

RESEARCH ARTICLE

Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption

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Abstract

Industrial biotechnology aims to develop robust microbial cell factories, such as *Saccharomyces cerevisiae*, to produce an array of added value chemicals presently dominated by petrochemical processes. Xylose is the second most abundant monosaccharide after glucose and the most prevalent pentose sugar found in lignocelluloses. Significant research efforts have focused on the metabolic engineering of *S. cerevisiae* for fast and efficient xylose utilization. This study aims to metabolically engineer *S. cerevisiae*, such that it can consume xylose as the exclusive substrate while maximizing carbon flux to biomass production. Such a platform may then be enhanced with complementary metabolic engineering strategies that couple biomass production with high value-added chemical. *Saccharomyces cerevisiae*, expressing xylose reductase, xylitol dehydrogenase and xylulose kinase, from the native xylose-metabolizing yeast *Pichia stipitis*, was constructed, followed by a directed evolution strategy to improve xylose utilization rates. The resulting *S. cerevisiae* strain was capable of rapid growth and fast xylose consumption producing only biomass and negligible amount of byproducts. Transcriptional profiling of this strain was employed to further elucidate the observed physiology confirms a strongly up-regulated glyoxylate pathway enabling respiratory metabolism. The resulting strain is a desirable platform for the industrial production of biomass-related products using xylose as a sole carbon source.

Introduction

Xylose is the most abundant pentose sugar in lignocellulosic feedstocks, including hardwoods and crop residues, and is the second most abundant monosaccharide after glucose (Olsson *et al.*, 2004). The demand for industrial biotechnology processes that leverage sustainable, environmentally favourable and cost-effective raw materials as alternatives to petrochemical feedstocks is receiving unprecedented research focus (Otero *et al.*, 2007; Stephanopoulos, 2010). *Saccharomyces cerevisiae* is a proven, robust, industrial production platform used for the expression of a wide range of therapeutic agents, food and beverage components, added value chemicals

(Ostergaard *et al.*, 2000; Chemler *et al.*, 2006; Kim *et al.*, 2012) and commodity chemicals (e.g. bioethanol) across large scales (> 50 000 L) (Olsson *et al.*, 2004; Kumar & Murthy, 2011). Wild-type *S. cerevisiae* is unable to efficiently utilize xylose as a primary substrate. The field has largely focused on metabolic engineering of *S. cerevisiae* for maximizing carbon flux from xylose to bioethanol under anaerobic conditions (Chu & Lee, 2007; Cai *et al.*, 2012); however, the use of *S. cerevisiae* is extended as microbial cell factory for a variety of added value chemicals (Chemler *et al.*, 2006). The design of a *S. cerevisiae* platform for broader biomass-coupled production from xylose would be favoured without the loss of carbon to overflow metabolites (ethanol, glycerol, xylitol) particularly in the case of

growth-associated production processes of non-secreted products that require simultaneous formation of biomass and target compound (e.g. poly-3-hydroxybutyrate, β -carotene and lycopene) (Yamano *et al.*, 1994; Tyo *et al.*, 2010).

Utilization of xylose in yeast and filamentous fungi occurs by a two-step pathway. First, xylose is reduced to xylitol via xylose reductase (XR, primarily NADPH consuming), and then xylitol is oxidized to xylulose via xylitol dehydrogenase (XDH, NADH producing) (Wang *et al.*, 1980). In bacteria, isomerization of xylose to xylulose occurs in a one-step reaction catalysed by xylose isomerase (Misha & Singh, 1993; Harhangi *et al.*, 2003). In yeast, fungi and bacteria, the final conversion of xylulose to xylulose-5P via xylulose kinase (ATP consuming) is conserved.

Recombinant *S. cerevisiae* strains expressing the *Pichia stipitis* xylose reductase (*PsXYL1*), and *P. stipitis* xylitol dehydrogenase (*PsXYL2*) has lead to transformants that can oxidatively and exclusively consume xylose, although resulting in significant xylitol production (Kötter *et al.*, 1990; Kötter & Ciriacy, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1995). While over-expression of the endogenous *XKS1* encoding xylulokinase improved the xylose utilization rate (Ho *et al.*, 1998; Eliasson *et al.*, 2000; Tiovari *et al.*, 2001), xylitol formation persisted. There is a redox imbalance, which results from recombinant co-expression of XR and XDH, and because of the lack of transhydrogenase activity in *S. cerevisiae*, and thereby inability to interconvert NADPH and NADH, there is a surplus formation of NADH and NADP⁺. Numerous metabolic engineering efforts employed to alleviate the redox imbalance are discussed above and to further improve the xylose consumption rate have been reviewed extensively (Amore *et al.*, 1991; Kuyper *et al.*, 2003, 2004, 2005; Hahn-Hägerdal *et al.*, 2007; van Maris *et al.*, 2007; Matsushika *et al.*, 2009; Van Vleet & Jeffries, 2009).

Among the possible bottlenecks investigated in xylose metabolism, several limiting steps have been identified. The reduced ability of *S. cerevisiae* to grow efficiently on xylose has been attributed to: (1) the inefficient xylose uptake (Amore *et al.*, 1991; Eliasson *et al.*, 2000; Kuyper *et al.*, 2003, 2004, 2005), (2) the insufficient level of expression of xylose transporters to enable significant sugar assimilation (Kötter & Ciriacy, 1993), (3) the redox imbalance generated in the first two steps of xylose metabolism involving the XDH and XR from *P. stipitis* (Kötter & Ciriacy, 1993; Roca *et al.*, 2003), (4) the level of aeration (Skoog & Hahn-Hägerdal, 1990; du Preez, 1994; Walfridsson *et al.*, 1995), (5) insufficient pentose phosphate pathway activity (Kötter & Ciriacy, 1993; Walfridsson *et al.*, 1995) and (6) the inability of pentose

sugar metabolism to activate the lower part of glycolysis (Boles *et al.*, 1993; Müller *et al.*, 1995).

Because of the previously described specificity of XR for NADPH and XDH for NAD⁺ and the resulting redox imbalance, xylose metabolism is partially regulated by the availability of oxygen in both native and metabolically engineered yeasts (Skoog & Hahn-Hägerdal, 1990; du Preez, 1994; Ho *et al.*, 1998). In the presence of oxygen, excess NADH produced via NAD-dependent XDH can be respired and the NADPH demand for the XR reaction provided by the oxidative part of the pentose phosphate pathway. The level of oxygenation determines the split in carbon flux between biomass and ethanol production under aerobic conditions where xylose is mainly converted into biomass, while ethanol production is favoured under anaerobic conditions (du Preez, 1994). The incomplete respiration of excess NADH under anaerobic conditions leads *S. cerevisiae* to produce and accumulate glycerol followed by xylitol. The xylose consumption rate and the assimilation to biomass increase with increasing aeration level, relieving the accumulation of NADH, yet still resulting in glycerol and xylitol formation (Müller *et al.*, 1995; Jin *et al.*, 2004).

This study aims to metabolically engineer *S. cerevisiae* such that it can consume xylose as the exclusive substrate while maximizing carbon flux to biomass production. Such a platform may then be enhanced with complementary metabolic engineering strategies that couple biomass production with high value-added chemicals. *Saccharomyces cerevisiae* CEN.PK 113-3C, expressing *PsXYL1* (encoding xylose reductase, XR), *PsXYL2* (encoding xylitol dehydrogenase, XDH) and *PsXYL3* (encoding xylulose kinase, XK) from the native xylose-metabolizing yeast *P. stipitis*, was constructed, followed by a directed evolution strategy to improve xylose utilization rates. The resulting strains were physiologically characterized under aerobic controlled batch fermentations supplemented with glucose and xylose. Transcriptional profiling was employed to further elucidate the strain physiology.

