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BIOENERGY/BIOFUELS/BIOCHEMICALS

Co-fermentation of xylose and cellobiose by an engineered *Saccharomyces cerevisiae*

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Abstract We have integrated and coordinately expressed in *Saccharomyces cerevisiae* a xylose isomerase and cellobiose phosphorylase from *Ruminococcus flavefaciens* that enables fermentation of glucose, xylose, and cellobiose under completely anaerobic conditions. The native xylose isomerase was active in cell-free extracts from yeast transformants containing a single integrated copy of the gene. We improved the activity of the enzyme and its affinity for xylose by modifications to the 5′-end of the gene, site-directed mutagenesis, and codon optimization. The improved enzyme, designated RfCO*, demonstrated a 4.8-fold increase in activity compared to the native xylose isomerase, with a K_m for xylose of 66.7 mM and a specific activity of 1.41 μmol/min/mg. In comparison, the native xylose isomerase was found to have a K_m for xylose of 117.1 mM and a specific activity of 0.29 μmol/min/mg. The coordinate over-expression of RfCO* along with cellobiose phosphorylase, cellobiose transporters, the endogenous genes *GAL2* and *XKS1*, and disruption of the native *PHO13* and *GRE3* genes allowed the fermentation of glucose, xylose, and cellobiose under completely anaerobic conditions. Interestingly, this strain was unable to utilize xylose or cellobiose as a sole carbon source for growth under anaerobic conditions, thus minimizing yield loss to biomass formation and maximizing ethanol yield during their fermentation.

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Introduction

Cellulosic ethanol is a promising alternative and renewable energy source that may significantly reduce greenhouse gas emissions and our reliance on oil. While ethanol production from the hexose sugars in sugarcane and corn starch by *Saccharomyces cerevisiae* are well-established processes, economical conversion of cellulosic feedstocks will also require rapid and efficient fermentation of xylose, the most abundant pentose sugar comprising up to 50 % weight of these feedstocks [1]. In addition, cellobiose is a β(1,4)-disaccharide of glucose that can accumulate during the enzymatic hydrolysis of cellulose and its subsequent hydrolysis by β-glucosidase liberates glucose that could potentially lead to catabolite repression of xylose fermentation [20]. Unfortunately, *S. cerevisiae* is unable to naturally ferment either xylose or cellobiose, and robust anaerobic fermentation of these sugars while maintaining the desirable properties of conventional yeast fermentation is essential for commercializing the next-generation cellulosic ethanol process.

There have been two main approaches to engineer xylose fermentation into yeast. The first has involved expression of a heterologous xylose assimilation pathway based on the sequential activities of NADPH-dependent xylose reductase, which catalyzes the conversion of xylose to xylitol, and a NAD^+-dependent xylitol dehydrogenase, which catalyzes the conversion of xylitol to xylulose [13]. Xylulose is then phosphorylated by the native xylulokinase to xylulose-5-phosphate, which enters glycolysis through

the pentose phosphate pathway. Yeast engineered with this pathway suffer a cofactor imbalance that results in the accumulation of xylitol, reduced ethanol yield, and a micro-aerobic oxygen requirement that could be difficult to maintain at a commercial scale. A second approach has involved expression of a cofactor-neutral pathway based on the introduction of xylose isomerase to catalyze the direct conversion of xylose to xylulose. While it may seem an obvious choice to engineer into yeast a single enzyme that avoids cofactor imbalance, it took enormous effort to identify xylose isomerases that are active in yeast [4, 15, 22, 30, 41] due to improper folding and insolubility of the enzyme in the yeast cytoplasm [6].

There have also been two approaches to engineer cellobiose utilization into yeast. The first has involved expression of heterologous genes encoding a cellobiose transporter and β-glucosidase catalyzing its hydrolysis to two molecules of glucose, which are subsequently phosphorylated to glucose-6-phosphate by the expenditure of ATP [7, 20, 28]. The development of yeast strains capable of cellobiose utilization under aerobic conditions has been reported recently [7, 12, 20, 24]. An alternative approach has involved coordinate expression of a cellobiose transporter and cellobiose phosphorylase, instead of β-glucosidase, that utilizes inorganic phosphate to hydrolyze the disaccharide, forming one molecule each of glucose and glucose 1-phosphate [29]. This latter pathway is hypothesized to be more energetically favorable as glucose-1-phosphate can be isomerized to glucose-6-phosphate without the expenditure of energy, thus reducing the overall ATP demand of cellobiose metabolism. This could be especially important under anaerobic fermentation conditions when ATP levels may be limiting.

