heat-shock. Plasmids were extracted and purified from *E. coli* strains using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). DNA sequencing was performed by STABVida (Oeiras, Portugal). Yeast transformation was performed by the lithium acetate method [15] and recombinant yeast strains were selected in YPD-agar plates supplemented with geneticin.

2.2.2. Plasmid construction

The *loxP-KanMX-loxP* cassette was excised from pUG6 [16] using *Sal*I and *Spe*I restriction enzymes, purified and sub-cloned in the multicloning site of the pMOS vector, generating pMLKr7. Vectors YIplac128 [17], YIpDR1 and YIpDR3 [7], were linearized with *Sma*I and dephosphorylated. The *loxP-KanMX-loxP* cassette was excised from pMLKr7 using *Sma*I and *Eco*RV, purified and ligated to the three vectors referred above, generating YIplac128-K, YIpDR1-K and YIpDR3-K. These vectors were linearized with *Eco*RV in *LEU2* locus and used to transform *S. cerevisiae*.

2.2.3. Real time PCR analysis

To determine the relative *GXF1* copy number, chromosomal DNA (from TMB3400, 34006 and 34007) was extracted by standard protocols from cells grown on YPD plates, and to determine relative *GXF1* mRNA levels, cells were grown under the same conditions as described below in Section 2.3.1. Total RNA was extracted (from TMB3400, 34006 and 34007) using Trizol Reagent (Invitrogen), purified with RNeasy Mini Kit (Qiagen). On-column DNase digestion with the RNase-Free DNase Set (Qiagen) was performed.

DNA or total RNA were used as template for Real-Time PCR reactions either to determine relative *GXF1* copy number or *GXF1* mRNA levels, using TAQurate GREEN Real-Time PCR Master Mix (Epicentre) or MasterAmp GREEN Real-Time RT-PCR kit (Epicentre), respectively. Quantitative Real-Time PCR reactions (3 min 94 °C; 40 cycles: 30 s 94 °C, 30 s 56 °C, 30 s 72 °C, preceded by 30 min 60 °C for reverse-transcriptase reaction when using total RNA as template) were performed in an RG-3000 Rotor-Gene apparatus (Corbett Research, Sydney, Australia). *GXF1*-specific primers CiGXFL1 and CiGXFR1 [18] were used to determine relative *GXF1* copy number and its mRNA levels. The *ACT1* gene or its mRNA were used as reference with Sc*ACT1*-specific primers, ActFor and ActRev [19]. All reactions were performed at least in duplicate. Determination of relative *GXF1* copy number and mRNA levels were performed with and without correction of amplification efficiency [20,21].

2.3. Strain characterization in defined media

2.3.1. Pre-culture conditions

S. cerevisiae strains were inoculated in shake flasks with YNB, 50 mM potassium hydrogen phthalate buffer, pH 5.5 [13] and 20 g L^{-1} D-glucose and incubated aerobically at 30 °C, 200 rpm, to an optical density at 640 nm ($OD_{640\,nm}$) of 1.0–1.5. Cells were harvested by centrifugation, 8000 × *g*, 4 °C, 5 min, washed twice with 9 g L^{-1} NaCl and resuspended.

2.3.2. Sugar transport

Cells of *S. cerevisiae* strains were prepared as described in the previous section. Initial uptake rates (zero-trans influx) were determined at 25 °C using D-[U-^{14}C]xylose to final concentrations of 5 mM for preliminary selection and between 1 and 100 mM for kinetic studies [22]. Kinetic parameters were estimated by nonlinear Michaelis–Menten regression analysis using Matlab (The MathWorks Inc., MA, USA).

2.3.3. Aerobic batch cultivation

Aerobic batch cultivation was performed in 50 mL D-xylose medium (4, 20 or 40 g L^{-1}) in 250 mL shake flasks, at 30 °C, 200 rpm and a starting $OD_{640\,nm}$ of 0.1. Growth was followed by measuring $OD_{640\,nm}$ and samples were collected for analysis of substrate and products. At least three biological replicates were performed.

2.3.4. Anaerobic batch cultivation

Anaerobic batch cultivation was performed in 40 mL D-glucose/D-xylose mixture (20 g L^{-1} each) in 100 mL Schott flasks sealed with a rubber septum. Air was replaced by argon prior to inoculation. Fermentation was performed at 30 °C, 200 rpm and a starting $OD_{640\,nm}$ of 12–15 (equivalent to approximately 4–5 g_{dw} L^{-1}). CO_2 outlet was assured by piercing a needle through the rubber septum. Samples were collected for analysis of substrate and products. At least two biological replicates were performed.

