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Expression of the Gxf1 transporter from *Candida intermedia* improves fermentation performance in recombinant xylose-utilizing *Saccharomyces cerevisiae*

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Abstract The glucose/xylose facilitator Gxf1 from *Candida intermedia* was expressed in the recombinant xylose-fermenting *Saccharomyces cerevisiae* strain TMB 3057. The new strain, TMB 3411, displayed approximately two times lower K_m for xylose transport compared to a control strain not expressing Gxf1. In aerobic batch cultivation, the specific growth rate was significantly higher at low xylose concentration, 4 g/L, when Gxf1 was expressed, whereas it remained unchanged at high xylose concentration, 40 g/L. Similarly, in aerobic-xylose-limited chemostat culture, the Gxf1-expressing strain consumed more xylose than the control strain at low dilution rates (low xylose concentration), whereas the situation was reversed at higher dilution rates (high xylose concentration). Also, under anaerobic conditions, the Gxf1-expressing strain showed faster xylose uptake and ethanol formation at low substrate concentrations. The results are discussed in relation to previous observations, which suggested that transport controlled xylose utilization in recombinant xylose-utilizing *S. cerevisiae* only at low xylose concentrations.

Keywords *Saccharomyces cerevisiae* · Xylose transport · *GXF1* · *Candida intermedia* · Lignocellulose

Prof. Isabel Spencer-Martins passed away during the final preparation of this manuscript.

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Introduction

Abundant research has been devoted to improve conversion of lignocellulosic biomass to fuel ethanol using baker's yeast *Saccharomyces cerevisiae* as the fermenting organism. In hard woods and agricultural residues, a major fraction of the raw material is the pentose sugar xylose. Since *S. cerevisiae* naturally lacks the ability to ferment pentoses, recombinant *S. cerevisiae* strains, able to utilize xylose and arabinose, have been generated (Hahn-Hägerdal et al. 2007a, b). In particular, xylose fermentation has been successfully achieved by integration of the xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) genes from *Pichia stipitis*, together with overexpression of the endogenous xylulokinase gene (*XKS1*; Eliasson et al. 2000; Xue and Ho 1990) or by expressing a fungal xylose isomerase (Kuyper et al. 2003).

S. cerevisiae lacks specific transporters for xylose, and this substrate enters the cell through nonspecific hexose transporters. The xylose transport properties of the endogenous hexose transporters have been individually characterized in terms of substrate affinity and maximum reaction rate (Hamacher et al. 2002; Saloheimo et al. 2007). Compared to glucose, these transporters have poor affinity for xylose, and it has been hypothesized that this step may control the rate of xylose utilization (Eliasson et al. 2000). In addition, the high-affinity hexose transporters responsible for xylose uptake show significant repression by glucose during co-fermentation of glucose and xylose (Sedlak and Ho 2004). To improve xylose uptake and potentially reduce repression by glucose, heterologous xylose transporters have been cloned and expressed in recombinant *S. cerevisiae*. The sugar transporter Sut1 from *P. stipitis* was successfully expressed in *S. cerevisiae* (Katahira et al. 2008), and a transporter homologue, Trxlt1,

was isolated from a cDNA library of *Hypocrea jecorina* (Saloheimo et al. 2007). The glucose/xylose facilitator Gxf1 was similarly identified from screening a cDNA library of *Candida intermedia* (Leandro et al. 2006). The Gxf1 transporter in particular was shown to improve xylose affinity approximately three times compared to native *S. cerevisiae* hexose transporters.

In this study, the glucose/xylose facilitator Gxf1 from *C. intermedia* (Gardonyi et al. 2003b; Leandro et al. 2006, 2008) was expressed in the xylose-fermenting *S. cerevisiae* strain TMB 3057 (Karhumaa et al. 2005). Although the Gxf1 transporter has been previously characterized in *S. cerevisiae* in terms of kinetic parameters, the physiological effect of the transporter under culture conditions remained to be determined. The fermentative performance of the recombinant strain expressing the Gxf1 transporter was assayed under aerobic and anaerobic conditions in batch and chemostat cultures. We here report an increased rate of xylose utilization and ethanol production due to improved xylose transport. The results are discussed in relation to the distribution of the flux control in the xylose-metabolizing pathway.