Materials and methods

Saccharomyces cerevisiae strain descriptions

All of the strains constructed in this study were derived from the reference *S. cerevisiae* strain, CEN.PK 113-7D (van Dijken *et al.*, 2000). The strains and plasmids used in this study are listed in Table 1. The strain that was modified using directed evolution is referred to as *evolved*.

Strain CMB.GS001 was derived from the *S. cerevisiae* CEN.PK 113-3C wild-type strain. This strain was transformed with the centromeric plasmid pRS314-X123,

Table 1. *Saccharomyces cerevisiae* strain and plasmid used in this study

Strain or plasmid	Relevant genotype	Origin/reference
CEN.PK 113-7D	<i>MATa URA3 HIS3 LEU2 TRP1 SUC2 MAL2-8^C</i>	SRD GmbH*
CEN.PK 113-3C	<i>MATa URA3 HIS3 LEU2 trp1-289 SUC2 MAL2-8^C</i>	SRD GmbH*
CMB.GS001	<i>MATa URA3 HIS3 LEU2 TRP1 SUC2 MAL2-8^C</i> <i>pTDH3-PsXYL1 pTDH3-PsXYL2 pTDH3-PsXYL3</i>	This study
CMB.GS010†	<i>MATa URA3 HIS3 LEU2 TRP1 SUC2 MAL2-8^C</i> <i>pTDH3-PsXYL1 pTDH3-PsXYL2 pTDH3-PsXYL3</i> <i>Evolved</i>	This study
pRS314-X123	<i>pTDH3-PsXYL1 pTDH3-PsXYL2 pTDH3-PsXYL3 (TRP1, Centromeric)</i>	Haiying <i>et al.</i> (2007)

*Scientific Research and Development GmbH, Oberursel, Germany.

†Single colony isolated after repetitive batch evolutionary process. The three final digits of the strain identifier indicate from which cycle in the directed evolution the strain originated, with the starting strain referred to as CMB.GS001.

expressing *TRP1* encoding for N-(5' phosphoribosyl)-anthranilate isomerase. Into the plasmid pRS314-X123 *PsXYL1* encoding xylose reductase (PsXRp), *PsXYL2* encoding xylitol dehydrogenase (PsXDhp) and *PsXYL3* encoding xylulokinase (PsXKp) all derived from *P. stipitis* were cloned under the glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) constitutive promoter and terminator (Haiying *et al.*, 2007).

Strains CMB.GS010 were evolved from CMB.GS001 after cycles of repetitive culture selection in shake flasks. The three final digits of the strain identifier indicate from which cycle in the repetitive culture the strain originated, with the starting strain referred to as CMB.GS001 (see Directed evolution and selection of strain CMB.GS010, for details).

Yeast strain transformation

Saccharomyces cerevisiae strain CEN.PK113-3C was transformed with plasmid pRS314-X123 (Haiying *et al.*, 2007) using a traditional lithium acetate treatment (Gietz & Woods, 2002). Transformants were selected using synthetic dextrose agar plates without tryptophan (ScD-trp).

Directed evolution and selection of strain CMB.GS010

Mutants of CMB.GS001 with higher specific growth rates on xylose were selected for by serial transfer of cells using repetitive cultures in shake flasks. Specifically, a 500-mL shake flask containing 100 mL of synthetic minimal medium with 20 g L⁻¹ xylose was inoculated with CMB.GS001. After 60 h, a new shake flask culture having the same medium composition was inoculated with cells from the preceding shake flask at an initial OD_{600 nm} of 0.025. This procedure was repeated for four iterations. Thereafter, the culture time was reduced to 48 h. This 48-h cultivation was repeated for six iterations after which strain CMB.GS010 was isolated. Cryovials of stock

cultures were prepared following every cycle of repetitive culture. Culture samples were streaked on plates with the same selective condition used throughout the evolution process (minimal media supplemented with 20 g L⁻¹ xylose) and growth at 30 °C. Three randomly selected single clones were re-streaked once and thereafter grown in shake flask (see Shake flask cultivation) when the late exponential phase was reached as determined by biomass optical density measurements at 600 nm (OD_{600 nm}), 25% (v/v) sterile glycerol was added, and 1.5 mL sterile cryovials were prepared and stored at -80 °C. From this final evolutionary cycle of the three isolates, the fastest growing strain was designed CMB.GS010 and used for further characterization.

Medium preparation

A previously described synthetic minimal medium containing trace elements and vitamins was used for all shake flasks and stirred tank cultivations (Verduyn *et al.*, 1992). Tryptophan was supplemented for the cultivations of CEN.PK113-3C to satisfy the auxotrophy.

The medium used for stirred tank batch cultivations had the following composition: 5 g L⁻¹ (NH₄)₂SO₄, 3 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 1 mL L⁻¹ trace element solution, 1 mL L⁻¹ vitamin solution, 0.5 mL L⁻¹ antifoam 204 (Sigma A-8311) and 1.25 mL L⁻¹ Ergosterol/Tween 80 solution (final concentration 0.01 g L⁻¹ Ergosterol and 0.42 g L⁻¹ Tween 80). The fermentation medium was pH adjusted to 5.0 with 2 M NaOH and autoclaved. For the cultivations on glucose, the concentration was 20 g L⁻¹, and for the cultivations on xylose, the concentration was 20 g L⁻¹. Both the sugar solutions were added by sterile filtration using a cellulose acetate filter (0.20 µm pore size Minisart®-Plus Satorius AG).

The medium used for shake flask cultivations had the same composition as described above, but the (NH₄)₂SO₄ concentration was increased to 7.5 g L⁻¹ and the

KH_2PO_4 to 14.4 g L^{-1} together with 20 g L^{-1} of glucose or xylose, and the pH was adjusted to 6.5 prior to autoclaving.

A solid synthetic minimal medium containing 5 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, 3 g L^{-1} KH_2PO_4 , 0.5 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL L^{-1} trace element solution, 1 mL L^{-1} vitamin solution, supplemented with 20 g L^{-1} xylose 20 g L^{-1} agarose was used to maintain and isolate the evolved mutant.

A yeast extract peptone dextrose (YPD) complex medium was used for yeast growth prior to transformation with the following composition (g L^{-1}): 10 yeast extract, 20 peptone, 20 glucose and 20 agar.

A synthetic dextrose minus tryptophan medium (ScD-trp) was used as selective media post-transformation with the following composition (g L^{-1}): 7.25 Dropout powder (J.T. Baker), 20 agar and 20 glucose.

Shake flask cultivation

Cultivations were carried out in 500-mL baffled Erlenmeyer flasks with two diametrically opposite baffles and side necks for aseptic sampling by syringe. The flasks were prepared with 100 mL of medium as previously described and cultivated in a rotary shaker at 150 r.p.m. with the temperature controlled at 30°C . The pH of the medium was adjusted to 6.5 with 2 M NaOH prior to sterilization.