We report here the metabolic engineering of an industrial strain of *S. cerevisiae* containing a novel xylose isomerase, two cellobiose transporters, and a cellobiose phosphorylase that is able to co-ferment both xylose and cellobiose to ethanol at high yield under completely anaerobic conditions.

Materials and methods

Strains and media

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. *E. coli* DH5α was used as the recipient for construction of recombinant plasmids. Competent cells were obtained from Monserate Biotechnology Group (San Diego, CA). *S. cerevisiae* BY4742 (*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) was obtained from the American Type Culture Collection (Manassas, VA, USA). The *S. cerevisiae* diploid strain Turbo, designated here as BF903, was acquired from HydroBrew (Oceanside, CA, USA). Yeast strains were cultured at 30 °C in either YPD (10 g/l yeast extract (BD, Franklin, NJ, USA), 20 g/l peptone (BD, Franklin, NJ), and 20 g/l glucose) or SCD-URA (6.7 g/l yeast nitrogen base (USB Swampscott, MA, USA), 20 g/l glucose and appropriate supplements). Intergenic sites for integration of expression cassettes were selected between neighboring convergent genes in transcriptionally active regions within the genome [23]. Intergenic sites were designated by adding ".5" to the standard systematic yeast gene name, e.g., YLR113.5 indicates integration between genes YLR113 and YLR114.

The BF903 diploid was sporulated and dissected using standard protocols [39]. Uracil-requiring auxotrophs were obtained following selection on 5-FOA, generating a *MATa* Δ*ura3* haploid strain, BF2513. The following sequential integrations/disruptions were constructed in BF2513 (Table 1). First, cellobiose phosphorylase from *Ruminococcus flavefaciens* FD-1 was integrated at site YGL054.5 under the control of the TEF1 promoter and CYC1 terminator, generating strain BF2901. Next, the cellobiose transporter encoded by the *CBT1* gene from *Neurospora crassa* (NCU00801) was codon optimized and synthesized by IDT (Coralville, IA, USA), then integrated at site YGR249.5 under the control of the TEF1 promoter and ADH1 terminator, generating strain BF3210. The *LAC12* gene encoding a fortuitous cellobiose transporter from *Kluyveromyces lactis* (KLLA0B14861g) was integrated at site YDR223.5 under the control of the TEF1 promoter and ADH1 terminator, generating strain BF3241. The native *GAL2* gene (YLR081W) encoding a fortuitous xylose transporter was integrated at site YDR275.5 under the control of the TEF1 promoter and CYC1 terminator, generating strain BF3539. An expression cassette containing two copies of the native *S. cerevisiae XKS1* gene (YGR194C) encoding xylulokinase in divergent orientation was integrated at site YER150.5, generating strain BF3579. Next, the *PHO13* gene (YDL236W) encoding a non-specific alkaline phosphatase was disrupted, generating strain BF3591. Then, the *GRE3* gene (YHR104W) encoding a non-specific aldose reductase was disrupted, generating strain BF3614. Finally, an expression cassette containing two copies of the xylose isomerase gene, *RfCO**, was integrated at site YOL108.5, generating strain BF3645. In addition, the *ura3* disruption in the parental strain BF2513 was repaired, generating strain BF3264.

