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BIOENERGY/BIOFUELS/BIOCHEMICALS

Engineering of *Saccharomyces cerevisiae* to utilize xylan as a sole carbohydrate source by co-expression of an endoxylanase, xylosidase and a bacterial xylose isomerase

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Abstract Xylan represents a major component of lignocellulosic biomass, and its utilization by *Saccharomyces cerevisiae* is crucial for the cost effective production of ethanol from plant biomass. A recombinant xylan-degrading and xylose-assimilating *Saccharomyces cerevisiae* strain was engineered by co-expression of the xylanase (*xyn2*) of *Trichoderma reesei*, the xylosidase (*xlnD*) of *Aspergillus niger*, the *Scheffersomyces stipitis* xylulose kinase (*xyl3*) together with the codon-optimized xylose isomerase (*xylA*) from *Bacteroides thetaiotaomicron*. Under aerobic conditions, the recombinant strain displayed a complete respiratory mode, resulting in higher yeast biomass production and consequently higher enzyme production during growth on xylose as carbohydrate source. Under oxygen limitation, the strain produced ethanol from xylose at a maximum theoretical yield of ~90 %. This study is one of only a few that demonstrates the construction of a *S. cerevisiae* strain capable of growth on xylan as sole carbohydrate source by means of recombinant enzymes.

Keywords Xylan degradation · Hemicellulose · Xylose isomerase · Bioethanol · *S. cerevisiae*

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Introduction

Alternative fuels need to be generated to complement or replace fossil based fuels in order to meet the growing energy demands of the world. One such alternative, bioethanol, has been the focus since the early 70s due to its contribution to energy security and its mostly positive environmental impact, with countries such as Brazil and the USA being the world's biggest producers of bioethanol. Microbial fermentation of lignocellulose (including agricultural residues, municipal solid waste, wood and dedicated energy crops) remains one of the most attractive options for the production of bioethanol [1, 2]. However, a high percentage of lignocellulose (fermentable carbon) conversion to ethanol is required for financial feasibility of bioethanol technologies implying that cellulose conversion alone will not be sufficient.

Xylan constitutes the major fraction of hemicellulose and can comprise up to 35 % of the total dry weight of plants [3, 4]. Xylan is a heteropolysaccharide consisting of a β-D-1, 4-linked xylopyranoside backbone, substituted with various sugar units and *O*-acetyl groups [3]. Enzymatic hydrolysis of xylan requires at least an endo-β-xylanase and a β-D-xylosidase, which hydrolyses xylan to free xylose units and substituent-linked xylooligos. Xylan-degrading *S. cerevisiae* strains have been reported that produce xylan-degrading enzymes extracellularly (free enzymes) and cell wall tethered [5–8]. Prior to microbial fermentation, pretreatment techniques are applied to the lignocellulose which yields water insoluble solids (WIS) containing mostly cellulose and a liquid fraction containing mostly xylose, oligosaccharides and furans [9]. The baker's yeast *S. cerevisiae* is well suited for bioethanol production and would therefore be ideal for the fermentation

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of pretreated lignocellulosic fractions. Unfortunately, this yeast is incapable of degrading xylan and cannot metabolize xylose.

The development of xylose assimilating *S. cerevisiae* strains have been achieved largely through the installation of the fungal oxidoreductive pathway using xylose reductase (XR) to reduce xylose to xylitol and subsequently xylitol dehydrogenase (XDH) which oxidizes xylitol to xylulose [10]. Xylulose is then phosphorylated to xylulose 5-phosphate via a xylulokinase [11] and is converted into glyceraldehyde 3-phosphate and/or fructose 6-phosphate via the pentose phosphate pathway (PPP). XR and XDH have different nucleotide co-factor preferences, NADPH and NAD respectively, which results in a redox imbalance in XR/XDH strains and leads to the accumulation of xylitol and glycerol under oxygen limitation [12–15]. As a result, the bacterial pathway has been considered more redox profitable since it converts xylose to xylulose in a single enzymatic reaction using a xylose isomerase (XI) which does not require nucleotide cofactors [16]. Early attempts at functional expression of xylose isomerases in *S. cerevisiae* have failed. Walfridsson et al. [17] was the first to report the functional expression of a xylose isomerase (thermophilic bacterium, *Thermus thermophiles*) in *S. cerevisiae*. More recently, xylose isomerases from bacteria [16, 18–20] and fungi [20–22] have been functionally expressed in *S. cerevisiae* with various degrees of success.

In this study, a xylan utilizing strain of *S. cerevisiae* had been constructed by co-expressing the *T. reesei* endoxylanase encoding gene (*xyn2*) [6], the *Aspergillus niger* xylosidase encoding gene [6], the *Bacteroides thetaiotaomicron* xylose isomerase encoding gene (*xylA*) [23] and the *Scheffersomyces stipitis* xylulose kinase gene (*xyl3*) [24]. Furthermore, to ensure minimal production of xylitol, the native *S. cerevisiae* non-specific aldose reductase (*GRE3*) was deleted [25]. The *S. cerevisiae* Y294[XYN2 XLO2] strain [6], expressing the *xlnD* and *xyn2* genes, was used as benchmark strain. This study demonstrated growth on xylose and xylan as well as ethanol production on xylose under anaerobic conditions.