Stirred tank batch fermentations

Stirred tank cultivations were performed in 2.2 L Braun Biotech Biostat B fermentation systems with a working volume of 2 L. The cultivations were operated at aerobic and anaerobic conditions with glucose or xylose as the carbon source. The fermenters were integrated with the Braun Biotech Multi-Fermenter Control System (MFCS) for data acquisition. The temperature was controlled at 30°C , and agitation was maintained at 600 r.p.m. Dissolved oxygen was monitored using an autoclavable polarographic oxygen electrode. During aerobic fermentations, the sparging flow rate of air was 2 vvm (volume per volume minute). During anaerobic cultivations, nitrogen containing $< 5 \text{ ppm O}_2$ was used for sparging at a flow rate of 2 vvm, with $< 1\%$ air-saturated oxygen in the fermenter as confirmed by the dissolved oxygen measurement and the off-gas analyser. The pH was controlled constant at 5.0 by automatic addition of 2 M KOH. Off-gas passed through a condenser cooled to 4°C to minimize evaporation, and oxygen and carbon dioxide concentrations were determined by the off-gas analyser as previously described (Christensen *et al.*, 1995). Fermentations were inoculated from shake flask precultures to a starting $\text{OD}_{600 \text{ nm}}$ 0.01.

Analysis

Cell mass determination

The optical density was determined at 600 nm using spectrophotometer (Shimadzu UV mini 1240). Dry cell weight measurements were determined as previously described (Nielsen & Olsson, 1997).

Extracellular metabolite analysis

Extracellular metabolite concentrations were determined by HPLC as previously described (Eliasson *et al.*, 2000).

Transcriptomics

RNA sampling and isolation

Samples for RNA isolation from the late exponential phase of glucose-limited and xylose-limited batch, and continuous cultivations were taken by rapidly sampling 25 mL of culture into a sterile tube with crushed ice. Cells were immediately centrifuged (5000 g at 0°C for 2.5 min), the supernatant was discarded and the pellet was frozen in liquid nitrogen. Total RNA was extracted using the RNeasy[®] Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA sample integrity and quality were determined prior to hybridization with an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit according to the manufacturer's instruction (Agilent, Santa Clara, CA).

Probe preparation and hybridization to DNA microarrays

Messenger RNA (mRNA) extraction, cDNA synthesis, labelling and array hybridization to Affymetrix Yeast Genome Y2.0 arrays were performed according to the manufacturer's recommendations (Affymetrix GeneChip[®] Expression Analysis Technical Manual, 2005–2006 Rev. 2.0). Washing and staining of arrays were performed using the GeneChip Fluidics Station 450 and scanning with the Affymetrix GeneArray Scanner (Affymetrix, Santa Clara, CA).

Microarray gene transcription analysis

Affymetrix Microarray Suite v5.0 was used to generate CEL files of the scanned DNA microarrays. These CEL files were then processed using the statistical language and environment R v2.9.1 (R Development Core Team, 2007, www.r-project.org), supplemented with BIOCONDUCTOR v2.3 (Bioconductor Development Core Team, 2008,

www.bioconductor.org) packages Biobase, affy, gcrma and limma (Smyth, 2005). The probe intensities were normalized for background using the robust multiarray average (RMA) method only using perfect match (PM) probes after the raw image file of the DNA microarray was visually inspected for acceptable quality. Normalization was performed using the qspline method, and gene expression values were calculated from PM probes with the median polish summary. Statistical analysis was applied to determine differentially expressed genes using the limma statistical package. Moderated *t*-tests between the sets of experiments were used for pair-wise comparisons. Empirical Bayesian statistics were used to moderate the standard errors within each gene, and Benjamini–Hochberg method was used to adjust for multi-testing. A cut-off value of adjusted $P < 0.01$ (referred to as P_{adjusted}) was used for statistical significance, unless otherwise specified (Smyth, 2005). Gene ontology process annotation was performed by submitting differentially expressed gene (adjusted $P < 0.01$) lists to the *Saccharomyces* Genome Database GO Term Finder resource and maintaining a cut-off value of $P < 0.01$ for hypergeometric testing of cluster frequency compared to background frequency (Ball *et al.*, 2000). Successively, the reporter feature algorithm (Patil & Nielsen, 2005) has been applied on the dataset to identify transcription factor analysis (TFs) around which the most significant changes occur. Metabolic pathway mapping was performed using Pathway Expression Viewer of the *Saccharomyces* Genome Database, where lists of differentially expressed genes ($P_{\text{adjusted}} < 0.01$, $|\log\text{-fold change}| > 1$) between two conditions were submitted (Ball *et al.*, 2001).

Results

Physiological characterization of CMB.GS001

Batch cultivations of the xylose-fermenting *S. cerevisiae* strain CMB.GS001 was investigated in synthetic medium supplemented with 20 g L^{-1} xylose. In contrast to the reference strain CEN.PK 113-7D, which cannot grow on xylose, the recombinant strain grew aerobically on xylose with a specific growth rate of 0.02 h^{-1} and a xylose consumption rate of $0.08 \text{ g (g dry cell weight)}^{-1} \text{ h}^{-1}$, $< 2 \text{ g L}^{-1}$ xylose was consumed (Fig. 1 and Table 2).

Directed evolution of CMB.GS001

Directed evolution was applied to select a spontaneous mutant with higher specific growth rate on xylose. The constructed xylose-fermenting strain CMB.GS001 was subjected to repetitive serial transfers in batch shake flask cultivations with minimal medium supplemented with

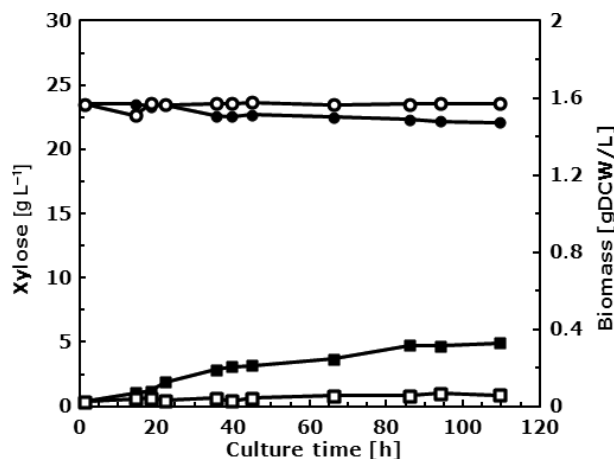


Fig. 1. Time course of aerobic batch culture on defined minimal medium supplemented with 20 g L^{-1} xylose of strain CEN.PK113-7D (empty symbols) and CMB.GS001 (filled symbols). Xylose, (circle) (g L^{-1}) and biomass (square) (g DCW L^{-1}) concentrations are presented as the functions of cultivation time. Data represent the average of three independent cultures.

20 g L^{-1} xylose. This approach targeted strain selection based on biomass formation rate, directly coupled to the xylose consumption rate. After four batch cultures, strain CMB.GS001 demonstrated an appreciable improvement in xylose consumption (Fig. 2a). After serial cultivations, over 10 cycles covering a period of 500 h (21 days) the xylose consumption for strain CMB.GS010 increased 15-fold to 20 g L^{-1} and the biomass production increased 52-fold to $9.37 \text{ (g dry cell weight)}^{-1} \text{ h}^{-1}$ (Fig. 2a). A total of 74 cell generations were produced across the ten cycles of directed evolution resulting in a doubling time decrease of sixfold from 34.7 to 5.42 h, with the final 50–74 generations not yielding any decrease in the doubling time (Fig. 2b).

In order to investigate the possible causes of the dramatic increase in the xylose consumption of CMB.GS010, the plasmid was removed and sequenced, but no mutation was detected compared to the original plasmid pRS314-X123 suggesting that the improved xylose consumption rate is a consequence of mutations in the genome level and not in the plasmid carrying the properties needed for xylose metabolism.