Plasmid construction

Each expression cassette consisting of a promoter, gene of interest, and terminator was ligated along with a *URA3* marker [2, 18] between the flanking regions of the targeted integration site and cloned into pUC19. The entire integration cassette was released from the vector backbone via

Table 1 Strains used in this study

Strain number	Genotype
BF903	Wild-type
BF2513	Δura3
BF2901	BF2513 + YGL054.5::P_{TEF1}−CBP−T_{CYC1} −URA3
BF3210	BF2901 + YGR249.5::P_{TEF1}−CBT1−T_{ADH1}−URA3
BF3241	BF3210 + YDR223.5::P_{TEF1}−LAC12−T_{ADH1}−URA3
BF3264	BF2513 + URA3
BF3539	BF3241 + YDR275.5::P_{TEF1}−GAL2−T_{CYC1}−URA3
BF3579	BF3539 + YER150.5:: P_{TEF1}−XKS1−T_{ADH1}−P_{TDH3}−XKS1−T_{CYC1}−URA3
BF3591	BF3579 + Δpho13−URA3
BF3614	BF3591 + Δgre3−URA3
BF3645	BF3614 + YOL108.5::P_{TEF1}−RfCO*−T_{CYC1}−P_{TDH3}−RfCO*−T_{ADH1}−URA3

Strain design explained in the "Materials and methods" section

PacI digestion and used in standard yeast transformation [39]. Integration cassettes contained two 300-mers that flanked the site of integration. Codon optimized sequences were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa) *S. cerevisiae* CAI score algorithm.

Phylogenetic analysis

Phylogenetic trees were constructed using the MEGA5 program [34]. Amino acid sequences of xylose isomerase homologs were obtained from NCBI with the following accession numbers: *Ruminococcus flavefaciens* 17, CAB51938; *Piromyces* sp. E2, CAB76571; *Clostridium phytofermentans* ISDg; YP_001558336; *Thermus thermophilus* HB8, P26997; *Orpinomyces* sp. ukk1, ACA65427; *Bacteroides thetaiotaomicron* VPI-5482, NP_809706; *Escherichia coli* str. K-12 substr. MG1655, NP_418022; *Thermoanaerobacter brockii* subsp. finnii Ako-1, YP_004186994; *Streptomyces rubiginosus*, P24300; *Thermotoga maritima* MSB8, NP_229467; *Thermotoga neapolitana* DSM 4359, YP_002534262; *Streptomyces murinus*, P37031; *Lactobacillus pentosus*, P21938; *Geobacillus stearothermophilus*, ABI49954; *Bacteroides uniformis* ATCC 8492, ZP_02069286; *Clostridium cellulolyticum* H10, YP_002507697; *Ruminococcus flavefaciens* FD-1, ZP_06143883; *Ruminococcus* sp. 18P13, CBL17278; *Clostridiales genomosp.* BVAB3 str. UPII9-5, YP_003474614; *Bacteroides stercoris* ATCC 43183, ZP_02435145. Sequences were aligned using ClustalW [17].

Xylose isomerase assays

Cells were inoculated to an initial OD_{600} of 0.1 and grown to an OD_{600} of 1.0 in 50 ml YPD, pelleted, and frozen at −80 °C. Cells were thawed then lysed with 2.5 ml/g cell paste of YPER-plus reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) for 30 min at room temperature on a rotating platform. Cell debris was removed by centrifugation at 3,000 rpm for 5 min at 4 °C and the supernatant was used for enzyme assays. Protein concentration was measured using a Bradford assay performed as described (Thermo Scientific Coomassie Plus Bradford Reagent; Rockford, IL, USA). Enzyme assays contained 100 mM Tris-HCl, pH7.5, 5 mM $MnCl_2$, 0.3 mM NADH, 2 U sorbitol dehydrogenase (Roche; Basel, Switzerland), and an amount of cell extract containing 25 µg protein. Reactions were started with the addition of 500 mM xylose and absorbance measured at 340 nm for 5 min at 30 °C. A linear range of enzymatic activity was tested from 1 to 25 µg xylose isomerase. The K_m was determined by varying the xylose concentration from 5 to 500 mM. Assays were performed in triplicate and normalized to the protein concentration [15].