2.3.5. Analysis

Substrate consumption (D-glucose and D-xylose) and product formation (xylitol, glycerol, acetate and ethanol) were analysed by High Pressure Liquid Chromatography (HPLC), using a Dionex HPLC system (Dionex, Sunnyvale, CA), a refractive index detector (LKB, Bromma, Sweden) and an Aminex HPX-87H column (Bio-Rad, Richmond, CA). Sulphuric acid at 5 mM was used as the mobile phase, at 0.5 ml/min flow rate, with an oven temperature of 65 °C. Culture samples were diluted (when needed) and filtered with 0.20-μm pore-size polyester filter (Chromafil PET-20/15 MS, Macherey-Nagel, Germany). Cell dry weight was determined in duplicates by filtering 10 ml of the culture through a 0.22-μm pore-size filter, followed by washing with three volumes of distilled water and drying at 80 °C.

2.4. Simultaneous saccharification and co-fermentation (SSCF)

2.4.1. Raw material and pretreatment

Wheat straw was pretreated as previously described [23]. The composition of the pretreatment slurry is summarized in Table 2. The water insoluble and liquid fractions were analysed using NREL (National Renewable Energy Laboratories) standard procedures [24,25]. The WIS (water insoluble solids) content of the pretreated slurry was 13%.

2.4.2. Cell cultivation

The recombinant xylose-fermenting strains *S. cerevisiae* TMB3400 and 34006 were used for SSCF of steam-pretreated wheat straw. Yeast cells were produced in aerobic batch cultivation on glucose, followed by aerobic fed-batch cultivation in wheat straw hydrolyzate liquid, to adapt the yeast cells to inhibitors in the steam-pretreated wheat straw [26]. According to a previously described protocol [23] cells were harvested by centrifugation in 700 mL flasks using a HERMLE Z 513K centrifuge (HERMLE Labortechnik, Wehingen, Germany). The pellets were resuspended in 9 g L^{-1} NaCl-solution to obtain a cell suspension with a cell mass concentration of 80 g_{dw} L^{-1}. The time between cell harvest and initiation of the SSCF was no longer than 3 h.

2.4.3. SSCF

SSCF was performed in 2.5 L bioreactors (Biostat A, B. Braun Biotech International, Melsungen, Germany and Biostat A plus, Sartorius, Melsungen, Germany) with a working broth weight of 1.4 kg. Batch experiments were performed with

Table 1
S. cerevisiae strains and plasmids used in this study.

Strains and plasmids	Description/relevant genotype	Reference
Plasmids		
pGXF1	Yeplac195 *GXF1*	[18]
pUG6	*loxP-KanMX-loxP*	[16]
pMOS	(Cloning vector with MCS)	GE Healthcare
pMLKr7	*loxP-KanMX-loxP*	This work
YIplac128	*LEU2*	[17]
YIpDR1	YIplac128 P_{tdh3}-*GXF1*-T_{cyc1}, *LEU2*	[7]
YIpDR3	YIplac128 P_{tef1}-*GXF1*-T_{cyc1}, *LEU2*	[7]
YIplac128-K	*LEU2*, *KanMX*	This work
YIpDR1-K	YIplac128 P_{tdh3}-*GXF1*-T_{cyc1}, *LEU2*, *KanMX*	This work
YIpDR3-K	YIplac128 P_{tef1}-*GXF1*-T_{cyc1}, *LEU2*, *KanMX*	This work
***S. cerevisiae* strains**		
TMB3400	*HIS3*::P_{pgk1}-*XYL1*-T_{pgk1}, P_{adh1}-*XYL2*-T_{adh1}, P_{pgk1}-*XKS1*-T_{pgk1} followed by chemical mutagenesis and selection in xylose	[9]
BH42	Resulting from breeding of recombinant xylose-fermenting strains (YIpXR/XDH/XK) – clone 42	[11]
BH46	Resulting from breeding of recombinant xylose-fermenting strains (YIpXR/XDH/XK) – clone 46	[11]
34006, BH426 and BH466	TMB3400, BH42 or BH46 and *LEU2*::P_{tdh3}-*GXF1*-T_{cyc1}, *KanMX* (YIpDR1-K)	This work
34007, BH427 and BH467	TMB3400, BH42 or BH46 and *LEU2*::P_{tef1}-*GXF1*-T_{cyc1}, *KanMX* (YIpDR3-K)	This work
34009, BH429 and BH469	TMB3400, BH42 or BH46 and *LEU2*::*KanMX* (YIplac128-K)	This work

Table 2
Composition of pretreated wheat straw.

Content in solid fraction (% of WIS)			Concentration in the liquid fraction (g L^{-1})				
Glucan	Xylan	Lignin	Glucose[a]	Xylose[a]	Acetic acid	HMF	Furfural
54.4	3.1	32.8	6.7	38.8	4.9	0.5	3.4

[a] Both monomeric and oligomeric forms included.

a WIS content of 7%. The desired WIS content was obtained by diluting the pretreated slurry with sterile deionized water. SSCF was performed at 34 °C for 96 h. pH was maintained at 5.0 with 3 M NaOH. The SSCF medium was supplemented with 0.5 g L^{-1} $(NH_4)_2HPO_4$, 0.025 g L^{-1} $MgSO_4{\cdot}7H_2O$ and 1.0 g L^{-1} yeast extract. An initial yeast concentration of 4 g_{dw} L^{-1} was used. Xylanase XL from SAF-ISIS (Sousston, France) with a cellulose activity of 43.7 FPU g^{-1} and a β-glucosidase activity of 37 IU g^{-1}, and Novozyme 188 (Novozymes A/S, Bagsvaerd, Denmark) with a β-glucosidase activity of 342 IU g^{-1} were used. The amount of enzyme added corresponded to a cellulase activity of 20 FPU (g glucan)$^{-1}$, and a total β-glucosidase activity of 43.1 IU (g glucan)$^{-1}$. Samples for HPLC-analysis were collected throughout the SSCF.