Materials and methods

Media and culture conditions

Bacterial cultivation (37 °C) took place in Luria–Bertani liquid medium or agar (5 g/L yeast extract, 10 g/L NaCl and 10 g/L tryptone) supplemented with 100 µg/mL ampicillin for selection. *S. cerevisiae* transformants were initially plated onto synthetic complete (SC) medium [3.4 g/L yeast nitrogen base without amino acids (Difco), 5 g/L $(NH_4)_2$ SO_4, 20 g/L glucose and yeast synthetic dropout medium supplements (Sigma-Aldridge, Germany)]. Liquid cultivation took place at 30 °C on an orbital shaker at 200 rpm and *S. cerevisiae* strains were routinely cultured in YP medium (10 g/L yeast extract, 20 g/L peptone) containing either 20 g/L glucose (YPD) or 20 g/L xylose (YPX) at 30 °C.

Strains and plasmid constructs

The relevant genotypes of all the bacterial and yeast strains, along with the plasmids used in this study, are listed in Table 1.

Plasmid construction

Standard protocols were followed for DNA manipulations [26]. The restriction enzymes and T4-ligase were purchased from Rosche Applied Science (Germany) and used according to manufacturer's instructions. Zymoclean Gel Recovery Kit (Zymo Research Corporation, USA) was used to elute DNA from agarose gel. Polymerase chain reactions (PCR) were carried out with a Perkin Elmer GeneAmp® PCR System 2400 (Perkin Elmer, USA) using Phusion DNA polymerase (Finnzymes) according to the supplier's specifications. The *B. thetaiotaomicron xylA* (Accession number AA075900.1) has previously been codon optimized and synthetically manufactured (GenScript Corporation) for expression in *S. cerevisiae* [27]. The *xylA* and the *xyl3* were cloned onto the YEp352 backbone to produce pDLG115 (Table 1). The *Sh ble* gene was amplified from pPICZ_A by means of PCR using the oligo primers ZCN-R (5′–GATCGGATCCT AATTCAGCTTGCAAATTAAA GCC–3′) and ZCN-L (5′–CTAGGGATCCGAATTCC CCACACACCATAGC–3′) and subsequently cloned into the *Eco*RI and *Spe*I sites of pDLG115, to create pMJM121.

Yeast transformations

The laboratory *S. cerevisiae* Y294 strain was transformed with the recombinant plasmids using electroporation as described by Cho et al. [28]. The pMJM121 was digested with *Cla*I to produce the expression cassette and *Sh ble* gene fragment flanked by the delta (*δ*) DNA sequences. This DNA fragment was then conferred to Y294[*XYN2 XLO2*] to generate *S. cerevisiae* Y294[YMX1]. Overexpression of the relevant cloned genes was facilitated by integration of the expression cassettes via homologous recombination with native *δ*-sequences distributed throughout the yeast genome [29]. Transformants were selected for growth on YPD plates containing 200 mg/L zeocin. The reference strain, *S. cerevisiae* Y294[YMXR] was generated by transferring pDLG1 to *S. cerevisiae* Y294. All relevant strains had been transformed to disrupt the *FUR1*

Table 1 Microbial strains and plasmids used in this study

Strains or plasmids	Genotype	Source/reference
Strains		
E. coli DH5α	*supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Life Technologies, Rockville, Md.
S. cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-299*	ATCC 201160
S. cerevisiae Y294[YMXR]	*bla URA3 fur1::LEU2 gre3::TRP1*; $ADH2_{PT}$	This study
S. cerevisiae Y294[YMX1]	*bla Sh Ble URA3 fur1::LEU2 gre3::TRP1*; $ADH2_P$-*xlnD*-$ADH2^a_T$; *URA3* $ADH2_P$-*xyn2*-$ADH2^a_T$; $ENO1_P$-*xylA*-$ENO1^b_T$; $ENO2_P$-*xyl3*-$ENO2^b_T$	This study
S. cerevisiae Y294[XYN2 XLO2]	*bla URA3 fur1::LEU2 gre3::TRP1* $ADH2_P$-*xlnD*-$ADH2_T$; $ADH2_P$-*xyn2*-$ADH2^b_T$	[6]
Plasmids		
YEp352	*bla URA3*	ATCC® 37673™
pDLG1	*bla URA3* $ADH2_{PT}$	[6]
pDLG56	*bla URA3* $ADH2_P$-*xyn2*-$ADH2_T$; $ADH2_P$-$MF\alpha1_s$-*xlnD*-$ADH2_T$	[6]
pDLG115	*bla δ-site* $ENO1_P$-*xylA*-$ENO1_T$; $ENO2_P$-*xyl3*-$ENO2_T$ *KanMX δ-site*	This study
pMJM121	*bla δ-site URA3* $ENO1_P$-*xylA*-$ENO1_T$ *Sh ble* $ENO2_P$-*xyl3*-$ENO2_T$ *δ-site*	This study
pPICZ_A	*bla Sh ble*	Invitrogen™

[a] δ-integrated expression

[b] Episomal multicopy expression

and *GRE3* genes with the *LEU2* [6] and the *TRP1* marker genes, respectively [25].