Cellobiose phosphorylase assays

The cellobiose phosphorylase gene from *Ruminococcus flavefaciens* FD-1, cloned under the control of the TEF1 promoter and CYC1 terminator, was integrated into site YGL054.5 in Turbo strain BF2513. Cells were inoculated to an initial OD_{600} of 0.1 and grown to an OD_{600} of 1.0 in 50 ml YPD, pelleted by centrifugation and frozen at −80 °C. Cells were thawed and resuspended in 1 ml 50 mM Tris-HCl. Lysis was achieved by adding 200 µl glass beads and using a Biospec Products (Bartlesville, OK) Mini beadbeater for 1.5 min. Cell debris was removed by centrifugation at 3,000 rpm for 5 min at 4 °C and the protein concentration of cell-free lysate measured by the Bradford assay (Thermo Scientific Coomassie Plus Bradford Reagent; Rockford, IL). Activity was assayed in 50 mM PIPES pH 6.8, 33 mM sodium-potassium-phosphate buffer pH 6.8, 3 mM $MgCl_2$, 1 mM ATP, 1 mM $NADP^+$, 5 U hexokinase, 5 U glucose-6-phosphate dehydrogenase and 20 mM cellobiose. A linear range of activity was determined by assaying 1–50 µg cell-free lysate. Reactions were initiated by the addition of cellobiose and absorbance measured at 340 nm for 5 min at 30 °C. The K_m was determined by varying the cellobiose concentration from 0 to 20 mM. Assays were performed in triplicate and normalized to the protein concentration.

Anaerobic growth on plates

Yeast nitrogen base lacking amino acids (6.7 g/l) and containing 20 g/l agar were supplemented with 2 % (w/v) glucose, 2 % (w/v) cellobiose, or 2 % (w/v) xylose, as

specified. The plates were incubated under anaerobic conditions in a BD Diagnostics (Sparks, MD) Gaspak EZ incubation chamber according to manufacturer's instructions. Inoculated plates were incubated at 30 °C under aerobic conditions for 3 days or anaerobic conditions for 4 days.

Anaerobic fermentations

Alcohol Fermentation Monitors, AFM, (Applikon, Foster City, CA, USA) containing 400 ml of 1 % (w/v) yeast extract, 2 % (w/v) peptone, 8 % (w/v) glucose, 4 % (w/v) xylose, and 2 % (w/v) cellobiose, were inoculated to an initial OD_{600} of 0.6 and incubated at 30 °C with an agitation rate of 300 rpm. Six 500-ml vessels were run in parallel for 96 h. One-milliliter samples were obtained in duplicate at 0, 8, 24, 48, 72, and 96-h time points. An Agilent (Santa Clara, CA, USA) 1100 HPLC equipped with a Rezex RFQ-Fast Fruit H+ (8 %) column (Phenomenex, Torrance, CA, USA) and ultraviolet detectors was used to measure fermentation metabolites. Sulfuric acid (25 mM) in Milli-Q water (EMD Millipore Corporation, Billerica, MA, USA) was used as mobile phase. The column temperature was held constant at 60 °C with an isocratic flow rate of 0.9 ml/min.

Results

Identification of a phylogenetically distinct xylose isomerase

A search of the NCBI database for known and putative xylose isomerases identified a phylogenetically distinct cluster of putative xylose isomerases from *Ruminococcus flavefaciens* 17, *Ruminococcus flavefaciens* FD-1, *Ruminococcus* sp. 18P13. The *Ruminococcus* proteins clustered closely together with amino acid identities ranging from 64–88 %, but showed clear phylogenetic distance from other known xylose isomerases (Fig. 1). For example, the xylose isomerase from *Ruminococcus flavefaciens* 17, which we designated XI-Rf, shared only 65, 50.2, and 23.1 % identity to the xylose isomerases from *Clostridium phytofermentans, Piromyces* and *Thermus thermophilus,* respectively, which have been shown previously to be active in yeast. A single copy of the XI-Rf gene was cloned under the control of the TEF promoter and CYC1 terminator and integrated at site YLR113.5 in *S. cerevisiae* BY4742 and found to be soluble and active in cell-free extracts with an activity of 0.29 U/mg protein (Table 2) and a K_m for xylose of 117.1 ± 8.3 mM. Despite this activity, the strain was incapable of growth on xylose as the sole carbon source.