2.4.4. Analysis

Cell mass was determined in duplicates from 10 mL samples centrifuged (1000 × *g*) for 5 min at 3000 rpm (Z200 A, HERMLE Labortechnik, Wehingen, Germany). Supernatants were discarded, and pellets were washed with a 9 g L^{-1} NaCl solution and centrifuged a second time. Pellets were dried at 105 °C overnight and weighed. Substrates and products from SSCF experiments were quantified by HPLC [23].

The ethanol yield, $Y_{E/S}$, was calculated based on the total amount of fermentable sugars added to the SSCF, i.e. the sum of available glucose and xylose present in the pretreatment slurry, including monomers, oligomers and polymers (glucan and xylan fibers).

2.4.5. Metabolic flux analysis

Metabolic flux analysis (MFA) was used to assess how the presence of the glucose/xylose facilitator Gxf1 influenced the initial xylose metabolism in the recombinant *S. cerevisiae* strains. A simple metabolic model including glycolysis, the pentose phosphate pathway, the heterologous xylose reductase (XR) and xylitol dehydrogenase (XDH) steps as well as the glycerol formation pathway was used [27]. The first 10 h of the SSCF were chosen for the analysis, since uptake rates were relatively constant during this period. Extracellular concentrations of glucose, xylose, glycerol, xylitol and ethanol were measured. The CO_2 and the intracellular compounds could not be measured due to the SSCF set-up. It was also assumed that the cells were not growing during the SSCF. In total 28 compounds and 26 reactions were included in the model. There were 22 intermediates assumed to be in pseudo-steady state, resulting in 4 degrees of freedom for the system. Hence, out of the six extracellular compounds, five compounds were measured and the extra measurement could be used to check the stability of the solution.

3. Results

The objective of this work was to investigate the significance of a heterologous xylose transporter in SSCF with xylose-fermenting *S. cerevisiae* strains of industrial origin. For this reason three xylose-utilizing *S. cerevisiae* strains were used to express the glucose/xylose facilitator gene *GXF1*. These strains (TMB3400, BH42 and BH46) already harboured a heterologous XR/XDH/XK pathway (Table 1). The effect of the introduced facilitator Gxf1 on xylose metabolism was assessed in aerobic batch culture and by zero-trans influx measurements. Strain 34006 was also assessed in SSCF of pretreated wheat straw.

3.1. Transport effects of Gxf1 expression

3.1.1. Expression of Gxf1 in different strains

GXF1, the glucose/xylose facilitator gene from *C. intermedia* PYCC 4715, was expressed in three xylose-fermenting *S. cerevisiae* strains, TMB3400, BH42 and BH46 of industrial origin. *GXF1* was expressed under the control of the *TDH3* or *TEF1* promoters, respectively (Table 1). Strains expressing *GXF1* under the control of *TDH3* promoter were named 34006, BH426 and BH466, and strains expressing *GXF1* under the *TEF1* promoter were named 34007, BH427 and BH467, respectively. The same *S. cerevisiae* terminator (*CYC1*) was used in all *GXF1*-expressing strains. Control strains (without the *GXF1* gene) displayed similar physiological and biochemical properties as the host strains (data not shown). Therefore, the *GXF1*-expressing strains were characterized directly in relation to their host strains, since these are better documented in the literature [1,2,10].

The presence of Gxf1 in TMB3400, BH42 and BH46 was assessed in aerobic batch culture (Fig. 1) and by zero-trans influx measurements (Fig. 2). In aerobic batch culture (4 g/L D-xylose) Gxf1 only significantly increased the growth of the TMB3400 transformants at low xylose concentrations (Fig. 1A and Table 3), whereas the expression had no or only marginal influence on growth in the BH42 and BH46 transformants (Fig. 1B and C). In contrast zero-trans influx measurements showed that the presence of the Gxf1 transporter significantly improved D-xylose transport in all three xylose-fermenting strains (Fig. 2). Thus the lack of improvement on growth in xylose medium with low sugar concentration in BH42 and BH46 transformants is likely to be caused by inadequate metabolic capacity downstream of transport.

Table 3
Initial specific growth rates (h^{-1}) of *S. cerevisiae* strains TMB3400 and 34006 in aerobic batch cultivations under different D-xylose concentrations (4, 20 and 40 g L^{-1}).