Enzyme activity assays

The endoxylanase activity was determined using the reducing sugar assay according to la Grange et al. [30]. The XylA activity was determined by harvesting *S. cerevisiae* Y294[YMX1] after 48–72 h of cultivation in 100 mL YPD. The cells were washed twice with 0.5 volumes cold 50 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)/NaOH (pH 7.5) before resuspending in 2 mL of the same buffer. Mechanical disruption of the cells was achieved using 0.4 µm glass beads and a FastPrep FP120 (Savant) at a speed of 50 oscillations/sec for 3 × 20 s with 1 min cooling intervals on ice. Cell debris was removed by centrifugation at 17,000×*g* for 10 min at 4 °C and the supernatant kept on ice.

Xylose isomerase activity assays were performed according to the resorcinol method described by Kulka [31] and modified as described by Schenk and Bisswanger [32]. The substrate was prepared in 50 mM citrate buffer (pH 7) and the assay performed at various temperatures to determine the temperature preference of the enzyme. The optimum pH was determined by using substrate prepared in 50 mM citrate buffer at varying pH values.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5 % polyacrylamide gel according to the method described by Laemmli [33]. Intracellular protein samples were prepared for electrophoresis by adding 5 µL of cell extract to 7 µL loading buffer (60 mM Tris.HCl (pH 6.8), 25 % glycerol, 2 % SDS, 0.1 % bromophenol blue, and 14 mM β-mercaptoethanol) and subsequently boiled at 100 °C for 3 min before loading the gel. After electrophoresis at 120 V, the gel was washed with dH_2O and subsequently stained with Coomassie brilliant blue R-250 (Bio-Rad) to visualize the protein species.

Zymogram analysis was carried out by the method described by Sapunova et al. [34]. Intracellular protein samples were prepared for electrophoresis as described above, with the exception that the loading buffer lacked β-mercaptoethanol. The samples were incubated at 80 °C for 3 min prior to loading the gel. After electrophoresis at 120 V, the gel was washed with dH_2O and subsequently submerged in 50 mM TES buffer (pH 7.5) containing 10 mM $MgSO_4 \cdot 7H_2O$ and 100 mM xylose for 20 min. The gel was washed with dH_2O and submerged in 0.1 % 2,3,5-triphenyltetrazolium chloride solution in 1 M NaOH at 30 °C for 1 min in darkness. The xylulose, product of the xylose isomerization reaction, oxidized the colorless 2,3,5-triphenyltetrazolium chloride to formazan, which results in a pink-red band on the polyacrylamide gel.

Aerobic growth on glucose and xylose as sole carbohydrate source

The recombinant strains were precultured in 5 mL of YPD at 30 °C overnight and inoculated into 100 mL of YPD or YPX in 500 mL baffled Erlenmeyer flasks to an initial optical density of 0.1 at OD_{600}. Samples were taken at regular intervals. Cell growth was measured spectrophotometrically at 600 nm. To obtain dry cell weight (DCW) measurements, calibration curves were setup that correlated OD_{600} with DCW. The relevant extracellular metabolites (ethanol, glycerol, xylitol and xylose) were quantified using HPLC. All shake flask experiments were performed on an orbital shaker at 200 rpm.

Anaerobic growth on xylose as the sole carbohydrate source

The recombinant strains were precultured in 5 mL of YPD at 30 °C overnight and inoculated into 100 mL of YPX in 120 mL serum bottles to an initial OD_{600} of 0.5. The anaerobic fermentation took place at 30 °C with shaking at 200 rpm with regular sampling over a period of 40 h. Cell growth was measured spectrophotometrically and converted to DCW, and the relevant extracellular metabolites (ethanol, glycerol, xylitol and xylose) quantified using HPLC, as described above.

Aerobic growth on xylan as the sole carbohydrate source

A 10 % (v/v) preculture (overnight culture in YPD) was used to inoculate 50 mL YPBX medium (10 g/L yeast extract, 20 g/L peptone, 50 g/L of Beechwood xylan as the sole carbohydrate source). Samples were obtained over a period of 28 days and cell numbers determined with the aid of a haemocytometer. Xylose, xylitol, glycerol, acetic acid, ethanol, xylotriose, xylobiose and oligosaccharide peaks [aldobiuronic acid (U4m2X) and aldotriuronic acid (U4m2XX)] were noted.