Materials and methods

Strains and cultivation conditions

Plasmids and *S. cerevisiae* strains used in this study are summarized in Table 1. *Escherichia coli* strain DH5α was used for sub-cloning and was grown on Luria–Bertani (LB) plates supplemented with 100 mg/L ampicillin. Defined mineral medium was used for *S. cerevisiae* cultivation and was composed of (per liter): carbon source (glucose or xylose); mineral salts ($(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g); buffer (potassium hydrogen phthalate, 50 mM pH 5.5, only in shake-flask cultivation); vitamins and trace elements (Verduyn et al. 1992). At the start of each experiment, yeast strains were streaked from 15% (*v*/*v*) glycerol stock and grown 2 days on yeast nitrogen base (YNB) glucose plates. A single colony was pre-grown overnight in mineral medium (glucose 20 g/L) and used for subsequent inoculation. Cultivation of *S. cerevisiae* was performed at 30°C.

Molecular biology methods and cloning

Standard molecular biology techniques were used for all cloning procedures (Sambrook et al. 1989). Fermentas GeneJet plasmid miniprep kit (Fermentas, Vilnius, Lithuania) was used for plasmid extraction, and Qiagen Gel Extraction Kit (Qiagen GMBH, Hilden, Germany) was used to extract DNA from agarose gels. E.Z.N.A Cycle-Pure Kit (Omega Bio-tek, Doraville, USA) was used for purification of polymerase chain reaction (PCR) products. Primers for PCR were ordered from MWG (MWG-Biotech AG, Ebersberg, Germany). All DNA-modifying enzymes, including restriction enzymes, *Taq/Pfu* polymerase, T4 DNA Ligase, and CIAP, were purchased from Fermentas (Fermentas, Vilnius, Lithuania). Sequencing of genetic constructs was performed using an ABI 3100 Genetic Analyzer and the Big Dye sequencing kit

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

Strains and plasmids	Relevant genotype	Reference
Plasmids		
pGXF1	YEplac195 *GXF1*	Leandro et al. 2006
pY7	*URA3 ADH1p-XYL1-ADH1t*, *PGK1p-XYL2-PGK1t*	Walfridsson et al. 1997
p426GPD	*TDH3* promoter	Mumberg et al. 1995
P426TEF	*TEF2* promoter	Mumberg et al. 1995
YIplac128	*LEU2*	Gietz and Sugino 1988
YIpDR1	YIplac128 *TDH3p-GXF1-CYC1t*	This work
YIpDR2	YIplac128 *TDH3p-GXF1-GXF1t*	This work
YIpDR3	YIplac128 *TEF1p-GXF1-CYC1t*	This work
S. cerevisiae strains		
TMB 3043	CEN.PK 2-1C Δ*GRE3*, *his3*::*PGK1p-XKS1-PGK1t*, *TAL1*::*PGK1p-TAL1-PGK1t*, *TKL1*::*PGK1p-TKL1-PGK1t*, *RKI1*::*PGK1p-RKI1-PGK1t*, *RPE1*::*PGK1p-RPE1-PGK1t*, *leu2*, *ura3*	Karhumaa et al. 2005
TMB 3411	TMB 3043, *leu2*::YIpDR1, pY7	This work
TMB 3412	TMB 3043, *leu2*::YIpDR2, pY7	This work
TMB 3413	TMB 3043, *leu2*::YIplac128, pY7	This work
TMB 3414	TMB 3043, *leu2*::YIplacDR3, pY7	This work

(Applied Biosystems, Weiterstadt, Germany). Transformation of *E. coli* was performed using the calcium chloride method (Dagert and Ehrlich 1979), and the lithium acetate method was used for transformation of *S. cerevisiae* (Gietz et al. 1995).