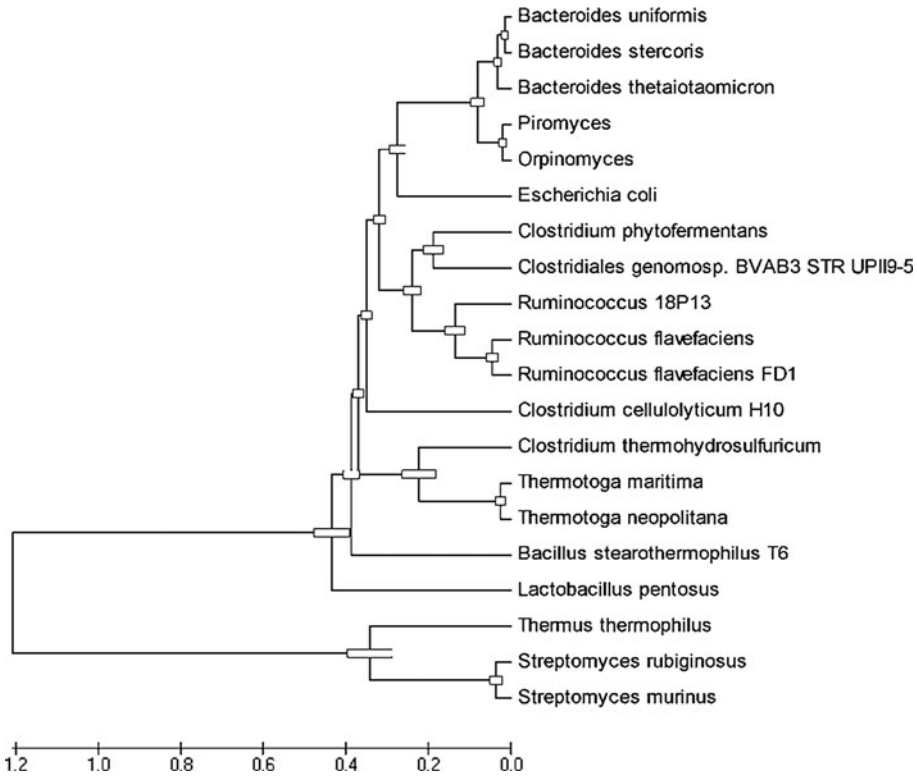

Fig. 1 Phylogenetic tree of xylose isomerase homologs obtained using MEGA5 software. NCBI numbers of sequences are listed in the "Materials and methods" section

Modifications to enhance xylose isomerase expression and activity

We set out to improve the xylose isomerase activity in yeast by increasing both the expression and activity of the enzyme. We hypothesized that the 5′ end of the gene and the corresponding N-terminus of the encoded protein would be important to efficient transcription and translation of XI-Rf. Thus, we replaced the first ten amino acids of the XI-Rf protein sequence with the corresponding amino acids from the *Piromyces* xylose isomerase, since it was previously shown to be highly expressed in *S. cerevisiae* [15]. This was accomplished by aligning the XI-Rf native amino acid sequence with the *Piromyces* xylose isomerase. The aligned sequences indicated that the N-terminus of the *Piromyces* gene was two amino acids longer than the corresponding XI-Rf sequence. Replacement of the first ten amino acids of the XI-Rf protein sequence with the corresponding 12 amino acids of the *Piromyces* sequence resulted in an 8.9 % increase in the in vitro xylose isomerase activity over the native XI-Rf protein (Table 2).

Next, we aimed to improve XI-Rf activity by site-directed mutagenesis. The XI-Rf amino acid sequence was aligned with homologs from *Pyromyces* and *Clostridium phytofermentans*, both known to be active in *S. cerevisiae*, and residues near the xylose binding site were compared. XI-Rf and *Clostridium phytofermentans* both contained a glycine at position 179, whereas this residue was an alanine in *Pyromyces*. The G179A mutation led to a 15.1 % increase in activity in cell-free extracts (Table 2). Interestingly, the G179A mutation combined with the 5′-P10 modification resulted in an additive increase in xylose isomerase activity in cell-free extracts (Table 2). The XI-Rf-P10-G179A was selected as our best-engineered XI-Rf, with a 26.8 % increase in activity over the wild-type enzyme. This sequence was then codon optimized using Integrated DNA Technologies (IDT, Coralville, IA, USA) *S. cerevisiae* CAI score algorithm, generating RfCO* with a CAI score [27] of 0.83 compared to 0.72 for the native XI-Rf. Codon optimization led to a substantial increase in expression resulting in a 385.2 % (4.8-fold) increase in activity in cell-free extracts over the native enzyme (Table 2). Interestingly, RfCO* also had a lower K_m for xylose, 66.7 ± 2.9 mM.