Analysis of extracellular metabolites

The concentrations of glucose, xylose, ethanol, glycerol, xylitol and acetic acid were determined using high performance liquid chromatography (HPLC), with a Waters 717 injector (Milford, MA, USA) and Agilent 1100 pump (Palo Alto, CA, USA). The compounds were separated on an Aminex HPX-87H column (Bio-Rad, Richmond, CA), at a column temperature of 60 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min and subsequently detected with a Waters 410 refractive index detector.

The concentrations of xylobiose, xylotriose, aldotriuronic acid and aldobiuronic acid were determined using a Dionex Ultimate 3000 system equipped with a Coulochem III electrochemical detector with a working gold electrode operating in the pulsed amperometric mode. Separation of the oligosaccharides was achieved by elution on a CarboPac PA1 (4 × 250 mm) analytical column coupled to a PA1 (4 × 50 mm) guard column and using 250 mM NaOH as mobile phase at a flow rate of 1 mL/min.

Results

Strain construction

The *xylA* and *xyl3* genes were obtained by means of PCR and cloned onto expression vectors where expression was directed by the *ENO1* and *ENO2* promoter sequences, respectively. The *S. cerevisiae* Y294[YMX1] strain, was constructed to harbor the *xlnD* and *xyn2* genes on an episomal plasmid whereas the *xylA* and the *xyl3* were integrated into native δ-sequences (low copy expression). The *GRE3* (nonspecific aldose reductase) gene was disrupted, in order to minimize the production of xylitol from xylose [25]. Autoselection of the *URA3* plasmid was obtained by disruption of the *FUR1* gene to allow for growth in complex media without the risk of losing the episomal plasmid [35]. The *S. cerevisiae* Y294[YMXR] reference strain lacked the *xlnD*, *xyn2*, and *xylA* genes and contained both *GRE3* and *FUR1* disruptions, whereas the *S. cerevisiae* Y294[XYN2 XLO2] strain harbored the *xlnD* and *xyn2* on an episomal plasmid with disrupted *GRE3* and *FUR1* genes.

Partial characterization of the *B. thetaiotaomicron* XylA

The recombinant XylA showed maximum activity at a temperature of 65 °C (Fig. 1a) and pH 7.5 (Fig. 1b) using resorcinol as substrate. SDS-PAGE revealed a protein species of approximately 50 kDa corresponding to the predicted molecular weight of the XylA monomer (Fig. 1c). Under non-denaturing conditions, an approximate 110 kDa species is apparent. Furthermore, zymogram analysis verified the approximate 110 kDa species to be the active XylA dimer (Fig. 1d).

Aerobic growth on glucose and xylose

The *S. cerevisiae* Y294[YMXR] and *S. cerevisiae* Y294[YMX1] strains were cultivated in YPD and YPX. Growth and concentration of the relevant metabolites were monitored over time (Fig. 2a, b, c, d). Sustained growth was achieved by the recombinant *S. cerevisiae* Y294[YMX1] strain when xylose was used as the sole