S. cerevisiae strains expressing *GXF1* from *C. intermedia*

The open reading frame of the *GXF1* gene was amplified from plasmid pGXF1 (Leandro et al. 2006), with and without the native terminator sequence, using primers containing flanking restriction sites for *Spe*I and *Sal*I. Amplicons were ligated into p426GPD (Mumberg et al. 1995) and transformed into *E. coli* DH5α cells. Expression cassettes (promoter + orf + terminator) were amplified from the resulting plasmids, using primers containing restriction sites for *Pst*I and *Bam*HI. The amplified cassettes were ligated into the multiple cloning site of the integrative plasmid YIplac128 (Gietz and Sugino 1988). The nucleotide sequences of the final constructs were verified before subsequent cloning. *S. cerevisiae* strain TMB 3043, carrying up-regulated genes for the nonoxidative pentose phosphate pathway and endogenous *XKS1*, as well as deletion of *GRE3* (Karhumaa et al. 2005), was used for expression of the *GXF1* construct (Table 1). In this strain, the transporter expression cassette was integrated by linearization of the integrative YIplac128 vector using *Eco*RV and transformation using the LiAc method (Gietz et al. 1995). The resulting strain was transformed with the multicopy plasmid pY7 (Walfridsson et al. 1997), carrying the genes for the initial xylose-metabolizing enzymes *XYL1* and *XYL2*. The resulting strains were named TMB3411 (*TDH3p-GXF1-CYC1t*) and TMB3412 (*TDH3p-GXF1-GXF1t*). Strain TMB 3414 was similarly constructed carrying a *TEF1* promoter from p426TEF (Mumberg et al. 1995), the *GXF1* gene, and the *CYC1* terminator (Table 1). A control strain (TMB 3413) was also constructed by integration of vector YIplac128 (lacking the *GXF1* gene) in TMB 3043 and transformation with the pY7 plasmid (Table 1). The control strain TMB 3413 was thus a homolog of the previously described xylose-utilizing laboratory strain, TMB 3057 (Karhumaa et al. 2005).

Transport kinetics

S. cerevisiae strains TMB 3411 and TMB 3413 were inoculated in 100 mL medium containing YNB (without amino acids) and D-glucose (5 g/L) and grown aerobically at 30°C, 180 rpm, to the exponential phase (OD_{640}=0.7–0.8). Initial uptake rates were determined at 25°C using D-[U-^{14}C] xylose to final concentrations between 0.2 and 100 mM (Fonseca et al. 2007). Kinetic parameters were estimated by nonlinear Michaelis–Menten regression analysis using the Graphpad Prism 3.0 software.

Aerobic batch cultivation in shake flasks

For aerobic growth rates on xylose, yeast strains were inoculated in 25 mL of xylose medium (4 or 40 g/L) in 250 mL baffled shake flasks at a starting OD_{620} of 0.1. Cells were grown at 30°C in an orbital shaker with 200 rpm rotation speed. The growth rate was followed by measuring the optical density at 620 nm. Cultivations were performed in biological triplicate, i.e., three independent cultivations, for each investigated strain.

Aerobic cultivation in bioreactor

Batch cultivation on xylose was performed in an instrumented bioreactor (Applicon Biotechnology, AC Schiedam, the Netherlands) with a working volume of 1 L and a starting OD_{620} of 0.4. The medium (xylose 4 g/L) was prepared as described above with the exception that buffering agent was not added. Antifoam (Dow Corning, Midland, USA) was added to the reactor to a final concentration of 0.5 mL/L. Temperature and agitation rate in the bioreactor were set to 30°C and 950 rpm, respectively. The pH was controlled at 5.5 through addition of 3 M KOH. The culture was sparged continuously with air at a flow rate of 1 L/min, and dissolved oxygen (DO) was monitored using a DO probe. Cultures were sampled for analysis of product formation and substrate consumption. Batch experiments were performed in biological duplicates.

Aerobic-xylose-limited chemostat

Continuous cultivation was performed as described for batch cultivation in bioreactor. Feed medium (xylose 15 g/L) was added continuously at a flow rate determined by the dilution rate. The CO_2 concentration in the off-gas was analyzed on-line using an INNOVA 1313 Fermentation Monitor (LumaSense Technologies A/S, Ballerup, Denmark). Steady state (four to five volume changes) was verified by constant biomass and CO_2 levels. At steady state, samples were taken for substrate and product analysis. Chemostat cultivation was performed in biological triplicate.