Physiological characterization of CMB.GS010

Batch xylose fermentation

Strain CMB.GS010 was physiologically characterized in aerobic batch fermentations supplemented with 20 g L^{-1} xylose or 20 g L^{-1} glucose. Xylose was completely consumed within 60 h with biomass ($62\% \text{ Cmol Cmol}^{-1}$ xylose) and carbon dioxide ($37\% \text{ Cmol Cmol}^{-1}$ xylose)

Table 2. Physiological parameters obtained during aerobic batch cultivation of strains CMB.GS001, CMB.GS010 and reference strain CEN.PK113-7D. Values represent the mean $\pm$ SD of two independent fermentations performed in triplicate ($n = 3$)

Strain	CMB.GS001	CMB.GS010			CEN.PK113-7D
Carbon source	Xylose	Xylose	Glucose	Glucose/Xylose*	Glucose†
Specific growth rate (h^{-1})	0.02 ± 0.02	0.18 ± 0.01	0.34 ± 0.01	0.32 ± 0.01	0.36
Sugar consumed (Cmol L^{-1})					
Glucose	–	–	0.64 ± 0.01	0.31 ± 0.02	0.66
Xylose	0.07 ± 0.02	0.53 ± 0.04	–	0.05 ± 0.01	–
Sugar consumption rate [$\text{g (g dry cell weight)}^{-1} \text{h}^{-1}$]					
Glucose	–	–	2.31 ± 0.06	2.62 ± 0.05	2.36
Xylose	0.08 ± 0.004	0.31 ± 0.02	–	0.21 ± 0.03	–
Biomass yield (Cmol Cmol^{-1})	0.48 ± 0.03	0.62 ± 0.01	0.16 ± 0.02	0.19 ± 0.01	0.15
Carbon recovery (%)	94.9 ± 3.5	100.1 ± 1.1	105.7 ± 2.5	103.6 ± 2.1	103.7
Productivities [$\text{g (g dry cell weight)}^{-1} \text{h}^{-1}$]					
CO ₂	n.a.	0.21 ± 0.01	0.53 ± 0.001	0.53 ± 0.004	0.49
Ethanol	n.a.	0.02 ± 0.002	0.76 ± 0.002	0.79 ± 0.001	0.96
Xylitol	n.a.	0	0	0	0
Glycerol	n.a.	0.002 ± 0.001	0.10 ± 0.002	0.17 ± 0.001	0.18
Acetate	n.a.	0	0.08 ± 0.003	0.04 ± 0.007	0.02

n.a., not available, because of the relative minimal growth.

*Values relative to the first phase of growth (phase I Fig. 4) when glucose with a small fraction of xylose are used.

†Values from Otero JM, unpublished.

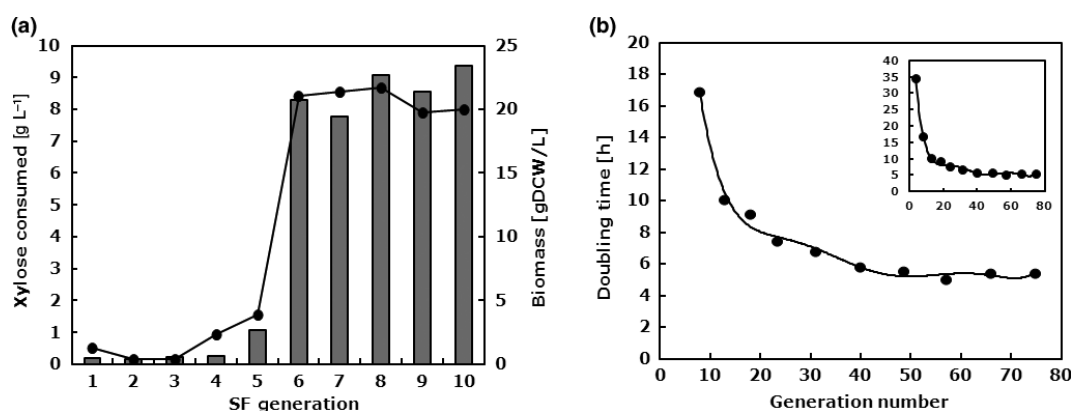


Fig. 2. (a) Comparison in xylose consumption (bars) and biomass production (line) during repetitive growth of *Saccharomyces cerevisiae* CMB.GS001 in shake flask cultures on synthetic medium with 20 g L⁻¹ xylose. Shake flask generation represents the number of specific shake flasks in the series of repetitive cultivations performed to select for mutants with higher specific growth rates and xylose utilization rates (for details, see Directed evolution and selection of strain CMB.GS010, Materials and methods). (b) Doubling time during the serial transfers of *S. cerevisiae* in shake flask cultures on synthetic medium with 20 g L⁻¹ xylose as a function of the number of cell generations. Each data point represents the doubling time of a single shake flask culture estimated from OD_{600 nm} measurements. The small plot in the top right represents all 10 cycles, noting the initial doubling time of CMB.GS001 of 35 h, and the rapid decrease to 10 h within < 20 cell generations. For cell generations 50–74, there was no significant improvement in the specific growth rate.

as the major fermentation products, noting the complete absence of xylitol during the culture (Table 2). The xylose consumption rate was highest 0.31 g xylose (g dry cell weight)⁻¹ h⁻¹ when the extracellular xylose concentration was above 10 g L⁻¹, as demonstrated by the biomass concentration and peak carbon evolution rate (Fig. 3a) subsequently decreasing to 0.08 g xylose (g dry cell weight)⁻¹ h⁻¹ until xylose exhaustion.

To further investigate whether xylose consumption is sensitive to changes in extracellular xylose concentration,

CMB.GS010 was cultivated with synthetic media supplemented with 10 g L⁻¹ xylose. Under this condition, the strain exhibits a maximum specific growth rate of 0.11 h⁻¹ compared with 0.18 h⁻¹ when supplemented with 20 g L⁻¹ xylose. The reduced extracellular concentration of xylose to 10 g L⁻¹ resulted in an increased lag phase (12–24 h) and maximum specific xylose consumption rate of 0.26 g xylose (g dry cell weight)⁻¹ h⁻¹.

Ability of CMB.GS010 to grow under anaerobic condition was tested in batch fermentation conditions with

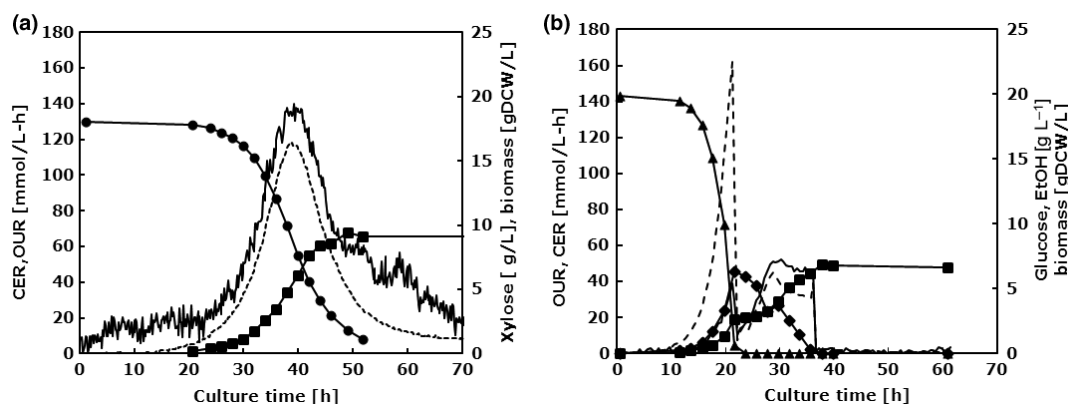


Fig. 3. Time course of aerobic batch culture on defined minimal medium supplemented with 20 g L⁻¹ xylose (a) and 20 g L⁻¹ glucose (b) of strain CMB.GS010 (evolved strain). For all plots presented, carbon evolution rate (CER) (dashed line), oxygen uptake rate (OUR) (solid line) (mM h⁻¹), and xylose (circle), glucose (triangle), ethanol (diamond) (g L⁻¹); and biomass (square) (g DCW L⁻¹) concentrations as the functions of cultivation time. Data represent the average of three independent cultures.