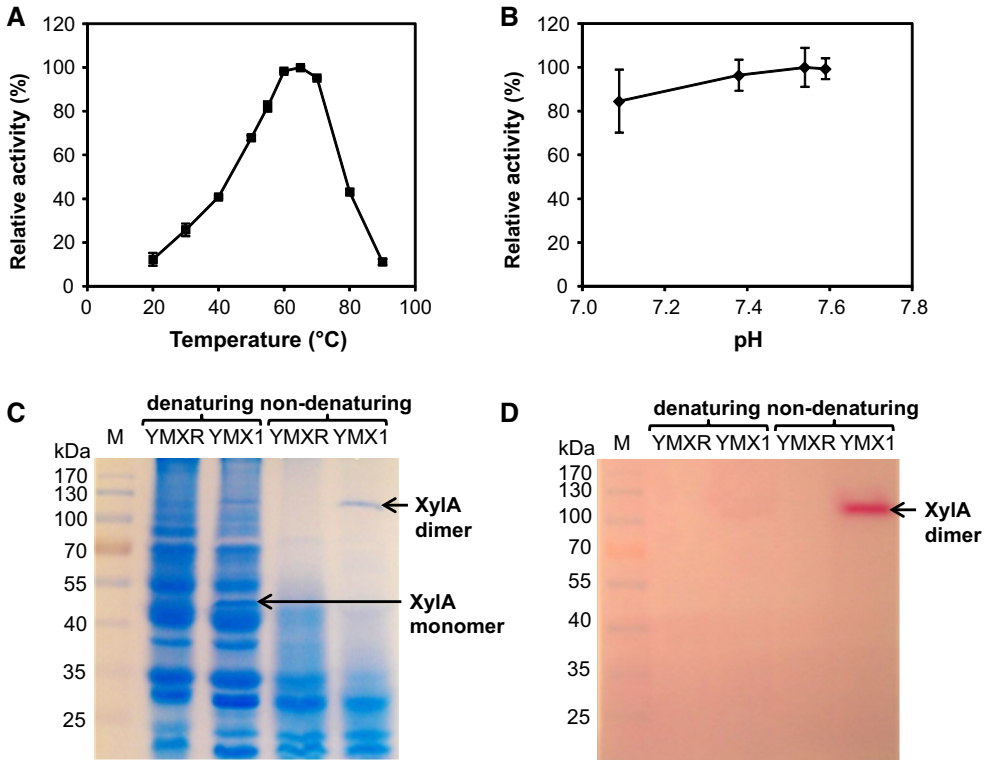

Fig. 1 The *B. thetaiotaomicron xylA* was expressed in *S. cerevisiae* Y294 and the **a** optimum temperature and **b** pH of the recombinant XylA determined using resorcinol as substrate. The **c** SDS-PAGE was performed on the intracellular protein fraction of the *S. cerevisiae* Y294[YMXR] and Y294[YMX1] strains with the corresponding (**d**) zymogram indicating the active XylA dimer. Lane *M* contains the protein molecular weight marker with the sizes depicted on the *left hand side*

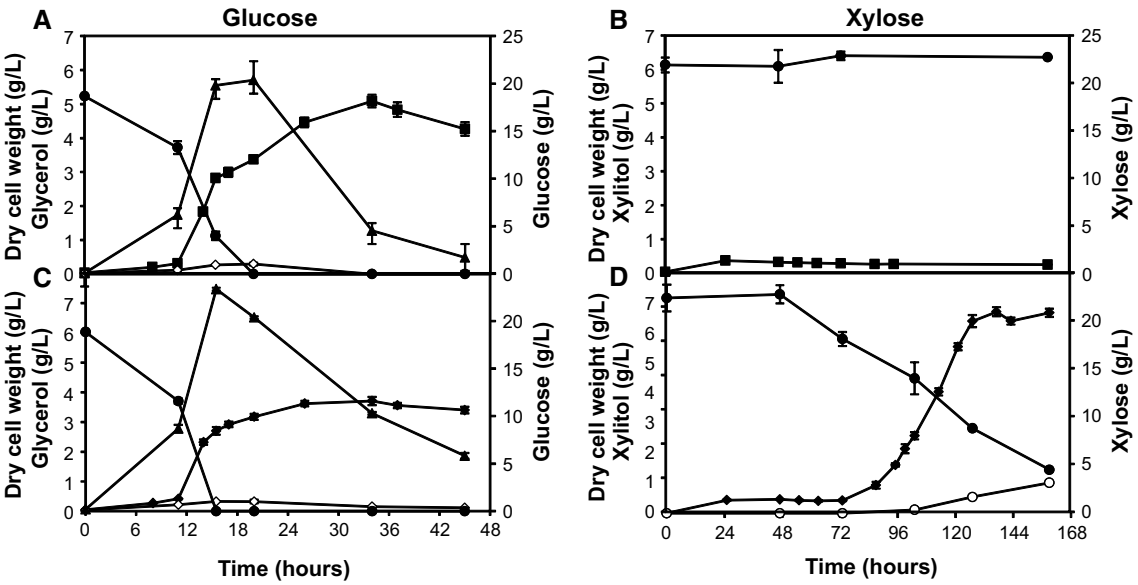

Fig. 2 The **a**, **b** (*filled squares*) *S. cerevisiae* Y294[YMXR] and **c**, **d** (*filled diamonds*) *S. cerevisiae* Y294[YMX1] strains were cultivated on glucose (YPD, *left hand side*) and xylose (YPX, *right hand side*) as sole carbohydrate source under aerobic conditions. The extracellular (*filled triangles*) ethanol, (*open diamonds*) glycerol and (*open circles*) xylitol accumulation as well as the residual (*filled circles*) glucose/xylose was monitored over time