Anaerobic batch cultivation

Anaerobic cultivation was performed in jacketed in-house-built fermentors with 150 mL working volume (no head space). Temperature was maintained at 30°C through circulation of water. Agitation with a magnetic

stirrer was set to approximately 100 rpm, and pH was monitored by a PHM61 Laboratory pH meter (Radiometer, Copenhagen, Denmark) and maintained at pH 5.5 through addition of 3 M KOH. Medium was prepared as described above but lacked buffering agent and was supplemented with 1.25 mL/L ergosterol for anaerobic growth (Andreasen and Stier 1953). At the beginning of each experiment, reactors were inoculated to a biomass concentration of 2 g/L. Samples were taken continuously for analysis of substrate and products. Carbon and degree of reduction balances were used to calculate ethanol concentration since about 10% ethanol evaporated. Cultivation was performed in biological duplicates for each investigated strain with less than 10% deviation in any single measurement.

Real-time reverse transcription-PCR measurements

Cells for transcription analysis were grown in duplicates as previously described for aerobic shake-flask cultivation (xylose 4 g/L). From each shake flask, two samples of approximately 5 mL of culture medium were removed and used for RNA extraction. A total of four independent samples were thus processed for each strain and each time point. After sampling, cells were immediately harvested by centrifugation (2 min, 5,000×*g*), and the cell pellet was stored at −20°C. Total RNA was extracted using a beadbeater (Biospecs products, Bartlesville, OK, USA) and phenol/chloroform (Sambrook et al. 1989). DNA contamination of the extracted RNA was reduced by using acid phenol. Prior to reverse transcription, isolated RNA was treated with DNase (Promega, Madison, WI, USA) to remove any remaining traces of DNA. Reverse transcription was performed according to the manufacturer's instructions, using the enzyme SuperScript II and 1.0 μg of DNA-free RNA as template (Invitrogen, Carlsbad, CA, USA). Primers for the reference gene *PDA1* (Wenzel et al. 1995) and the target gene *GXF1* were used together in generation of cDNA. Quantification of transcription levels were performed in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using *Tth*-polymerase and CybrGreen fluorescent dye (Roche Diagnostics, Mannheim, Germany). Conditions in the amplification reaction were as follows: 1× *Tth* buffer; 0.2 mM deoxyribonucleotide triphosphates; 7 mM $MgCl_2$; 0.2 μM primers (reference or target gene); 0.05 U *Tth*-polymerase and 1× CybrGreen. Conditions for the target gene differed in that 3 mM $MgCl_2$ was used instead of 7 mM. The following LightCycler amplification protocol was used for both reference and target gene: initial denaturation for 1 min at 95°C followed by 40 cycles with denaturation for 0 s at 95°C, annealing for 20 s at 59°C, and elongation for 20 s at 72°C. The crossing point (CP) was determined according to the maximum derivative method. Relative expression (RE) was calculated according to Eq. 1 (Pfaffl 2001).

$$\mathrm{RE} = \frac{(E_{\mathrm{target}})^{\Delta \mathrm{CP}_{\mathrm{target}}(\mathrm{control}-\mathrm{sample})}}{(E_{\mathrm{ref}})^{\Delta \mathrm{CP}_{\mathrm{ref}}(\mathrm{control}-\mathrm{sample})}} \quad (1)$$

Analysis of substrate and products

Glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined by high performance liquid chromatography (HPLC), using a Waters HPLC system (Milford, MA, USA). An Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, CA, USA) and a refractive index detector (RID-6a, Shimadzu, Kyoto, Japan) were used for separation and detection, respectively. The column temperature was 45°C, and 5 mM H_2SO_4 was used as a mobile phase at a flow rate of 0.6 mL min^{-1}. Cell dry weight was determined by filtering the sample through a dried and preweighed 0.45-μm pore membrane (Pall Corporation, New York, USA), washing with distilled water, and drying in a microwave oven for 8 min at 350 W.

Results

A total of three *S. cerevisiae* strains expressing the *GXF1* gene from *C. intermedia* were constructed and investigated in the current study. In two strains, different promoter regions, *TDH3* or *TEF1*, were employed. The third strain was constructed carrying the original *GXF1* terminator sequence instead of the endogenous *CYC1* terminator. The effect of alternative terminator sequences was, however, observed to be insignificant, and results from the latter strain are therefore not presented.