20 g L⁻¹ xylose as the sole carbon source. After 100 h, no growth or xylose consumption was observed. To ensure that the absence of growth was a direct consequence of the anaerobic environment, a recovery experiment was performed, where the culture was aerated quickly from anaerobic to aerobic condition. Growth was immediately restored to the above-described aerobic physiology (data not shown).

Batch glucose fermentation

The aerobic and anaerobic physiology of CMB.GS010 was evaluated in glucose-supplemented batch fermentations to quantify the possible effects of directed evolution on the maximum specific growth rate and the product yields compared to the reference strain CEN.PK113-7D. During aerobic conditions, strain physiology was comparable to previous results with CEN.PK 113-7D (Fig. 3b). The main differences were a small reduction in the maximum specific growth rate and a fourfold higher acetate production, and a small reduction in ethanol production (Table 2). Anaerobic cultivation also showed similar results. Similarly, a reduction in the maximum specific growth rate and in the ethanol yield was observed (data not shown).

Batch mixed substrate fermentation

In order to investigate the proprieties of the strain CMB.GS010 with respect to mixed sugar utilization, the strain was grown aerobically in a mixture containing 10 g L⁻¹ glucose and 10 g L⁻¹ xylose. The results show that both sugars were completely consumed, however with glucose remaining the preferred substrate. Three different growth phases can be identified (Fig. 4). During the first growth

phase (0–21 h), cells consumed 10 g L⁻¹ glucose and 1.6 g L⁻¹ xylose in the same period (16% more carbon resulting from xylose consumption). The maximum specific growth rate was slightly lower compared with the growth on glucose only (Table 2). Following glucose exhaustion, there was a second growth phase (21–32 h) where the remaining xylose, 8 g L⁻¹ (0.27 Cmol L⁻¹), was consumed in conjunction with the re-assimilation of ethanol produced during the glucose consumption phase. During this phase, 0.17 Cmol L⁻¹ xylose and 0.15 Cmol L⁻¹ ethanol were consumed. In this phase, the maximum specific growth rate decreased 2.5-fold from 0.32 to 0.13 h⁻¹. The maximum xylose consumption rate during the first growth phase on glucose was 0.21 g (g dry cell weight)⁻¹ h⁻¹. Once glucose was depleted, the maximum xylose consumption rate was 0.18 g (g dry cell weight)⁻¹ h⁻¹. After ethanol re-assimilation, the xylose consumption continued until all the sugar was consumed in the third and final growth phase (> 32 h) with a reduced maximum consumption rate of 0.06 g (g dry cell weight)⁻¹ h⁻¹. In contrast to the glucose consumption phase, the xylose–ethanol phase was characterized by a large production of biomass, corresponding to a 28% increase in biomass yield (Cmol Cmol⁻¹).

The fermentation characteristics of strain CMB.GS010 were also investigated under anaerobic growth on a medium containing 10 g L⁻¹ glucose and 10 g L⁻¹ xylose. However, only glucose was fully consumed (data not shown).

Transcriptome characterization

Transcriptome characterization was performed with the evolved strain (CMB.GS010) cultivated in batches with xylose and glucose as carbon sources, and the un-evolved

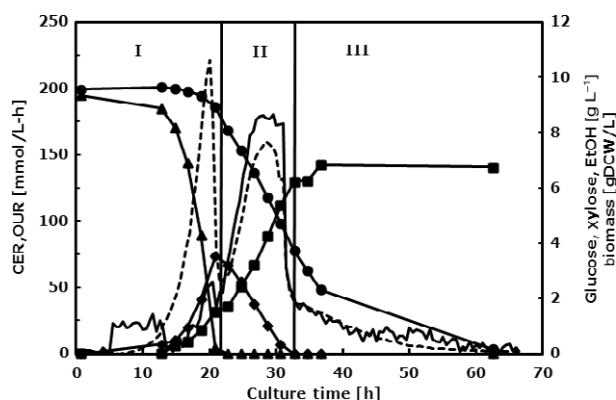


Fig. 4. Time course of aerobic mixed substrate batch cultivation on defined minimal medium supplemented with 10 g L^{-1} xylose and 10 g L^{-1} glucose of strain CMB.GS010 (evolved strain). Carbon evolution rate (CER) (dashed line), oxygen uptake rate (OUR) (solid line) (mM h^{-1}), and xylose (circle), glucose (triangle), ethanol (diamond) (g L^{-1}) and biomass (square) (g DCW L^{-1}) concentrations as functions of cultivation time. Vertical line identifies the three different growth phase (I, II and III). Data represent the average of three independent cultures.

strain (CMB.GS001) with glucose as the sole carbon source in batch cultivations. This different cultivation conditions were selected to elucidate overall carbon flux distributions observed in CMB.GS010 compared to CMB.GS001. The specific comparisons made were focused on identifying fermentative vs. respiro-fermentative metabolism for growth on the different carbon sources. Similar to other studies, the direct comparison with the un-evolved strain on xylose was not possible because of the poor and un-exponential growth on minimal media (Salusjärvi *et al.*, 2006).

Principal component analysis (PCA) of the expression data after normalization showed that the evolved strain grown on xylose in batch conditions clustered with clear separation from the evolved and un-evolved strain grown on glucose (data not shown). Gene ontology (GO) term enrichment analysis from the significant differential gene expressions between the evolved strain (CMB.GS010) grown on xylose or glucose and the un-evolved (CMB.GS001) grown on glucose was performed. A schematic representation of the GO process term identified (P -value < 0.01) is represented in Fig. 5a. The terms 'citrate cycle (TCA cycle)', 'glyoxylate and dicarboxylate metabolism', 'peroxisome' and 'oxidative phosphorylation' were among the most represented functional category with respect to metabolism of the evolved strain on xylose compared with the un-evolved or evolved strains grown on glucose. In contrast, processes related to biosynthesis of several amino acids were repressed. TF analysis was used to analyse whether the evolved mutant physiology was affected at a global regulatory level. Figure 5b represents