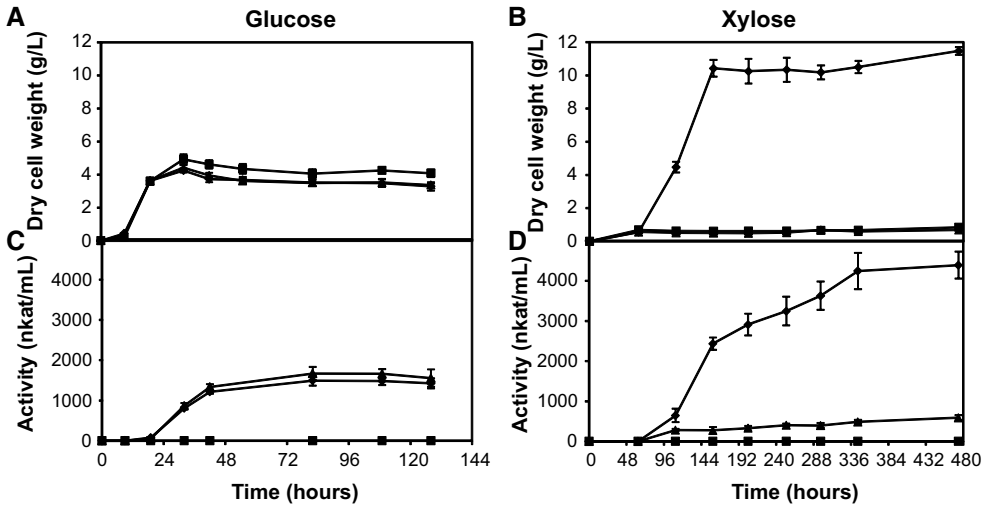

Fig. 3 The (*filled diamonds*) *S. cerevisiae* Y294[YMX1], (*filled squares*) *S. cerevisiae* Y294[YMXR] and (*filled triangles*) *S. cerevisiae* Y294[XYN2 XLO2] strains were cultivated on glucose (YPD, *left hand side*) and xylose (YPX, *right hand side*) as sole carbohydrate source under aerobic conditions. The **a**, **b** cell density and **c**, **d** extracellular endoxylanase activity were measured at regular intervals

carbohydrate source, though it was evident that the growth rate on xylose was slow compared to growth on glucose (Fig. 2c, d). Low levels of xylitol were produced (~0.9 g/L) despite the deletion of the *GRE3*. A higher biomass yield was obtained during cultivation on xylose compared to cultivation on glucose.

The biomass yield of *S. cerevisiae* Y294[YMX1] at the end of 32 h on glucose (~ 0.21 g DCW/g glucose, Fig. 3a) was significantly lower than on xylose at 159 h (~0.52 g DCW/g xylose, Fig. 3b), similar to the results obtained in Fig. 2. A significant increase in Xyn2 activity was observed which corresponds with the increase in cell mass (Fig. 3c, d). The *S. cerevisiae* Y294[YMX1] strain produced ~198 and ~234 nkat/mL/g biomass on glucose after 32 h and xylose after 159 h, respectively. The Xyn2 activity was ~422 and ~404 nkat/mL/g biomass on glucose (after 82 h) and xylose (after 345 h), respectively.

Anaerobic fermentation on xylose as the sole carbohydrate source

The *S. cerevisiae* Y294[YMX1] and *S. cerevisiae* Y294[YMXR] strains were evaluated for xylose conversion under anaerobic conditions (Fig. 4). The *S. cerevisiae* Y294[YMX1] strain was able to consume the xylose (~20 g/L) and produce ~9 g/L of ethanol after 40 days. The formation of by-products i.e. xylitol, glycerol and acetic acid, were relatively low following xylose fermentation.

Aerobic growth on xylan as the sole carbohydrate source

The *S. cerevisiae* Y294[YMX1], *S. cerevisiae* Y294[YMXR] and *S. cerevisiae* Y294[XYN2 XLO2] strains were aerobically cultivated on YPBX. The *S. cerevisiae* Y294[YMX1] strain was able to grow on polymeric xylan as sole carbohydrate source, although slow (Fig. 5).

Xylan degradation profile reveals high initial release of trisaccharides (xylotriose and aldotriuronic acid (U4m2XX) [36]) and disaccharides (xylobiose, and aldobiuronic acid (U4m2X) [36]) for the *S. cerevisiae* Y294[YMX1] and Y294[XYN2 XLO2] strains (Fig. 5b, c). Trisaccharides indicated a gradual decrease over time; however, the concentration remained slightly lower for that produced by *S. cerevisiae* Y294[YMX1] compared to *S. cerevisiae* Y294[XYN2 XLO2]. An increase in endoxylanase activity can be expected (as was the case in Fig. 3d) as a result of the biomass increase of *S. cerevisiae* Y294[YMX1] (Fig. 5a). On the other hand, it appears that the release of disaccharides was similar for both *S. cerevisiae* Y294[YMX1] and *S. cerevisiae* Y294[XYN2 XLO2] and accumulated in the medium (Fig. 5c). Xylose accumulated to ~12.7 g/L for *S. cerevisiae* Y294[XYN2 XLO2], while low levels could be detected for *S. cerevisiae* Y294[YMX1], suggesting that xylose was consumed for biomass production (Fig. 5d). As expected, no ethanol was detected and the production of xylitol, glycerol and acetic acid were negligible (data not shown).

