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Employing a combinatorial expression approach to characterize xylose utilization in *Saccharomyces cerevisiae*

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ABSTRACT

Fermentation of xylose, a major constituent of lignocellulose, will be important for expanding sustainable biofuel production. We sought to better understand the effects of intrinsic (genotypic) and extrinsic (growth conditions) variables on optimal gene expression of the *Scheffersomyces stipitis* xylose utilization pathway in *Saccharomyces cerevisiae* by using a set of five promoters to simultaneously regulate each gene. Three-gene (xylose reductase, xylitol dehydrogenase (XDH), and xylulokinase) and eight-gene (expanded with non-oxidative pentose phosphate pathway enzymes and pyruvate kinase) promoter libraries were enriched under aerobic and anaerobic conditions or with a mutant XDH with altered cofactor usage. Through characterization of enriched strains, we observed (1) differences in promoter enrichment for the three-gene library depending on whether the pentose phosphate pathway genes were included during the aerobic enrichment; (2) the importance of selection conditions, where some aerobically-enriched strains underperform in anaerobic conditions compared to anaerobically-enriched strains; (3) improved growth rather than improved fermentation product yields for optimized strains carrying the mutant XDH compared to the wild-type XDH.

1. Introduction

Biological synthesis of liquid transportation fuels provides an attractive route for sustainably meeting the growing demands of an increasingly expanding global economy (Balat and Balat, 2009). Bulk production of commodity biofuels requires engineered microbes to efficiently convert inexpensive biomass-derived substrates. The first generation of biofuel production has relied on fermentation of the glucose, often sourced from sugar cane and corn; however, as the demand for biofuels grows, it will become increasingly critical to utilize lignocellulosic feedstocks (Hayes, 2009; Somerville, 2007). Lignocellulose is primarily comprised of lignin, a complex aromatic polymer, and two sugar biopolymers: cellulose, a polymer of glucose molecules, and hemicellulose, a heterogeneous polymer representing approximately a third of biomass by dry weight (33% for corn stover, 27% for *Miscanthus*, and 32% for hardwoods) of which the pentose xylose is the major constituent (Pauly and Keegstra, 2008). Compared to cellulose, hemicellulose is more readily hydrolyzed to its component monosaccharides, (Hayes, 2009), but its use is limited by the metabolism of most microbes, which have not evolved to rapidly utilize xylose (or at all, in some cases). Thus, rapid xylose utilization is an important engineering target for efficient and commercially viable microbial conversion of diverse feedstocks into various compounds, such as sustainable biofuels.

Saccharomyces cerevisiae has historically been the consensus choice as a production host for biofuels for a number of reasons: it has a high tolerance to toxic intermediates produced during most lignocellulose pretreatments (Olsson and Hahn-Hägerdal, 1993); it can naturally ferment an isomer of xylose, xylulose (Deng and Ho, 1990); it exhibits high tolerance to low pH and the fermentation product ethanol (Yomano et al., 1998); and it has well-developed large-scale fermentation protocols. While there are many yeast species that can natively utilize xylose (Skoog and Hahn-Hägerdal, 1988), *S. cerevisiae* lacks this capability. To confer the ability to convert xylose into fermentable xylulose in *S. cerevisiae*, two enzymes, xylose reductase (XR) and xylitol dehydrogenase (XDH) must be heterologously expressed (Fig. 1A). These are often derived from the natural xylose fermenting yeast, *Scheffersomyces*