the increase in the expression of genes regulated by different TFs. The main carbon catabolite repressor regulator *SNF1* and several of its known targets *PIP2*, *OAF1*, *CAT8* and *MIG1*, the carbon source responsive *ADR1* and the four subunits of the global respiratory regulator *HAP* complex (*HAP1*, *HAP2*, *HAP3* and *HAP4*) were among the identified over-represented TFs in the evolved strain grown on xylose vs. the un-evolved strain grown on glucose. Interestingly, expression of genes regulated by TFs linked to the glucose sensor *RGT2*, such as *STD1*, and *SNF1* target TFs like *INO2* and *INO4* (involved in the regulation of lipid metabolism) were down-regulated when the evolved and un-evolved strains were compared on glucose. The GO and TF results were consistent with the differences in metabolism expressed by the evolved strain grown on the two sugars. Further, detailed analysis was performed to analyse the change of gene expression at the metabolic pathway level (Fig. 6). The significant mRNA up-regulation of genes encoding enzymes of the central carbon metabolism often correlating with respiration in the TCA cycle and glyoxylate pathways correlates well with the physiological observations that growth on xylose is dominated by respiratory metabolism. The glyoxylate pathway (*ICL1*, *MLS1*, *MDH2*, *AGX1* encoding isocitrate lyase, malate synthase, malate dehydrogenase and glyoxylate aminotransferase) was significantly up-regulated in the evolved strain grown on xylose compared to the evolved strain grown on glucose or the un-evolved strain grown on glucose. This pathway had a significantly higher log-fold change than succinate dehydrogenase, α -ketoglutarate dehydrogenase and succinyl-CoA ligase (*SDH1*, *SDH2*, *SDH3*, *SDH4*, *KGD1*, *KGD2* and *LSC2*, respectively), suggesting that it plays an important role during xylose respiratory metabolism of *S. cerevisiae* as found from studies in chemostat cultures (Regenberg *et al.*, 2006). Finally, *IDP2* and *IDP3* (encoding two isocitrate dehydrogenase) were up-regulated significantly in batch xylose cultivations with the evolved strain (Fig. 6).

The evolved strain, when cultivated on xylose in a batch mode, is able to utilize the glyoxylate bypass to efficiently respire the carbon source. Similar to previous report, the up-regulation of *HXXK1* and *GLK1* (encoding hexokinase isoenzyme 1 and glucokinase) supports the hypothesis that xylose is identified as a non-fermentable carbon source and therefore respired (Herrero *et al.*, 1995; Jin *et al.*, 2004; Salusjärvi *et al.*, 2008; Runquist *et al.*, 2009). Furthermore, the expression levels of *MDH2*, *PCK1* and *FBP1* (encoding malate dehydrogenase, phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase, respectively) were up-regulated in the evolved strain cultivated on xylose compared to the evolved or un-evolved strain cultivated on glucose, indicating some glyconeogenic activity. It should be

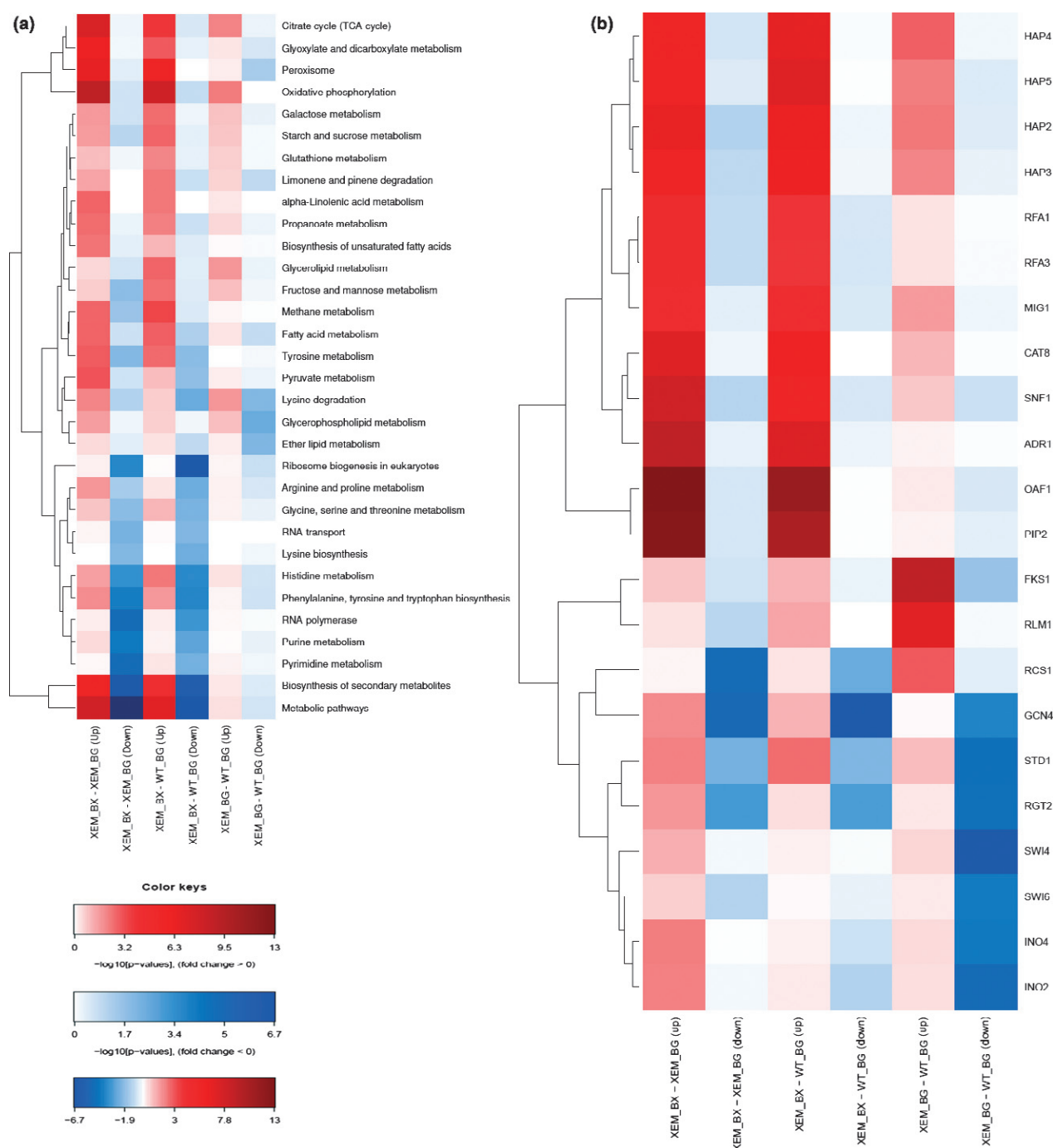


Fig. 5. Integrated analysis of gene ontology (GO) process terms (a) and TFs analysis (b), the cluster frequency is presented on the y-axis. Compared conditions are the evolved strain CMB.GS010 strain cultivated on batch xylose (XEM_BX), evolved strain CMB.GS010 cultivated in batch glucose (XEM_BG) and the un-evolved strain CMB.GS001 cultivated on batch glucose (WT_BG). Colour key indicates the different expression in log-fold change ($P_{\text{adjusted}} < 0.01$).

mentioned though that these genes are also up-regulated at low dilution rates in glucose-limited chemostat cultures (Regenberg *et al.*, 2006), and expression of these genes may therefore be associated with respiratory metabolism.