Strain	Initial D-xylose concentration (g L^{-1})		
	4	20	40
TMB3400	0.022 ± 0.003	0.092 ± 0.004	0.082 ± 0.001
34006	0.039 ± 0.000	0.101 ± 0.001	0.085 ± 0.001

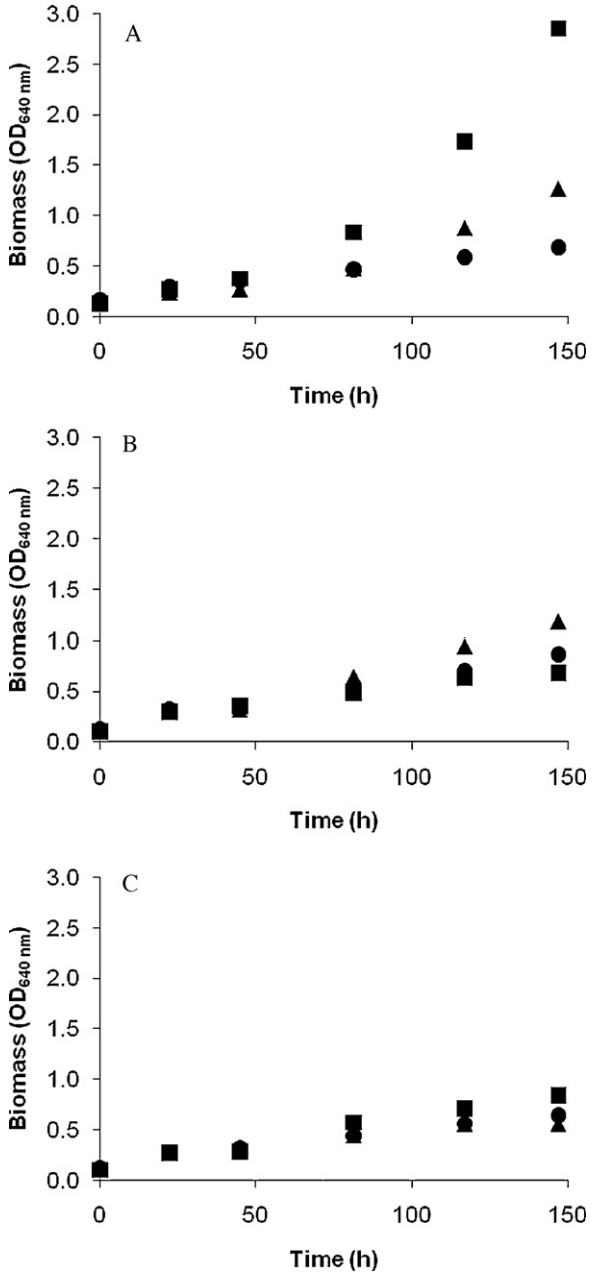

Fig. 1. Aerobic batch cultivations in 4 g/L D-xylose with *S. cerevisiae* strains showing (A) TMB3400 and derived strains, (B) BH42 and derived strains and (C) BH46 and derived strains. Control strains TMB3400, BH42 and BH46 (●), P_{tdh3}-*GXF1* strains 34006, BH426 and BH466 (■), and P_{tef1}-*GXF1* strains 34007, BH427 and BH467 (▲).

3.1.2. *GXF1* copy number and relative mRNA levels

Strain 34006 expressing *GXF1* under the control of the *THD3* promoter grew significantly faster and to higher cell density than strain 34007 where *GXF1* was expressed under control of the *TEF1* promoter (Fig. 1A). This also was observed when *GXF1* was expressed under control of these promoters in a laboratory CEN.PK strain [7]. The relative *GXF1* copy number in strains 34006 and 34007 was the same (ratio = 1.0 ± 0.4) as determined by Real-Time PCR, using the housekeeping *ACT1* gene as reference. In contrast, relative *GXF1* mRNA levels determined by Real-Time PCR with *ACT1* as reference showed that the expression of *GXF1* was 2.5 to 3-fold higher in 34006 than in 34007, when pre-grown in 20 g L^{-1} glucose. This agrees with previous observations that the *TDH3* promoter is stronger than the *TEF1* promoter [28], but contradicts the results obtained in a laboratory CEN.PK strain where relative *GXF1* mRNA levels were significantly higher when *TEF1* was used as promoter [7].

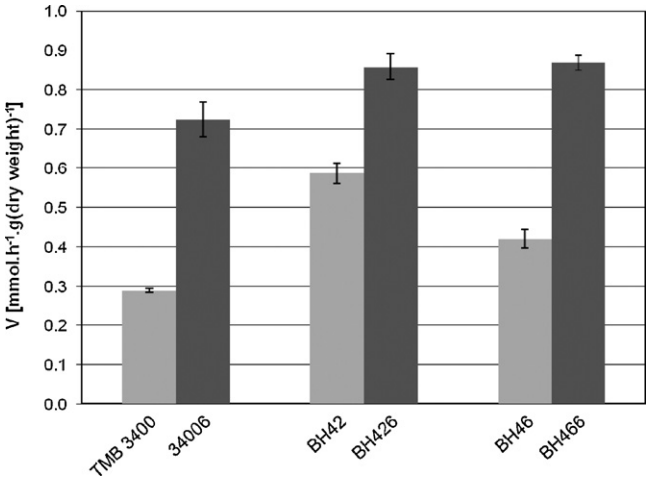

Fig. 2. D-[U-^{14}C]Xylose (5 mM) initial uptake rates in D-glucose-grown cells of industrial recombinant xylose-fermenting *S. cerevisiae* strains (light grey – control strains; dark grey – *GXF1* expressing strains with *TDH3* promoter).