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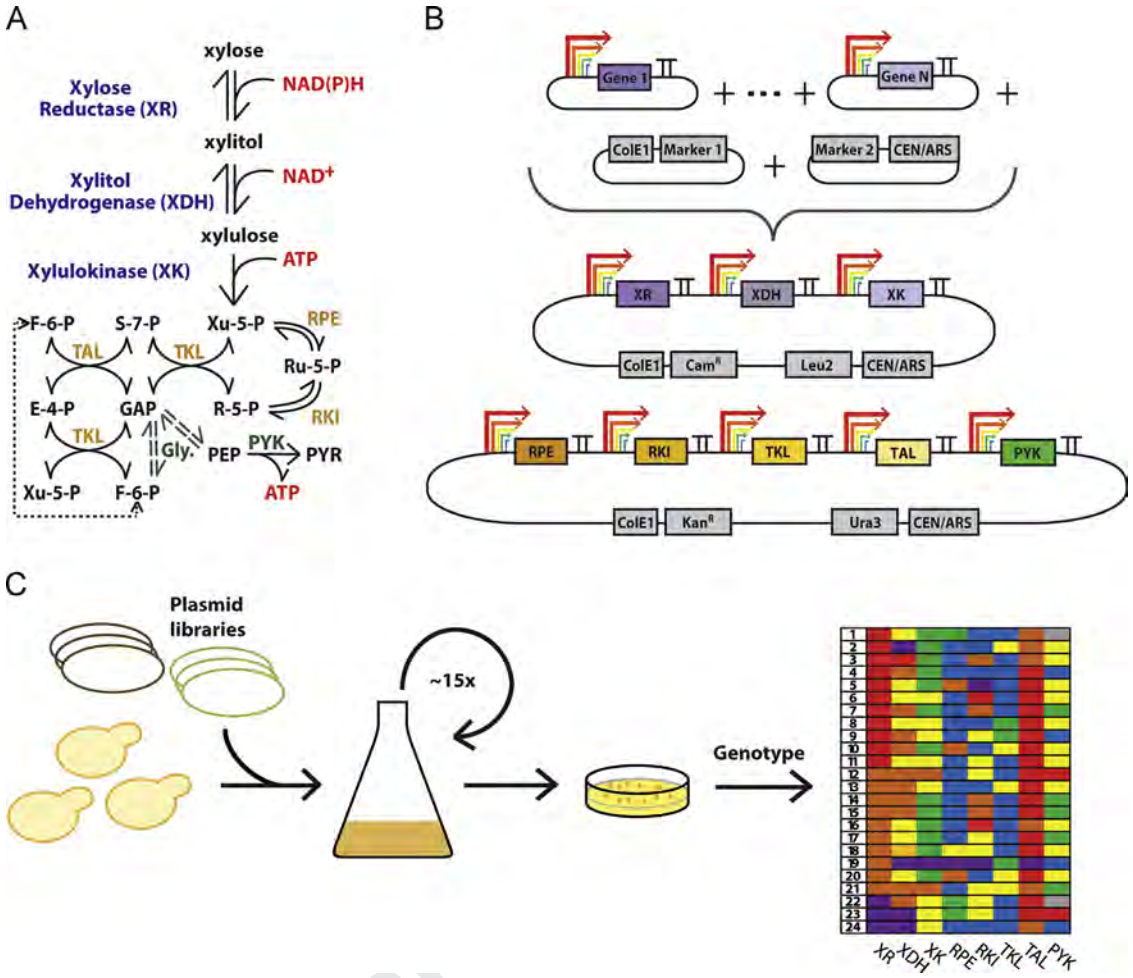

Fig. 1. Experimental design for optimization of xylose metabolism using a combinatorial promoter library. (A) Xylose catabolism to pyruvate. The fungal catabolic pathway (blue) feeds into glycolysis (green) via the non-oxidative pentose phosphate pathway (gold). Nomenclature is in accordance with the Saccharomyces Genome Database (http://www.yeastgenome.org). (B) Plasmid-based promoter library assembly scheme. A mixture of backbone and cassette plasmids with various promoters and *tADH1* are assembled in a one-pot golden gate reaction. (C) Cartoon depicting the library enrichment protocol. *S. cerevisiae* cells are transformed with the promoter library plasmids and grown on xylose as the sole carbon source in the desired conditions. After iterative dilutions, individual colonies are isolated and genotyped. Genotypes for colonies after 29 days of aerobic growth are shown (sorted by XR promoter), where red represents *pTDH3*, orange represents *pTEF1*, yellow represents *pRPL18B*, green represents *pRNR2*, blue represents *pREV1*, grey indicates no detected promoter and purple indicates a mixed signal in the TRAC sequencing reaction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