The mRNA expression profile of the evolved strain cultivated on xylose suggests a strong flux towards glucose-6-phosphate, requiring inspection of the pentose phosphate pathway (PP pathway). The comparison of differential gene expression in the PP pathway is represented

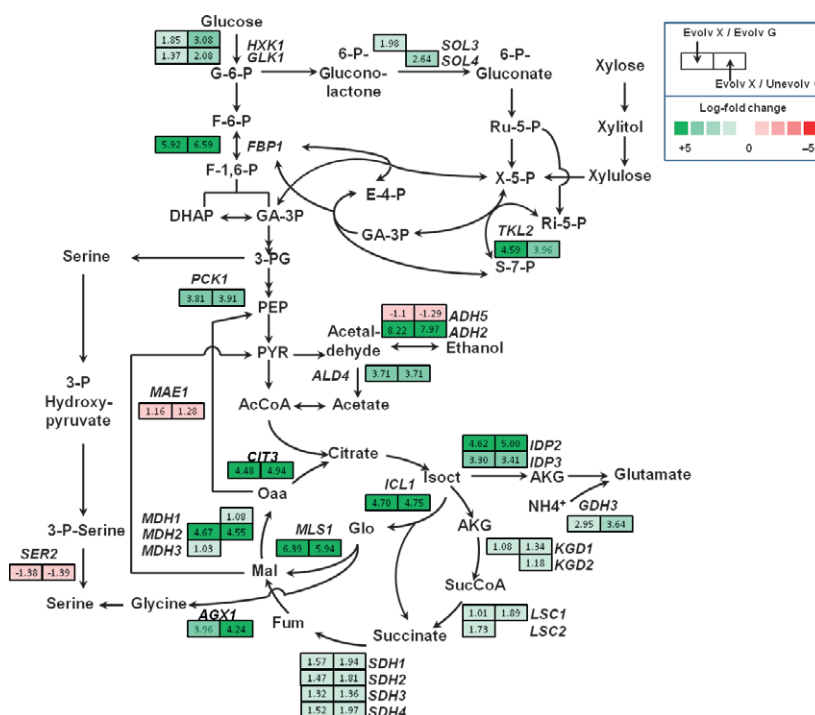


Fig. 6. Gene expression levels of central carbon metabolic pathways: tricarboxylic acid (TCA) cycle, glyoxylate pathway, glutamine/glutamate synthesis and pentose phosphate (PP) pathway are presented. The comparative conditions evaluated include CMB.GS010 cultivated on batch xylose vs. CMB.GS010 cultivated on batch glucose (Evolv X/Evolv G), shown on the left side box; CMB.GS010 cultivated on batch xylose vs. CMB.GS001 cultivated on batch glucose (Evolv X/Unevolv G), shown on the right side box. The log-fold change of significantly differentially expressed genes ($P_{\text{adjusted}} < 0.01$, $|\log\text{-fold change}| > 1$) is indicated inside each box next to the gene name; boxes are coloured according to log-fold colour scale. If no gene is shown for a given comparative condition, then no significant differential expression changes were detected. The terms evolved and CMB.GS010, and un-evolved and CMB.GS001, are used interchangeably. The pathway intermediate abbreviations are the follows: G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6-biphosphate; GA-3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetonephosphate; 3-PG, 3-phospho-glycerate; PEP, phosphoenol-pyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; Isoct, isocitrate; AKG, alpha-keto-glutarate; SucCoA, succinyl-CoA; Fum, fumarate; Mal, malate; Oaa, oxaloacetate; Ru-5-P, ribulose-5-phosphate; X-5-P, xylulose-5-phosphate; S-7-P, sedoheptulose-7-phosphate; E-4-P, erythrose-4-phosphate.

in Fig. 6. Independent of whether the evolved strain was cultivated on batch xylose or glucose, the *SOL* genes were significantly up-regulated (*SOL3* and *SOL4* encode 6-phosphogluconolactonase) compared to growth on glucose. The evolved strain cultivated on xylose, when compared to the evolved and un-evolved on glucose, exhibited significant up-regulation of transketolase (encoded by *TKL2*, log-fold change 4.59 and 3.96). Transketolase (encoded by major isoform *TKL1* and minor isoform *TKL2*) in combination with transaldolase (encoded by *TAL1*) enables a reversible link between the non-oxidative PP pathway and glycolysis, allowing the cells to adapt their NADPH production and ribose-5-phosphate production to biomass demands (Senac & Hahn-Hägerdal, 1991). The over-expression of *TAL1* and *TKL1* in *S. cerevisiae* over-expressing *PsXYL1* and *PsXYL2* has been previously demonstrated, and there was no influence on growth under either aerobic or anaerobic fermentation conditions in the *TKL1* over-expressed mutant (Walfridsson

et al., 1995). *TKL2* over-expression was not considered, and the authors concluded that transaldolase expression in *S. cerevisiae* is insufficient for the effective utilization of PP pathway metabolites (Walfridsson *et al.*, 1995). Furthermore, *TKL2* up-regulation has been correlated with carbon-limited chemostat culture (Boer *et al.*, 2003), but this was not observed here.

Discussion

The strain constructed in this work (CMB.GS010) was obtained through a combination of genetic modification (plasmid introduction) and the application of selective pressure (shake flask repetitive cultivation). Because of the native inability of *S. cerevisiae* to metabolize xylose, three essential genes for xylose uptake from *P. stipitis* were introduced. Using repetitive batch cultivation technique, a strain capable of fast aerobic xylose metabolism was obtained in a relatively short period of time. The

10-fold increase in the specific growth rate on xylose under aerobic growth conditions in only 21 days through repetitive shake flask cultures is the evidence of the efficiency and simplicity of the method. Different evolutionary studies conducted using nutrient limitation as selective pressure has highlighted the high level of adaptability of *S. cerevisiae* (Ferea *et al.*, 1999; Sonderegger & Sauer, 2003; Jansen *et al.*, 2005; Kuyper *et al.*, 2005; Pitkänen *et al.*, 2005). The evolutionary profile observed in this study during the selection period displayed a rapid adaptation to the new fast xylose growing condition rather than a gradual process. Similarly, recent studies reported that when strong selective pressure is applied to yeast cultures in laboratory conditions, adaptation occurs in few steps involving only limited number of mutation (Hong *et al.*, 2011) and the early phase of evolution plays a critical role in the adaptation process (Gresham *et al.*, 2008). Furthermore, plasmid recovery confirms that the genetic modifications during adaptive evolution are present chromosomally in the host rather than any modifications in the plasmid. The *S. cerevisiae* strain CMB.GS010 exhibited a specific growth rate and a xylose consumption rate among the highest reported for *S. cerevisiae* strains metabolically engineered for xylose assimilation with XR, XDH and XK genes and a xylose consumption rate on minimal media under aerobic conditions (Sonderegger & Sauer, 2003; Wahlbom *et al.*, 2003a, b; Jin *et al.*, 2005; Karhumaa *et al.*, 2005; Pitkänen *et al.*, 2005; Parachin *et al.*, 2010). Strain CMB.GS010 clearly exhibited a respiratory metabolism on this sugar. Xylose utilization was almost entirely oxidative as indicated by the respiratory coefficient ($RQ = CER/OUR$), which remains close to 1 during the entire cultivation time (data not shown), and the high carbon fraction of xylose converted to biomass as compared to glucose metabolism. Furthermore, the physiological observations were supported by transcriptome data analysis at global and metabolic level. The most over-represented gene families in the evolved strain were related to functions or features linked to respiratory process. Consistently, TFs enrichment analysis identified factors primarily involved in carbon catabolite repression response mechanism and regulation of the respiration. Mainly, the significantly enriched TFs in the evolved strain represent transcriptional activator of gene involved in non-fermentative metabolism (Sculler, 2003). Among them is *SNF1* that is a major regulator of carbon metabolism together with several related TFs known to be involved in the generation of precursors of energy and linked to the activation of peroxisomal proteins (*PIP2*, *OAF1*) (Karpichev & Small, 1998) and in the metabolism of non-fermentable carbon sources (*CAT8*, *MIG*) (Usaite *et al.*, 2009). Recent transcriptome studies on recombinant *S. cerevisiae* strain engineered for xylose consump-