Transport kinetics

In order to confirm functional expression of the Gxf1 transporter, *S. cerevisiae* strains TMB 3411 and TMB 3413 were characterized in terms of initial D-[U-^{14}C] xylose uptake rates. Both strains present saturation kinetics with the possible presence of multiple transport systems for D-xylose, as denoted by the nonlinear Eadie–Hofstee plots (not shown). The heterologous expression of Gxf1 affected the overall kinetics of D-xylose transport by increasing the uptake rates to approximately twice their values in the control strain (TMB 3413) for all the xylose concentrations tested. Apparent kinetic parameters were calculated for the transformant and the reference: TMB 3411: V_{max}=30.8±0.7 mmol h^{-1} (g dw)$^{-1}$, K_m=88±4 mM; TMB 3413: V_{max}=22.2±0.8 mmol h^{-1} (g dw)$^{-1}$, K_m=146±8 mM.

Aerobic growth rates

Aaerobic growth rates on xylose were determined for TMB 3411 (expressing Gxf1) and TMB 3413 (control strain) under substrate-saturated and transport-limited conditions. Under substrate-saturated conditions (xylose 40 g/L), the growth rate of the control strain (μ_{max}=0.197 h^{-1}±0.002) was at least as high as the strain overexpressing the Gxf1 transporter (μ_{max}=0.189 h^{-1}±0.005). Under transport-limited conditions (xylose 4 g/L) on the other hand, the growth of the Gxf1-expressing strain was significantly improved (Fig. 1). Since growth rates at nonsaturating substrate concentrations are nonconstant, a linear rather than exponential growth profile was observed for both strains. While the control strain reached OD 3 after 150 h, the Gxf1-expressing strain grew to approximately OD 5 in the same time period.

Aerobic batch cultivation was also performed in an instrumented bioreactor under transport-limited conditions (xylose 4 g/L). These cultivations were analogous to corresponding cultivations in shake flask and similarly showed significantly faster xylose consumption rate in the strain expressing the Gxf1 transporter (Fig. 2). Whereas the control strain was unable to consume xylose at substrate concentrations below 2 g/L, the Gxf1-expressing strain continued to consume xylose throughout the entire fermentation.

Influence of expression levels

The Gxf1-expressing strain TMB 3411 was also compared in terms of aerobic growth rate under transport-limited conditions (xylose 4 g/L) to strain TMB 3414. In this strain, the *GXF1* gene was expressed under control of the *TEF1* promoter rather than the *TDH3* promoter (Table 1). The purpose of this setup was to compare the effects of varying

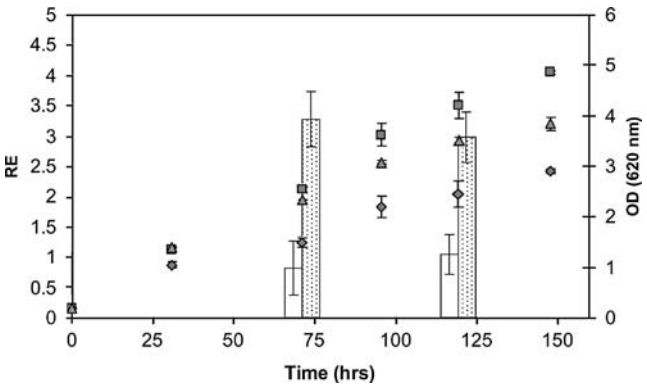

Fig. 1 Aerobic batch growth on 4 g/L xylose and RE of the *GXF1* gene. OD_{620}: *closed squares* TMB 3411; *closed diamonds* TMB 3413; *closed triangles* TMB 3414. RE: *open columns* TMB 3411; *shaded columns* TMB 3414