stipitis (Kotter et al., 1990). Although redox balanced, the cofactor usage of these two enzymes is asymmetric, with XR preferring NADPH and XDH exclusively using NAD^+. Mutant XR and XDH enzymes with altered cofactor preference have been developed in an attempt to resolve this asymmetry, but the effects of these mutations have been confounded by the simultaneous alteration of cofactor usage and enzyme kinetics (Jeppsson et al., 2006; Bengtsson et al., 2009; Khoury et al., 2009; Watanabe et al., 2005, 2007; Krahulec et al., 2010). A third enzyme, xylulokinase (XK), is usually overexpressed to convert xylulose into the pentose phosphate pathway (PPP) intermediate xylulose-5-phosphate (Johansson et al., 2001; Kim et al., 2013; Van Vleet et al., 2008; Scalcinati et al., 2012; Ho et al., 1998; Karhumaa et al., 2005), which is further metabolized by native PPP enzymes into substrates for glycolysis.

Despite vigorous engineering efforts over the past few decades, xylose catabolism in *S. cerevisiae* has not reached industrially viable efficiencies, in part because the ideal combination of enzyme expressions in any particular strain is unclear. Most previous studies have used arbitrary overexpression of a subset of enzymes chosen from the heterologous xylose utilization enzymes (XR, XDH, and XK) and pentose phosphate enzymes ribulose-5-phosphate epimerase (RPE), ribulose-5-phosphate isomerase (RKI), transketolase (TKL), and transaldolase (TAL) (Matsushika et al., 2009a). Improvements were often achieved with overexpression of many of these enzymes individually as well as in combination, suggesting that not only are high amounts of the heterologous xylose utilization enzymes required, but also increased PPP enzyme activity (Kim et al., 2012; Jin et al., 2005; Bera et al., 2011; Kuyper et al., 2005; Karhumaa et al., 2007; Lu and Jeffries, 2007). This might be expected since the PPP, especially in *S. cerevisiae,* has not evolved to handle the elevated flux required when xylose is used as the sole carbon source (Fiaux et al., 2003; Blank et al., 2005). In addition, conflicting findings for the optimal expression of some of these enzymes, such as xylulokinase, has resulted in confusion in understanding pathway design principles. In some studies, the highest overexpression of XK produced the fastest growing strains (Parachin et al., 2011); however, in other studies, intermediate expression levels were determined to be ideal, suggesting toxicity, perhaps due to ATP depletion (Rodriguez-Pena et al., 1998; Jin et al., 2003). It is quite possible that intermediate expression levels of other pathway enzymes would be optimal as well. To this end, combinatorial expression engineering has been applied in two studies to various enzymes in

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the xylose utilization pathway. Lu and Jeffries (2007) combinatorially sampled two expression levels of three *S. cerevisiae* genes – the PPP genes *TKL1* and *TAL1* and the glycolytic enzyme *PYK1* – and observed the best ethanol titers when *TKL1* and *PYK1* were expressed with the stronger promoter and *TAL1* with the weaker promoter (Lu and Jeffries, 2007). Du and colleagues placed the upstream genes – XR, XDH, and XK – under control of three promoter mutant libraries to identify fast-growing genotypes in both a laboratory and industrial strain. Interestingly, in this study, the optimal ratio of enzyme activities changed in the two strain backgrounds (Du et al., 2012). This finding is highly representative of an issue that this field faces: different strain backgrounds, growth media, and conditions used all impact the metabolic context of this pathway, which complicates the integration of knowledge from various studies for strain engineering.