tion with the oxoreductive pathway (XR, XDH and XK) indicated the role of partial repression of xylose on TCA and glyoxylate cycle (Salusjärvi *et al.*, 2008), and the physiology of the strain employed during this study differ substantially from the evolved mutant reported here, showing that on batch cultivation, the xylose consumed was partially fermented to ethanol and acetate beside high xylitol production. In contrast, a xylose consuming mutant carrying the oxoreductive pathway with a mutated XR and additionally engineered on the PPP pathway exhibits a full physiological respiratory response without ethanol or xylitol overflow metabolites formation during xylose batch cultivation that correlated with the up-regulation of the TCA cycles at the transcriptional level (Runquist *et al.*, 2009), which is consistent with our findings. Moreover, physiological characterization under continuous cultivation conditions of mutagenesis isolated strains capable of fast growth on xylose shown a clear Crabtree-negative characteristics (Souto-Maior *et al.*, 2009). the observed up-regulation of the glyoxylate pathway in the evolved strain grown on xylose compared to growth on glucose, or the un-evolved strain grown on glucose is in line with observations made at low dilution rates in glucose-limited chemostat cultures in wild-type *S. cerevisiae* (Regenberg *et al.*, 2006). As an extension of the glyoxylate pathway, *IDP2* and *IDP3* were up-regulated significantly in the evolved strain grown on xylose. Xylose metabolism requires the pentose phosphate pathway (PPP), and the first step of the PPP involves the conversion of glucose-6-phosphate to 6-phosphogluconate, catalysed by glucose-6-phosphate dehydrogenase (*ZWF1*). The PPP is essential for the generation of biomass precursors and NADPH cofactor for anabolic reactions (Jeffries, 2006). While the non-oxidative PPP satisfies biomass precursor demands, cytosolic NADPH must still be generated, and the oxidative part of the pathway is bypassed during growth on xylose. Cytosolic isocitrate dehydrogenase (*Idp2*) catalyses the oxidation of isocitrate to α -ketoglutarate and is $NADP^+$ -specific (Cherry *et al.*, 1997). On both fermentable and non-fermentable carbon sources, *Zwf1p* is constitutively expressed while *Idp2p* levels are glucose-repressed (Thomas *et al.*, 1991; Minard *et al.*, 1998), whereas *Idp2p* levels have been demonstrated to be elevated on non-fermentable carbon sources and during the diauxic shift as glucose is depleted (Loftus *et al.*, 1994; DeRisi *et al.*, 1997; Minard *et al.*, 1998). Furthermore, in $\Delta zwf1 \Delta adh6$ *S. cerevisiae* mutants, it was demonstrated that *Idp2* is up-regulated and generates enough NADPH to satisfy biomass requirements, noting that the $NADP^+$ -specific cytosolic aldehyde dehydrogenase (*Adh6p*) catalysing acetaldehyde conversion to acetate is the other major cytosolic source of NADPH (Minard & McAlister-Henn, 2005). In the evolved strain, *IDP2* and

IDP3 likely provide a source of NADPH to satisfy biomass requirements.

The native xylose-fermenting strain *P. stipitis*, which is the source of the heterologous expressed enzymes, XR and XDH, does not produce xylitol during xylose fermentations (Skoog & Hahn-Hägerdal, 1990). Extensive xylitol formation has been observed in all the *S. cerevisiae* xylose consuming strains expressing these enzymes (Kötter & Ciriacy, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1995; Ho *et al.*, 1998; Eliasson *et al.*, 2000; Tiovary *et al.*, 2001). The production of xylitol has been shown to be the direct result of a redox imbalance of the NAD(P) co-factors between the XR and XDH (Eliasson *et al.*, 2000; Wahlbom & Hahn-Hägerdal, 2002; Jeppsson *et al.*, 2003; Roca *et al.*, 2003; Träff-Bjerre *et al.*, 2003; Verho *et al.*, 2003; Watanabe *et al.*, 2007). This imbalance has recently been successfully avoided by direct conversion of xylose to xylulose via the introduction of a bacterial isomerase (Kuyper *et al.*, 2003, 2004). Xylitol formation is often described as being the major drawback of the XR-XDH strategy; however, in the engineered strain selected in this study, the formation of xylitol was completely absent during all the xylose fermentations.

The absence of xylitol accumulation under oxidative conditions may be interpreted as a result of complete xylitol oxidation. Consistent with this assumption is that reduction of xylose to xylitol by XR is limited by the availability of NADPH. Perhaps, as the data in this study suggest, up-regulation of *IPD2* ensures sufficient NADPH production necessary for the *P. stipitis* NADPH-preferring XR to drive xylose catabolism, whereas the NADH surplus produced is reduced through the respiration eliminating the NADP⁺/NAD⁺ imbalance. Thus, the xylose consumption relies on the external NADH dehydrogenases (Ned1p or Ned2p) or the mitochondrial glycerol-3-phosphate/dihydroxyacetone phosphate shuttle (Gut2p and Gpd1p or Gpd2p) for the re-oxidation of the excess cytosolic NADH (Luttik *et al.*, 1998; Overkamp *et al.*, 2000). This hypothesis is consistent with the previously reported effect of aeration on the reduction of xylitol formation during xylose fermentation (Winkelhausen & Kuzmanova, 1998) and could explain the inability of the evolved strain to consume xylose under anaerobic conditions.

Co-utilization of both sugars, glucose and xylose, is essential for an economically feasible conversion of lignocellulose to industrially relevant bioproducts. Xylose is predominantly consumed after glucose exhaustion. This could be explained a competitive inhibition model for the uptake of the two sugars. Until now, no transporters have been found in *S. cerevisiae* that can exclusively and specifically transport xylose. Nevertheless, it is known that xylose competes with glucose for the same transporters albeit their affinity for xylose is lower (Kötter & Ciriacy,

1993; van Zyl *et al.*, 1999), and so xylose uptake proceeds slower compared to glucose (Leandro *et al.*, 2009). Sugar uptake rate has been related to the carbon catabolite repression and has a role in determining the control the switch between fermentative vs. respirative metabolism (Goffrini *et al.*, 2001; Elbing *et al.*, 2004; Daphne *et al.*, 2012). The evolved mutant displayed sensitivity to different extracellular xylose concentration. In xylose consuming mutant, sugar transport constitutes an important step in determining the fermentation performances (Saloheimo *et al.*, 2007; Jojima *et al.*, 2010). However, the evolved mutant retains a fermentative ability towards glucose comparable to the un-evolved that prevail during mixed sugar cultivation suggesting that the metabolic response of the evolved strain was exclusive to xylose. During glucose-xylose mixed cultivation, the observed poor xylose consumption in the presence of glucose has been attributed to the glucose repression effect (Belinchon & Gancedo, 2003). Recently, several studies propose elegant approaches to overcome the sequential utilization of glucose and xylose, acting on bypass the glucose repression effect on xylose uptake and allowing the co-fermentation of the two sugars (Nakamura *et al.*, 2008; Ha *et al.*, 2010).

Although the efficiency of the evolutionary approach presented in this work is promising, the underlying genetic change that has likely taken place during the direct evolution process remains unclear. Further studies are necessary to gain insight into the possible mutations that contributes to the observed physiology. A detailed genome-wide investigation would offer the opportunity to investigate the genetic basis that result in the ability of the selected strain to consume efficiently xylose as sole carbon source as demonstrated recently in a study on galactose metabolism (Hong *et al.*, 2011).

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Authors' contributions

G.S. and J.M.O. contributed equally to this research.

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