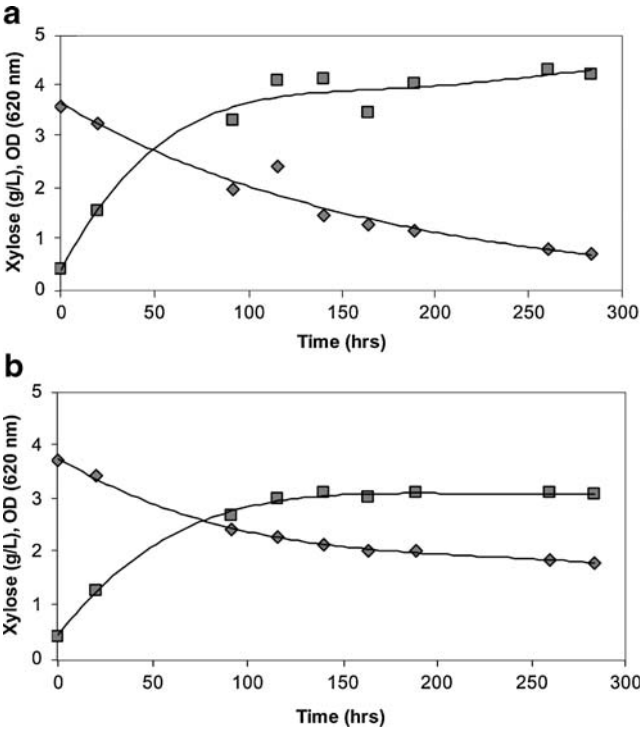

Fig. 2 Xylose consumption and biomass formation in aerobic batch growth on 4 g/L xylose. **a** The Gxf1-expressing strain TMB 3411. **b** The control strain TMB 3413. *Closed squares* OD_{620}; *closed diamonds* xylose (g/L)

Gxf1 expression levels. During the experiments, transcription levels of the *GXF1* gene were analyzed using quantitative reverse transcription-PCR. The TEF strain TMB 3414 grew slower than the TDH strain TMB 3411, however still faster than the control strain TMB 3413 (Fig. 1). The TEF strain showed three times higher transcription level of the *GXF1* gene compared to the TDH strain. Thus, increased expression of the Gxf1 transporter did not yield any additional improvement of the growth rate.

Aerobic-xylose-limited chemostat

The influence of the Gxf1 transporter on xylose metabolism was next investigated in aerobic-xylose-limited chemostat. In the chemostat setup, the residual amount of substrate reflects the concentration required to push a rate-controlling reaction. Thus, a lower steady-state substrate concentration indicates a higher overall affinity for the particular substrate. Cultures were grown at increasing dilution rates (D=0.03 h^{-1} to D=0.12 h^{-1}), and the steady-state biomass and substrate concentration were monitored (Fig. 3). In contrast to glucose-limited chemostat cultures of *S. cerevisiae*, complete substrate consumption of xylose was not possible at any dilution rate. At low dilution rates (D=0.03 h^{-1}), the xylose concentration in the medium was lower for the Gxf1-

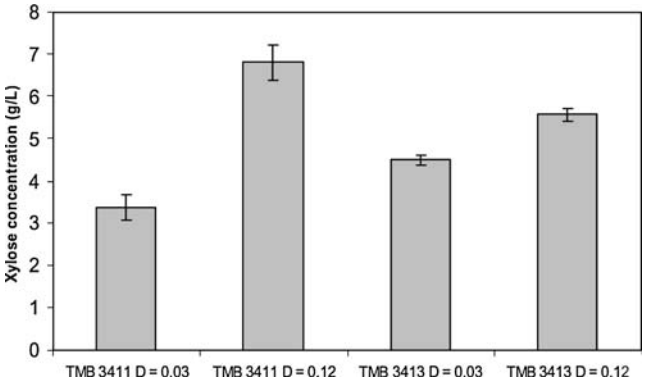

Fig. 3 Steady-state concentration of xylose in aerobic-xylose-limited chemostat. Strains with (TMB 3411) and without the Gxf1 transporter (TMB 3413) were grown at different dilution rates

expressing strain TMB 3411 than for the control strain TMB 3413, indicating that the transporter improved xylose affinity at low substrate concentrations. However, with increasing dilution rates, this effect gradually disappeared, and at D=0.12 h^{-1}, the relation between TMB 3411 and the control strain was reversed so that a higher steady-state substrate concentration was seen in the Gxf1 culture. The results thus complement the results from the aerobic batch cultivation, where an effect of the Gxf1 transporter only was seen at low substrate concentrations.