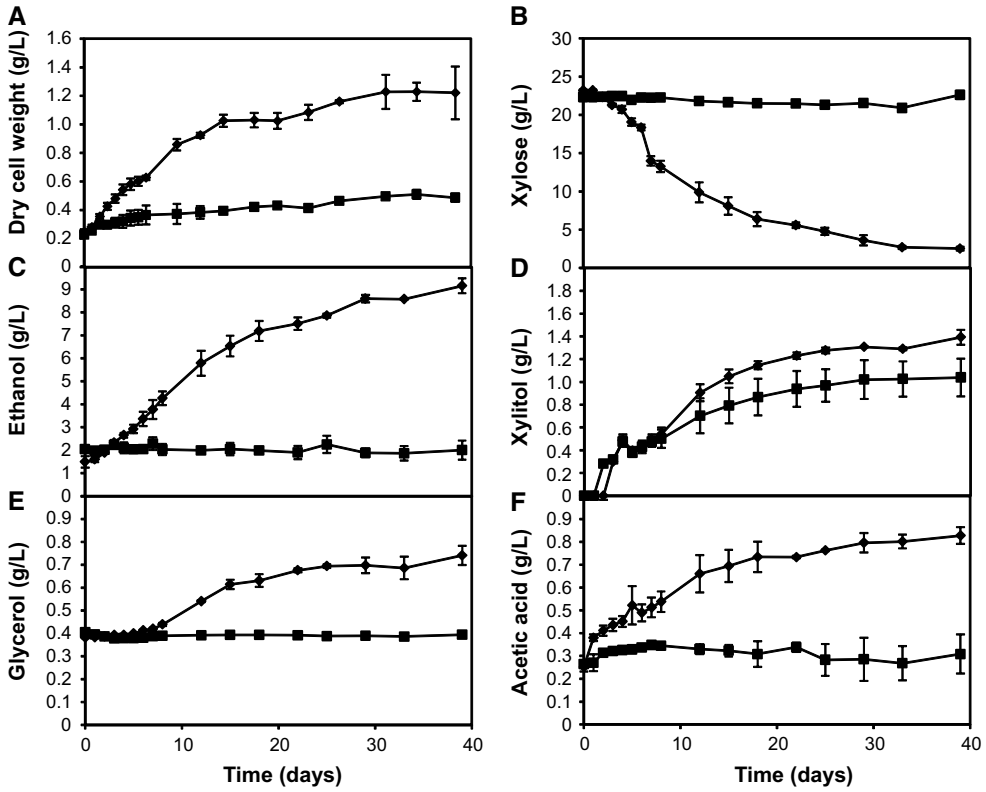

Fig. 4 The (*filled diamonds*) *S. cerevisiae* Y294[YMX1] and (*filled squares*) *S. cerevisiae* Y294[YMXR] strains were cultivated on xylose as sole carbohydrate source (YPX) under oxygen-limited conditions and the **a** cell density, the extracellular **b** xylose, **c** ethanol, **d** xylitol, **e** glycerol and **f** acetic acid concentrations were monitored over time

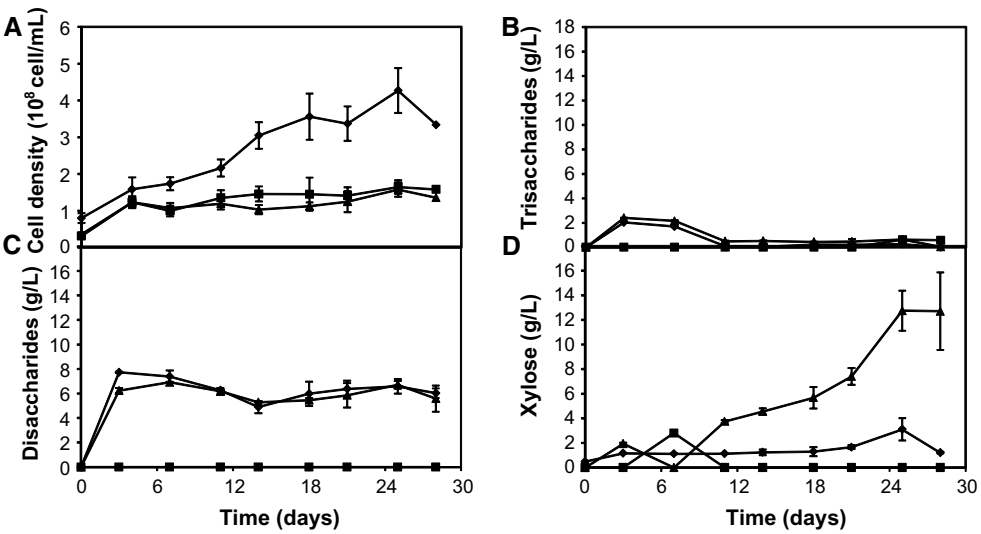

Fig. 5 The (*filled diamonds*) *S. cerevisiae* Y294[YMX1], (*filled squares*) *S. cerevisiae* Y294[YMXR] and (*filled triangles*) *S. cerevisiae* Y294[XYN2 XLO2] strains were aerobically cultivated on beechwood xylan (YPBX) as sole carbohydrate source and the **a** cell density, **b** trisaccharides, **c** disaccharides and **d** xylose concentrations monitored over time

Discussion

In order to obtain maximum xylose yields from hemicellulose fractions, harsh pretreatment methods should be avoided due to negative effect on xylose degradation. Unfortunately, milder pretreatment methods lead to residual xylan, which reduces the accessibility of the cellulose portion to hydrolytic enzymes [37]. Therefore, developing xylan metabolizing *S. cerevisiae* strains would be ideal to reduce pretreatment inputs, maximize intact xylose recovery for metabolic conversion as well as providing a more accessible cellulose portion. To date, only a few *S.*

cerevisiae strains had been documented that can utilize xylan [5, 7, 8] primarily due to the difficulties involved in the utilization of xylose [16, 38]. Xylose isomerases directly convert xylose to xylulose, which can be utilized by *S. cerevisiae* using the pentose phosphate pathway. The *B. thetaiotaomicron* xylose isomerase [16] is one of only a few xylose isomerases that had been successfully expressed in *S. cerevisiae* [4].