Here we present a study where we simultaneously titrated expression of eight genes involved in xylose utilization. In accordance with much of the previous work on this pathway (Karhumaa et al., 2005; Eliasson et al., 2000; Ho et al., 1998; Wahlbom et al., 2003; Matsushika and Sawayama, 2008), we chose to include the three heterologous enzymes XR, XDH, and XK from *S. stipitis* in our library. Because the PPP does not typically need to support high flux in *S. cerevisiae* (Blank et al., 2005), we included additional copies of these enzymes (RPE, RKL, TKL, TAL). Like the xylose catabolic enzymes, we elected to express *S. stipitis* homologs of these genes under the assumption that they have evolved to support high flux in the natively xylose-consuming yeast. Finally, although we did not expect glycolytic enzymes to be metabolically limiting, we also included *S. stipitis* pyruvate kinase (PYK) based on previous characterization of the *S. cerevisiae* homolog, *PYK1*, which was shown to determine the glycolytic rate when driven by a weak promoter (Pearce et al., 2001) and improved xylose fermentations when expressed highly during combinatorial expression experiments of *TKL1*, *TAL1* and *PYK1* (Lu and Jeffries, 2007).

Using growth on xylose as a selection, we probed the role of both intrinsic variables –changes made directly to the starting strain such as number and variants of pathway genes expressed – and extrinsic variables – changes to the external selection pressures applied to the strain such as oxygenation – on optimal gene expression. We included such a large number of genes to investigate the possibility of local optima when only a fraction of the pathway enzymes are optimized. In this way, we were able to identify important factors in xylose utilization.

2. Materials and methods

2.1. Strains and media

Single gene (cassette) plasmids were transformed in chemically competent TG1 cells grown in LB containing spectinomycin (50 mg/L). All multi-gene plasmid assemblies were transformed into TransforMax EPI300 (Epicentre) electrocompetent *E. coli*. Transformed cells were selected on LB plates containing antibiotics chloramphenicol (34 mg/L) or kanamycin (25 mg/L). The *S. cerevisiae* strain background for all experiments in this paper was BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). Dry cell weight conversion for this strain was determined to be 0.19 ± 0.01 g cell/L at OD_{600}=1 as described previously (Sonderegger and Sauer, 2003). In all instances, *S. cerevisiae* strains were grown at 30 °C. Wild-type yeast cultures were grown in YPD (10 g/L Bacto Yeast Extract; 20 g/L Bacto Peptone; 20 g/L Dextrose). Excluding library transformations, yeast transformed with plasmids containing the *LEU2* and *URA3* auxotrophic markers were selected and grown on synthetic drop-out media (6.7 g/L Difco Yeast Nitrogen Base w/o Amino Acids; 2 g/L Drop-out Mix Synthetic minus Leucine and Uracil, w/o Yeast Nitrogen Base (US Biological); 20 g/L Dextrose or 20 g/L Xylose).

2.2. Pathway construction and combinatorial library assembly

All pathway enzymes, except the mutant *SsXDH* (D207A/I208R/F209S/N211R), were cloned by PCR from the *S. stipitis* genome using primers listed in Table S1. Four of the genes: *SsXK*, *SsRPE*, *SsRKI*, and *SsTKL* were cloned by SOEing PCR (Horton, 1995) to introduce silent mutations to mutate internal BglII or BsmBI sites. The mutant XDH was synthesized by GenScript and included flanking BglII and XhoI sites. Cassette plasmids were assembled through standard restriction-ligation subcloning using restriction enzymes BglII and either SpeI or XhoI into backbones (pML530-4 and pML557). Plasmid libraries or plasmids with specific promoter genotypes were assembled in a BsmBI golden gate reaction using 20 fmol of each cassette plasmid template and two backbone plasmids (pML634-638) (Engler et al., 2009). Plasmid information is summarized in Table S2 and backbone vector construction details are available upon request.

For library plasmids, golden gate reactions were electroporated, cells were recovered in LB for one hour, plated on 241 mm × 241 mm plates and grown overnight. The resulting colonies (over 35,000) were scraped into 15 mL of ddH_2O and treated as a liquid culture for plasmid purification with a HiSpeed Plasmid Maxi Kit (Qiagen) following manufacturer's instructions. Plasmid libraries were test digested to confirm correct assembly. Individual plasmids were also isolated from single colonies and confirmed by test digest.