Anaerobic batch fermentation

The Gxf1-expressing strain TMB 3411 and the control strain TMB 3413 were grown under anaerobic conditions to evaluate the effect of the Gxf1 transporter under oxygen-free conditions (Fig. 4). Under these conditions, TMB 3411 consumed xylose and produced ethanol significantly faster than the control strain (Fig. 4a, b). By-product formation in terms of xylitol production was also higher for TMB 3411, although acetate and glycerol production were slightly lower. At the end of the fermentation, the final ethanol concentration was similar for the two strains. This indicates that, although more xylose was consumed at a faster rate in the Gxf1 strain, a significant portion was excreted as xylitol rather than metabolized to ethanol. When the xylose uptake rate was compared against the xylose concentration in the fermentor, an approximately linear function was seen for both strains (Fig. 4c). Although no kinetic information can be derived from this plot, it is noteworthy that the slope of the curve is significantly steeper for the Gxf1-expressing strain, indicating a higher affinity for the substrate. The difference between the maximum uptake rate (V_{max}=30.8 mmol h^{-1} $(g\ dw)^{-1}$), and the one recorded in Fig. 4 (rXylose=0.8 mmol h^{-1} $(gDW)^{-1}$) reflects the low influence of transport on xylose uptake rate at higher substrate concentrations.

Discussion

Although xylose transport in recombinant *S. cerevisiae* has been extensively investigated, studies have so far yielded moderate improvement of fermentative capacity (Hamacher et al. 2002; Saloheimo et al. 2007; Sedlak and Ho 2004). Out of the native *S. cerevisiae* transporters, Hxt4, 5, and 7 and Gal2 were able to support growth on xylose in an *S. cerevisiae HXT* null strain (Hamacher et al. 2002). However, overexpressing any of these transporters in a xylose-utilizing strain did not improve growth or ethanol production. Similarly, individual expression of either Hxt1, 2, 4, or 7 supported xylose growth, albeit at a much lower rate than a positive control expressing all transporters

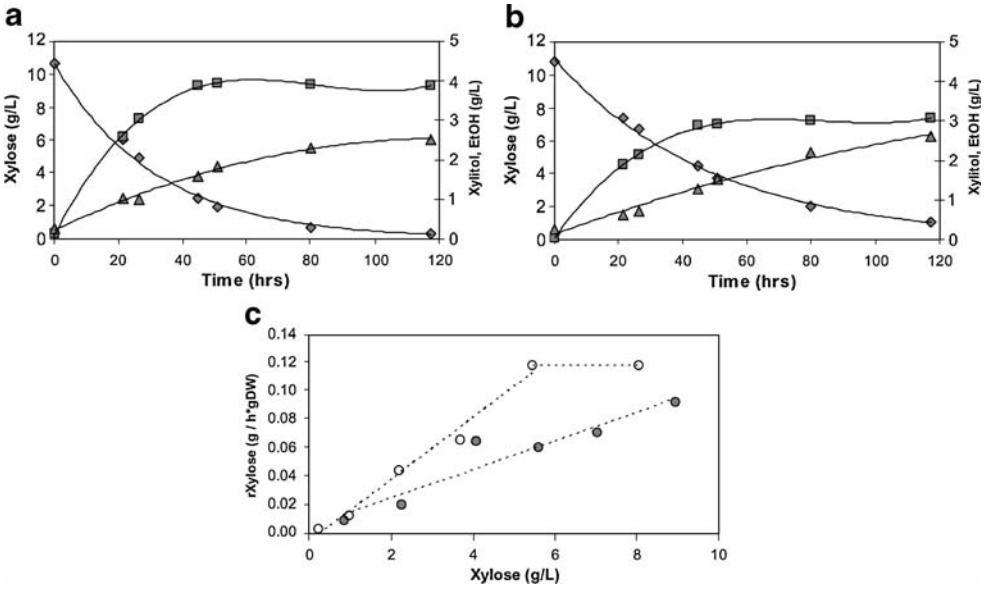

Fig. 4 Anaerobic batch fermentation. **a** The Gxf1-expressing strain TMB 3411. **b** The control strain TMB 3413. *Closed diamonds* xylose; *closed squares* xylitol; *closed triangles* ethanol. **c** Rate of xylose consumption. *Open circles* TMB 3411; *closed circles* TMB 3413