3.1.3. Estimation of overall xylose transport affinity

Overall kinetics of D-xylose transport were determined by measuring initial D-[U-^{14}C]xylose uptake rates in strain TMB3400 and its Gxf1 transformants 34006 and 34007 (Fig. 3). The expression of *GXF1* reduced the estimated overall K_m, as denoted by the slopes ($-K_m$) in the Eaddie-Hofstee plots to 130 ± 13 mM and 210 ± 25 mM for 34006 and 34007, respectively, compared with 300 ± 12 mM for TMB3400. V_{max} values for D-xylose uptake were hardly affected and only marginally higher in the *GXF1* expressing strains 34006 and 34007, 20 ± 1 and 21 ± 2 mmol $(g_{dw})^{-1}$ h^{-1}, respectively, compared to 18 ± 1 mmol $(g_{dw})^{-1}$ h^{-1} for TMB3400.

The kinetic parameters represent the sum of the contribution from the native transporters (Hxts and Gal2) and, in the case of 34006 and 34007, also the heterologous glucose/xylose facilitator Gxf1. Therefore the values reflect both cultivation conditions and strain background. Nevertheless, qualitatively the present results are in accordance with similar data obtained in laboratory strains expressing the Gxf1 transporter when compared with their parent strains [6,7]. Also the approximate contribution of the Gxf1 transporter to the total xylose uptake in strain 34006, as estimated by subtracting the xylose uptake of the control strain TMB3400, agreed with data obtained for laboratory strains [7]. In 34006 Gxf1 contributed to the total xylose uptake with approximately 50–60%, while the corresponding contribution to xylose uptake in a laboratory strain was 66–70% [7].

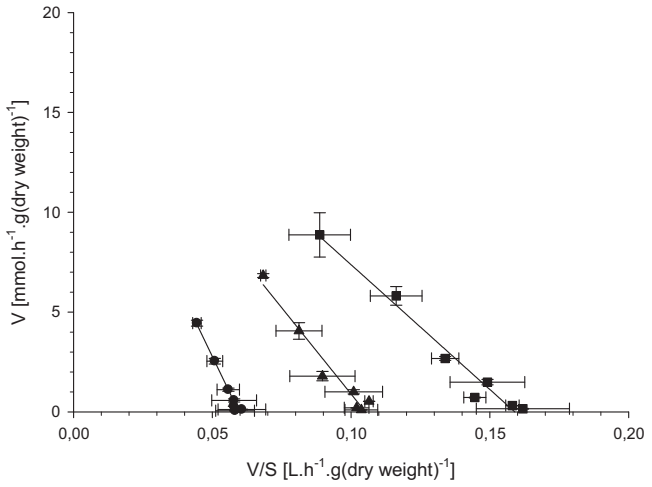

Fig. 3. Kinetics of D-xylose uptake. Eaddie-Hofstee plot of D-[U-^{14}C]xylose initial uptake rates in D-glucose-grown cells of *S. cerevisiae* TMB3400 (●), 34006 (■) and 34007 (▲).