In this study, a laboratory *S. cerevisiae* Y294 strain was engineered for co-expression of the *T. reesei* endoxylanase encoding gene (*xyn2*) and the *A. niger* xylosidase encoding gene (*xlnD*) for xylan hydrolysis, and the *B. thetaiotaomicron* xylose isomerase encoding gene (*xylA*) and the *Scheffersomyces stipitis* xylulose kinase gene (*xyl3*) for xylose metabolism. The previous attempt at expression of the native *xylA* was suboptimal [16], hence the decision to codon optimize the gene for expression in *S. cerevisiae* for this study. The resulting *S. cerevisiae* Y294[YMX1] strain was evaluated for its ability to utilize xylose as sole carbohydrate source and subsequent ethanol production. The μ_{max} of ~0.06 g/g/h on xylose compares well relative to other reported *xylA*-expressing *S. cerevisiae* [39]. The recombinant *S. cerevisiae* Y294[XYN2 XLO2] strain, constructed by La Grange et al. [6], was used as benchmark strain.

The *GRE3* gene encodes for the major aldose reductase in *S. cerevisiae*. It was therefore disrupted to prevent loss of carbon due to the formation of xylitol. Despite the disruption of the *GRE3* in the *S. cerevisiae* Y294[YMX1] strain, xylitol could still be detected (Figs. 2, 4) suggesting the existence of additional aldose reductases in *S. cerevisiae* [40, 41]. Cultivation on xylose also resulted in more biomass being produced than with cultivation on glucose. The lack of ethanol production under aerobic conditions suggests that *S. cerevisiae* does not recognize xylose as a Crabtree positive sugar making more carbon available for the biomass production. In addition, the utilization of the xylose is preceded by a long lag phase. This might be explained by the low-level expression of the *xylA* resulting in a slow accumulation of the XylA monomers preventing dimer formation. The lengthy lag phase might be addressed by multicopy expression of the *xylA* gene. Under oxygen-limited conditions the *S. cerevisiae* Y294[YMX1] strain produced 90 % of the theoretical ethanol yield after 40 days of cultivation on xylose (Fig. 4). This time consuming fermentation is most probably also related to the XylA activity and should benefit from overexpression of the *xylA* gene.

The overexpression of multiple genes has a negative impact on biomass production (Figs. 2a, c, 3a) probably due to a metabolic burden [42, 43]. The Xyn2 activity was not affected by the choice of carbon source, but rather increased proportionately with the increase in cell mass (Fig. 3d). This study demonstrated the successful growth of *S. cerevisiae* Y294[YMX1], expressing xylan-degrading and xylose-assimilating genes, on polymeric glucuronoxylan (Fig. 5). The initial high levels of disaccharides and trisaccharides suggest the accumulation of aldotribiuronic acid and aldotriuronic acid [36], (Fig. 5b, c). For complete utilization of beechwood xylan, the *S. cerevisiae* Y294[YMX1] strain would benefit from the addition of an α-glucuronidase to remove the glucuronic acid substituents from the main chain [3].

This study contributes to the repertoire of *S. cerevisiae* strains capable of utilizing xylose as sole carbohydrate source. Sun et al. [8] was the first to report on xylan conversion to ethanol using a *S. cerevisiae* strain that produces a minihemicellulosome and using the XR and XDH combination. Yet, this is the first report of the efficient xylose fermentation and xylan utilization by *S. cerevisiae* secreting free enzymes. To our knowledge, this has been the first report of the use of the codon-optimized *xylA* of *B. thetaiotaomicron* in *S. cerevisiae* for the production of bioethanol from beechwood xylan. The XylA proved to work well and provides an alternative to the xylose reductase and xylulose dehydrogenase combinations traditionally used. This is also the first report of a xylan utilizing *S. cerevisiae* strain that is an improvement on the xylan hydrolyzing *S. cerevisiae* Y294[XYN2 XLO2] strain originally constructed by la Grange et al. [6].

The rate of bioethanol production is directly influenced by the level of xylose and the rate of xylose conversion. The xylose isomerase is required to prevent xylose accumulation, which would result in product inhibition of the xylosidase enzyme. It is therefore paramount that the xylose isomerase activity needs to be high enough to prevent it from being the rate-limiting step. Therefore, much still needs to be done in terms of improving the *S. cerevisiae* Y294[YMX1] strain. Future work will focus on bioreactor studies (using xylose or xylan feeds) to improve the cell growth rate and the xylose consumption rate of the *S. cerevisiae* Y294[YMX1] strain through directed evolution strategies. It remains, however, a good candidate for further manipulation directed toward the conversion of lignocellulosic substrates to ethanol.

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