Two copies of RfCO* in divergent orientation were integrated into intergenic site YOL108.5 in BF3645. One copy was placed under the transcriptional control of the TEF1 promoter/CYC1 terminator, and the other under the TDH3 promoter/ADH1 terminator. It was found to have a specific activity of 1.2 ± 0.07 μmol/min/mg.

Table 2 Specific activities of xylose isomerases integrated in single copy in *S. cerevisiae* BY4742 at site YLR113.5

Xylose isomerase	Specific activity (U/mg)	% increase over native
XI-Rf native	0.29 ± 0.008	0.0
XI-Rf-P10	0.32 ± 0.013	8.9
XI-Rf-G179A	0.34 ± 0.013	15.1
XI-Rf-P10-G179A	0.37 ± 0.007	26.8
RfCO*	1.41 ± 0.029	385.2

One unit of activity is defined as 1 μmol/min/mg of xylose isomerase activity

Cellobiose phosphorylase activity assays

A single copy of the CBP1 gene encoding cellobiose phosphorylase from *Ruminococcus flavefaciens* FD-1 was integrated at intergenic site YGL054.5 of BF2513 and assayed for in vitro activity. It was found to have a specific activity of 0.75 ± 0.1 μmol/min/mg protein and a K_m for cellobiose of 0.55 ± 0.04 mM.

Growth on xylose and cellobiose

BF3264 and BF3645 were inoculated onto YNB plates containing 2 % (w/v) glucose, 2 % (w/v) xylose, or 2 % (w/v) cellobiose and incubated at 30 °C under aerobic conditions for 3 days or anaerobic conditions for 4 days (Fig. 2). Both strains were capable of luxuriant growth on glucose as the sole carbon source under aerobic or anaerobic conditions. BF3645 was capable of growth on cellobiose as the sole carbon source, but only under aerobic incubation conditions. It was incapable of growth on xylose as the sole carbon source under either incubation condition.

Anaerobic fermentation

Strains BF3264 and BF3645 were tested for their ability to ferment glucose, xylose, and cellobiose to ethanol under anaerobic conditions. Each strain was inoculated into 400 ml of YP media containing 8 % (w/v) glucose, 4 % (w/v) xylose, and 2 % (w/v) cellobiose, in 500 ml AFM vessels (Table 3). Time-course data is shown in Fig. 3. Whereas both strains consumed all of the glucose, only BF3645 also consumed 19.0 g/l of xylose and 9.8 g/l of cellobiose in 96 h. Fermentation of xylose and cellobiose continued even after the complete exhaustion of glucose from the fermentation media and was presumably simultaneous as neither sugar was fully depleted by the end of the fermentation. In contrast, the control strain BF3264 consumed only 3.9 g/l of xylose, and produced 0.7 g/l xylitol. BF3645 consumed five times more xylose, but produced only slightly more xylitol, 1.3 g/l. BF3645 produced 48.6 g/l ethanol, with a final ethanol yield of 0.44 ± 0.01 g/g based on consumption of glucose, xylose