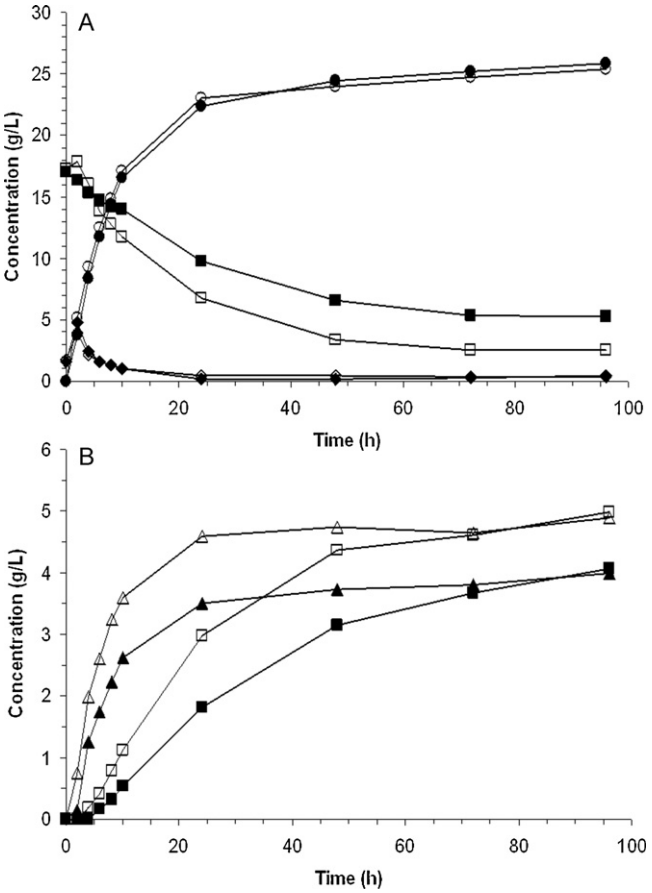

Fig. 4. Measured concentrations during SSCF of wheat straw with 34006 (open symbols) and TMB3400 (filled symbols) at 7% WIS showing (A) glucose (♦), xylose (■) and ethanol (●) and for (B) xylitol (■) and glycerol (▲).

3.2. Evaluation in SSCF

3.2.1. SSCF of pretreated wheat straw

Based on the results reported above strain 34006 was selected for further assessment in SSCF of pretreated wheat straw (Table 2). Strains TMB3400 and 34006 were compared in batch SSCF in biological duplicates at 34 °C with a WIS loading of 7% (Fig. 4 and Table 4). Both strains were cultivated in hydrolyzate liquid prior to SSCF in order to adapt the yeast to the hydrolyzate [26]. The results showed that strain 34006 took up significantly more xylose than strain TMB3400, 88% compared to 76% (Fig. 4A and Table 4). This clearly demonstrates that the presence of the Gxf1 transporter in a strain of industrial origin can significantly improve xylose uptake in SSCF. However, despite the increased xylose uptake, the ethanol yield did not increase. The ethanol yield was 0.38 g g^{-1} for both strains with a final ethanol concentration just above 25 g L^{-1}. Instead, the additional xylose taken up by strain 34006 was converted to glycerol and xylitol (Fig. 4B and Table 4). In both strains glycerol formation was initially more rapid than xylitol formation, but xylitol formation continued for a longer time.

3.2.2. Metabolic flux analysis

To understand the underlying mechanism of the increased by-product formation caused by the xylose uptake in the Gxf1 expressing strain 34006 during SSCF, a metabolic flux analysis (MFA) was made (Fig. 5). The first 10 h of the SSCF were chosen for the analysis, since uptake rates were relatively constant during this period. Compared with TMB3400 a larger fraction of sugars taken up by 34006 was converted to glycerol and xylitol. Furthermore, more glycerol than xylitol was formed during this phase of the SSCF. Co-factor balancing in the MFA suggests that the Gxf1 expressing strain 34006 uses NADPH to a larger extent than TMB3400 for the reduction of xylose to xylitol (i.e. reaction 1 and 2 in Fig. 5). The increased demand for NADPH causes carbon loss as CO_2 when NADPH is regenerated in the oxidative part of the PPP (i.e. reactions 10–11 in Fig. 5). Similarly, the increased uptake of xylose by the *GXF1* expressing strain results in an increased demand for NAD^+ for the oxidation of xylitol to xylulose (reaction 3 in Fig. 5), which is met by increased glycerol formation (reaction 18 in Fig. 5). In addition, more xylitol was shown to be excreted due to the increased xylitol formation (reaction 26 in Fig. 5), which is an alternative way for the cell to handle the limited supply of NAD^+.

3.2.3. Evaluation in anaerobic batch

To further elucidate the shift in product formation observed in SSCF, apparently caused by the presence of Gxf1 in strain 34006, anaerobic batch fermentation of mixture of 20 g L^{-1} xylose and 20 g L^{-1} glucose in defined medium was performed with strains TMB3400 and 34006 (Table 5). Similar to SSCF the ethanol yield was the same for both strains. The xylose uptake increased marginally for strain 34006 but resulted merely in a higher xylitol yield, while the glycerol yield was unchanged. Even though the conditions of the anaerobic fermentation do not fully reflect the conditions of the SSCF – among others the anaerobic fermentation started with an initially higher glucose concentration – the results point towards increased xylose uptake by the presence of Gxf1. However, surplus xylose is excreted as xylitol rather than fermented to ethanol.

4. Discussion

For the first time the influence of a heterologous xylose transporter in a xylose-fermenting *S. cerevisiae* strain has been assessed in fermentation of non-detoxified lignocellulose. The gene *GXF1* encoding the glucose/xylose facilitator Gxf1 from *C. intermedia* was chromosomally integrated into the genome of three different xylose-utilizing *S. cerevisiae* strains of industrial origin. The presence of Gxf1 in strain 34006 significantly increased the xylose uptake from 76% to 88% in a simultaneous saccharification and co-fermentation (SSCF) set-up using pretreated wheat straw as substrate. It was also found that the additional xylose taken up by the cell was stoichiometrically converted to xylitol and glycerol rather than to the final fermentation product ethanol. Our observations suggest that the xylose catabolic reactions down-stream of

Table 4
Final yields and concentrations from SSCF of wheat straw with TMB3400 and 34006 after 96 h. (Mean values with standard deviations of duplicate experiments.).