(Saloheimo et al. 2007). In the same study, expression of the Trxlt1 transporter from *H. jecorina* yielded growth on xylose (after adaptation), however at a lower rate than the control strain. In contrast, it was found that single expression of Hxt7 increased the xylose uptake rate by 91% compared to a control strain expressing all transporters (Sedlak and Ho 2004). However, the high substrate concentrations employed (70 g/L glucose and 35 g/L xylose) and the low K_m and V_{max} of Hxt7 impedes the interpretation of these results (Hamacher et al. 2002; Saloheimo et al. 2007). Recently, the expression of the Sut1 transporter from *P. stipitis* (Katahira et al. 2008) and the At5g59250 transporter from *Arabidopsis thaliana* (Hector et al. 2008) have also been shown to improve xylose utilization by recombinant *S. cerevisiae*. In both of these cases, improvement of substrate uptake was seen for both glucose and xylose. Although these results are interesting, different strain backgrounds and experimental setups prevent a direct comparison with the current study.

In the present study, improved xylose transport was demonstrated to increase the xylose uptake rate, growth rate, and ethanol production under fermentative conditions in xylose-utilizing *S. cerevisiae*. The increased fermentative capacity due to expression of the Gxf1 transporter was evident during batch (Figs. 1 and 4) and chemostat (Fig. 3) cultivation, under aerobic and anaerobic conditions. The effect of the transporter was clearly dependent on substrate concentration. Only at low xylose concentrations (~4 g/L) did the expression of Gxf1 improve growth rate and substrate uptake rate. Transport kinetics, on the other hand, showed that the in vitro transport capacity of TMB 3411 was considerably higher than the control strain over the entire investigated substrate range. This apparent discrepancy is a central theme in metabolic control analysis, which describes the substrate uptake rate as a function of the whole pathway rather than a single reaction step (Fell 1992, 1997). Thus, although our results unambiguously confirmed that expression of Gxf1 improved xylose uptake, the physiological effect was restricted to a limited substrate range.

Several reports of overexpressing native or heterologous transporters in recombinant *S. cerevisiae* have failed to see improvement in xylose growth or fermentation (Hamacher et al. 2002; Saloheimo et al. 2007). While these results may be a consequence of the particular transporters, it may also reflect the properties of the host strains. Previous transport studies have typically used xylose-utilizing strains with comparatively poor growth rates and fermentation performance. In such strains, transport only controlled xylose utilization at very low (<0.5 g/L) substrate concentration (Gardonyi et al. 2003a). However, it was also seen that improving the downstream pathway led to increased flux control of the transport step. By using a host strain with high xylose-assimilating capacity (μ_{max}=0.2) within a restricted substrate range in the current study, it is possible that effects of increased transport were observed that otherwise may have been overlooked. In addition, our results using different promoters showed that the expression level of the transporter influences the physiological effects (Fig. 1). Analogous to observing flux control at different substrate concentrations, the effect of expressing the *GXF1* gene is progressively smaller with increasing expression levels. The metabolic burden, on the other hand, is directly related to the extent of heterologous protein expression (Gorgens et al. 2001). Consequently, expressing the Gxf1 permease under nontransport-limiting conditions slightly reduced xylose uptake (Figs. 1 and 3).

The current study clearly demonstrates the influence of the Gxf1 transporter on xylose uptake rate and ethanol production. During fermentation of lignocellulose hydrolysate using industrial recombinant yeast strains, it has been frequently observed that the xylose uptake rate (and consequently ethanol formation) is significantly reduced at low xylose concentrations towards the end of fermentation (Ohgren et al. 2006; Olofsson et al. 2008). Our results indicate that a Gxf1-expressing strain would be of advantage under such circumstances. It will thus be of interest to further study the Gxf1 transporter in an industrial-strain background under industrial conditions.

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