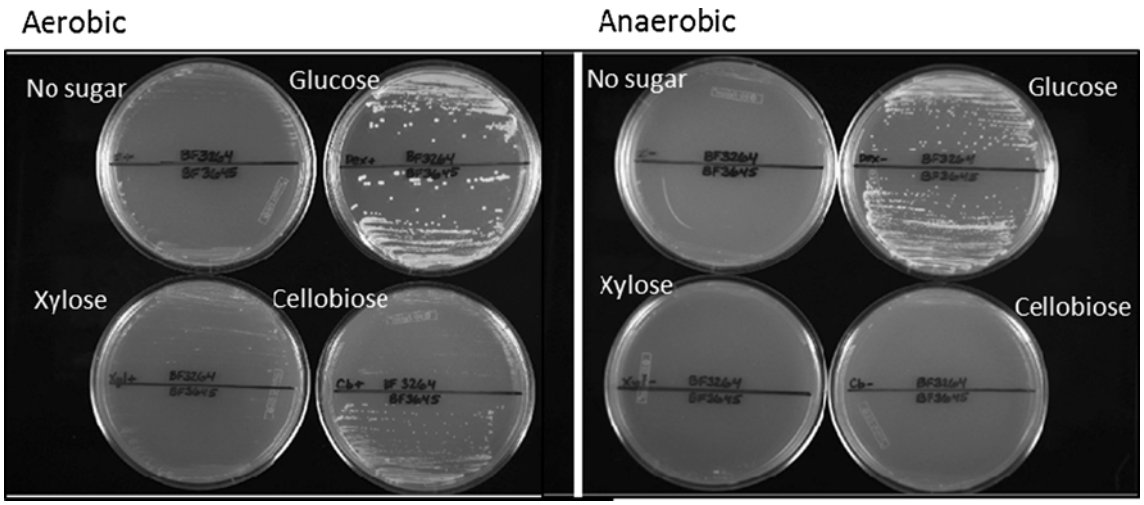

Fig. 2 Growth on glucose, xylose, or cellobiose as sole carbon sources. Inoculated plates were incubated at 30 °C under aerobic conditions for 3 days or anaerobic conditions for 4 days. Each plate is divided in half with BF3264 struck out on the top and BF3645 struck out on the bottom. Plates on *left* were incubated under aerobic conditions; plates on *right* were incubated under anaerobic conditions. Order of plates is (*top*, *left* to *right*): YNB (no sugar), YNB + glucose, and (*bottom*, *left* to *right*) YNB + xylose and YNB + cellobiose

Table 3 AFM fermentation results with standard deviation of three replicates per strain, grown in YP media with 8 % (w/v) glucose, 4 % (w/v) xylose, and 2 % (w/v) cellobiose

Strain	Xylose consumed (g/l)	Cellobiose consumed (g/l)	Glycerol produced (g/l)	Xylitol produced (g/l)	Ethanol produced (g/l)	Ethanol yield (g/l/g sugar consumed)	Ethanol production rate (g/l/h)
BF3264	3.9 ± 0.71	0.4 ± 0.23	3.4 ± 0.34	0.7 ± 0.02	37.7 ± 0.25	0.45 ± 0.01	0.4 ± 0.00
BF3645	19.0 ± 0.81	9.8 ± 2.19	4.3 ± 0.05	1.3 ± 0.22	48.6 ± 0.99	0.44 ± 0.01	0.5 ± 0.01

and cellobiose. BF3264 produced only 37.7 g/l with a final ethanol yield 0.45 ± 0.01 g/g based solely on the consumption of glucose and a very minor amount of xylose. Both strains produced small amounts of glycerol (3.4–4.3 g/l), while neither produced any significant amounts of lactate, acetate, or succinate.

Discussion

There have been several attempts to engineer yeast to produce ethanol from cellulosic feedstocks. Our approach has been to construct a single *Saccharomyces cerevisiae* strain capable of fermenting glucose, xylose, and cellobiose under completely anaerobic conditions. Our strategy is based on the coordinate expression of a xylose isomerase and a cellobiose phosphorylase from *Ruminococcus flavefaciens*, unlike previous reports that used a combination of xylose reductase, xylitol dehydrogenase [7, 20] and either an extracellularly displayed β-glucosidase [24] or an intracellularly expressed β-glucosidase [7, 20]. Although a xylose metabolic pathway based on the sequential activities of xylose isomerase and xylulokinase is cofactor neutral, engineering a xylose fermenting strain required several additional genetic modifications. Since the native GAL2p has been shown to function as a fortuitous xylose transporter [8, 31–33], we integrated a second copy of the gene under the control of a strong constitutive TEF1 promoter that is not regulated by glucose. We also deleted the *GRE3* gene, encoding a non-specific aldose reductase, previously shown to minimize xylitol production [3, 11, 14, 16, 19, 26, 35, 37] and prevent potential competitive inhibition of xylose isomerase [38]. Indeed, BF3645 containing the *gre3* deletion produced little or no xylitol during xylose fermentations. Xylose enters the pentose phosphate pathway via the action of xylulokinase [36], which is known to be limiting during growth on xylose [3, 9–11, 21, 26], thus we also over-expressed this gene in our strain. We also deleted the *PHO13* gene encoding a phosphatase that has been shown to increase growth on xylose while increasing expression of TAL1 and TKL1, although the mechanism is not fully understood [5, 25, 40, 41]. Additionally, we integrated the cellobiose transporters encoded by the *K. lactis LAC12* [29] and *N. crassa CBT1* [20] genes.