Strain	Ethanol yield ($g\,g^{-1}$)	Xylitol yield[a] ($g\,g^{-1}$)	Glycerol yield ($g\,g^{-1}$)	Xylose uptake[b] (%)	Ethanol ($g\,L^{-1}$)
TMB3400	0.38 ± 0.01	0.24 ± 0.01	0.06 ± 0.01	76	25.8 ± 0.3
34006	0.38 ± 0.01	0.26 ± 0.00	0.07 ± 0.00	88	25.4 ± 0.3

[a] Based on consumed xylose.
[b] Based on total amount of xylose (both monomeric xylose and xylan).

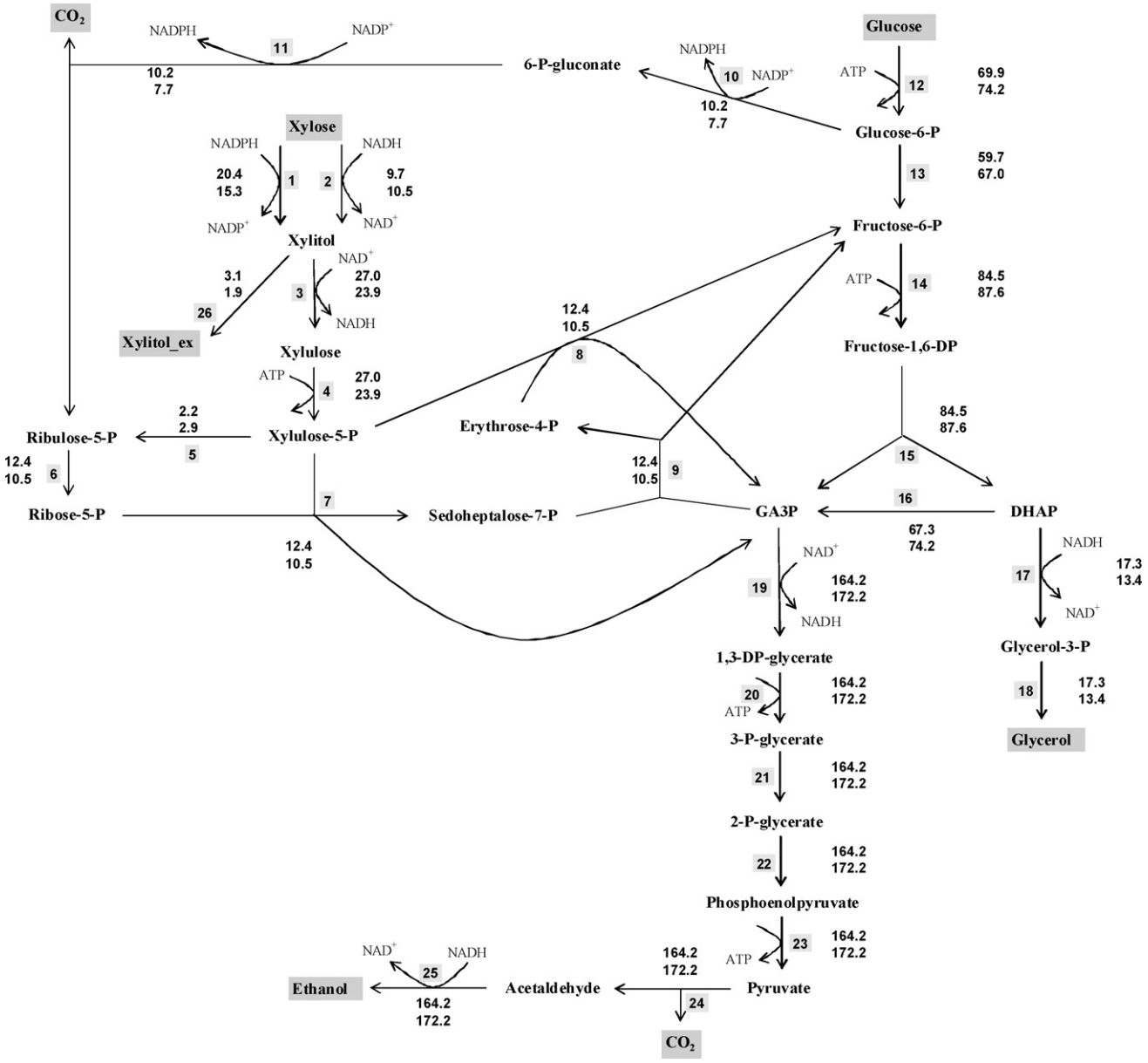

Fig. 5. Metabolic flux analysis showing mean fluxes during the first 10 h of SSCF. The sum of glucose and xylose flux was normalized to 100 in both experiments. Flux values shown next to the reactions refer to 34006 (upper value) and TMB3400 (lower value). Numbers next to reaction arrow represent the reaction number as referred to in the text.