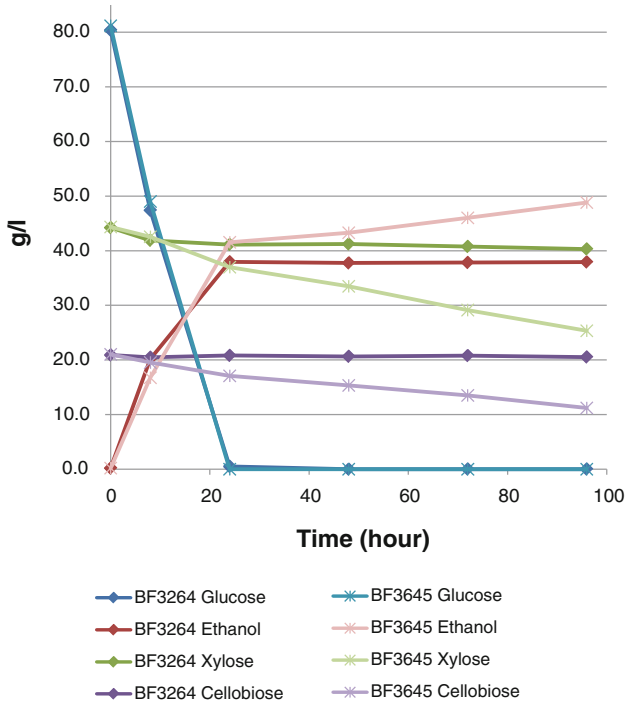

Fig. 3 Fermentation time course profile of AFM data. $n = 3$ for each strain

We increased in vitro xylose isomerase activity of XI-Rf by engineering the 5′-end of the gene, introducing a site-specific mutation, G179A, and codon optimization. Overall these modifications resulted in a 4.8-fold increase in xylose isomerase activity in cell-free extracts, and also decreased the K_m for xylose. Surprisingly, this strain was incapable of growth on xylose as the sole carbon source under aerobic or anaerobic conditions. While a K_m of 66.7 mM is comparable to other xylose-assimilation enzymes, including xylose reductases, it is still higher than the micromolar K_m common for hexokinases involved in the first step of glucose utilization. It is rational to suspect that this low affinity for xylose may be a key hurdle for rapid and efficient utilization of xylose comparable to the utilization of glucose. We suspect that metabolism of xylose or cellobiose alone does not generate sufficient ATP to support the growth of BF3645 on either substrate as the sole carbon source [32] when plated on minimal media and incubated under anaerobic conditions, particularly since transport of xylose depends on expenditure of ATP. In contrast, xylose and cellobiose consumption and fermentation to ethanol took place under anaerobic conditions, but in liquid media also containing glucose and yeast extract (1 % w/v) to support the fermentation. Note that xylose and cellobiose consumption and fermentation to ethanol continued even after the complete exhaustion of glucose from the fermentation media. We suspect xylose fermentation under these fully anaerobic conditions is supported by the ATP generated via cellobiose metabolism. From a practical perspective, the inability to grow on either substrate under anaerobic fermentation conditions is perhaps fortuitous by minimizing yield loss to biomass formation and increasing the ethanol yield based on consumed sugar. While it has been hypothesized that a cellobiose fermentation pathway based on cellobiose phosphorylase may be energetically favorable over one based on β-glucosidase, it is clearly not sufficient, by itself, to allow growth on cellobiose under anaerobic conditions. It is possible that increasing cellobiose phosphorylase activity may alleviate this constraint, or that a passive cellobiose transporter may be necessary to confer anaerobic growth on cellobiose. Interestingly, both BF3264 and BF3645 had similar ethanol yields despite BF3645 consuming far more cellobiose and xylose sugars, indicating that BF3645 is capable of co-fermenting xylose and cellobiose at the same efficiency as it ferments glucose.

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