Table 5
Final yields in anaerobic batch cultivations of a mixture of 20 $g\,L^{-1}$ xylose and 20 $g\,L^{-1}$ glucose. (Mean values with standard deviations of duplicate experiments.).

Strain	Ethanol yield ($g\,g^{-1}$)	Xylitol yield[a] ($g\,g^{-1}$)	Glycerol yield ($g\,g^{-1}$)	Xylose uptake[b] (%)	Biomass yield ($g_{dw}\,g^{-1}$)
TMB3400	0.34 ± 0.01	0.20 ± 0.00	0.14 ± 0.01	98	0.04 ± 0.00
34006	0.34 ± 0.00	0.24 ± 0.03	0.14 ± 0.01	98	0.04 ± 0.00

[a] Based on consumed xylose.
[b] Based on total amount of xylose.

transport require further engineering to meet the increased influx of xylose governed by the presence of the heterologous xylose transporter Gxf1.

S. cerevisiae does not harbour known specific xylose transporters. Xylose is instead transported by the hexose transporters Hxt1-17 and Gal2, which have approximately two orders of magnitude lower affinity for xylose than for glucose [5]. Several endogenous [29–31] as well as heterologous [7,18,32,33] transporters have been expressed in *S. cerevisiae* with the aim to improve xylose uptake. In a comparison of known heterologous xylose transporters in an isogenic laboratory strain background the Gxf1 transporter from *C. intermedia* [6,18,34] was singled out as being superior with respect to transport capacity and xylose growth [6].

The present study also demonstrated that the influence of an additional heterologous xylose transporter on fermentation performance is highly dependent on strain background. Both xylose uptake and growth on xylose clearly benefited from the presence of the heterologous Gxf1 transporter in strain TMB3400. However, this was not observed for strains BH42 and BH46. These two strains are hybrid mutants obtained by interbreeding of a number of laboratory and industrial xylose-utilizing strains [10,11]. The strains were isolated based on selection for improved xylose catabolism. The fact that the presence of Gxf1 had none or only marginal influence on xylose growth at low sugar concentration may reflect that BH42 and BH46 have significantly lower xylose reductase (XR) activity than TMB3400 as reported for BH42 [10]. Metabolic control analysis (MCA) has previously shown that xylose transport only exercises control over the initial xylose catabolism in recombinant xylose-utilizing strains of *S. cerevisiae* when these are supplied with additional copies of the *XYL1* gene encoding XR [8]. Thus a strain with high initial xylose catabolic activity is required to make full justice to the glucose/xylose facilitator Gxf1 evaluated in the current investigation.

SSCF is advantageous for co-fermentation of glucose and xylose [35–37]. A metabolic model including kinetic and molecular aspects of glucose/xylose transport in *S. cerevisiae* [38] partly explains this positive effect. In SSCF the hemicellulose fraction is to a large extent hydrolysed already in the acid catalysed steam pretreatment step [12]. Xylose is therefore present in the liquid phase already at the beginning of the SSCF, whereas glucose is continuously released from glucan polymers throughout the process as the cellulose fraction is enzymatically hydrolysed. Enhanced co-utilization of xylose and glucose in SSCF is attributed to the high xylose/glucose ratio, where the low glucose concentration supports xylose transport by inducing high-affinity transporters, while it also reduces the competition for transport [39,40]. The model showed that low glucose concentrations (~0.5 g/L) supported xylose transport – primarily by inducing high affinity transporters, which more than compensated for the kinetic competition between xylose and glucose [38]. The modelling results are supported by numerous observations both in defined media and lignocellulose hydrolyzates where it has been demonstrated that co-consumption of glucose increases xylose uptake if the glucose concentration is deliberately controlled at a sufficiently low level [36,40–44].

Even though SSCF already favours simultaneous uptake of xylose and glucose, the presence of the transporter Gxf1 enabled further xylose uptake. However, anaerobic batch fermentation in defined medium and metabolic flux analysis (MFA) of SSCF showed that the xylose catabolic reactions down-stream of transport were not dimensioned for the increased influx of substrate. Instead increased xylose uptake in the Gxf1 expressing strain (34006) translated into a deficiency of NADPH and NAD^+ in the XR and XDH reactions, respectively (Fig. 5). The increased NADPH demand was met by increased activity of the oxidative pentose phosphate pathway during which carbon was lost as CO_2, whereas the increased demand for NAD^+ in the XDH reaction led to increased glycerol formation and excretion of xylitol. Significantly more glycerol was produced in defined media fermentation ($0.14\,g\,g^{-1}$) than in SSCF ($0.07\,g\,g^{-1}$). A likely explanation to this previously reported phenomenon is the presence of additional electron acceptors in the hydrolyzate, which change the cofactor balance and reduce glycerol production [36]. The ethanol yield remained unchanged in the Gxf1 expressing 34006 strain even though significantly more xylose was taken up. Thus, as the flux control by transport was reduced by expression of Gxf1, cofactor regeneration re-emerged as the primary metabolic control of the pathway.

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