Eight gene library transformations into *S. cerevisiae* followed standard Lithium acetate transformation protocol (Gietz and Woods, 2006) scaled to a 500 mL culture and transformed using 100 μg of each library plasmid. Following heatshock, cells were recovered in 50 mL of YPD (250 mL baffled flask, 200 rpm) for one hour before being pelleted, washed in SX-LU and then resuspended in 500 mL SX-LU under either aerobic (2 L baffeled flask) or anaerobic (media supplemented with 0.01 g/L ergosterol, 0.43 g/L Tween 80 and 2.8 g/L ethanol; 1 L Erlenmeyer screw cap flask flushed with $N_{2(g)}$) conditions with an aliquot plated on SD-LU for sampling of initial library coverage and diversity. Three gene library transformations followed the above procedure using an 'empty' plasmid containing markers and origins only, instead of the five-gene PPP plasmid; 1/100 the listed masses and volumes were used in 50 mL culture tubes or serum vials.

2.3. Library enrichments

Transformed libraries were grown 4–7 days on SX-LU until the OD_{600} of the library increased twofold over the initial OD_{600}. Library cultures were subsequently diluted into 50 mL of fresh SX-LU carrying over at least 1×10^7 cells into either a 250 mL baffled Erlenmeyer flask (aerobic, 200 rpm) or 250 mL serum vial (anaerobic, 100 rpm). After this, cells were similarly diluted in late log-phase (OD_{600} 5–10, aerobic; 1.5–2.5, anaerobic) into fresh media every 1–4 days, depending on growth rate. During select dilutions, aliquots of cells were also plated on SD-LU, grown for 2–3 days and genotyped according to the TaqMan-based TRAC protocol previously developed in our lab (Lee et al., 2013). Primer sequences used for TRAC genotyping reactions are listed in Table S1.

2.4. Growth curves and fermentations

Following plasmid transformation into yeast, cells were grown on SD-LU agar plates for 2–3 days. Colonies were picked into SD-LU (3 mL), grown for 24 h and then diluted into SX-LU (3 mL) by transferring an aliquot of grown cells into fresh SX-LU. After 48 h

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of aerobic growth in 24-well blocks, OD_{600} was measured and cells were diluted into a larger volume of SX-LU and shaken at either 100 or 200 rpm depending on the growth conditions. At designated time points, aliquots were taken from the cultures to measure OD_{600} and media was stored at −20 °C for later analysis by HPLC.

2.5. Metabolite quantification

Media aliquots were pelleted, and supernatant was transferred to GC/MS vials for sampling. From each sample, 10 μL was analyzed by refractive index on a Shimadzu LC20AD HPLC equipped with a Rezex RFP-fast acid H^+ column (100 × 7.8 mm, 55 °C) run with 1 mL/min 0.01 N H_2SO_4 mobile phase. Metabolite concentrations were determined by comparing to a standard curve.

2.6. Shotgun proteomics

Cells were grown following the same procedure used during a growth curve. After 24 (aerobic) or 48 (anaerobic) hours of growth in the final, larger SX-LU culture, OD_{600} was measured for each culture and 10 OD units (where 1 OD unit = the cells in 1 mL of culture at 1 OD_{600}) of cells were pelleted, washed in 1 mL PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), and immediately frozen at −80 °C until later use.

Cell lysates (100 μg total protein) were precipitated in 20% TCA at −80 °C overnight, pelleted at 4 °C, washed three times with ice cold 0.01 N HCl/90% acetone, dried at 25 °C and then resuspended in 8 M urea. ProteaseMax (0.1%, Promega) was added, vortexed and the reaction was diluted to 100 μL with NH_4HCO_3 (70 mM). Protein was reduced by incubated with TCEP (10 mM) for 30 min at 55 °C and then alkylated with Iodoacetamide (12.5 mM) for 30 min in the dark with shaking. The reaction was brought up to a final volume of 234 μL with PBS and ProteaseMax (0.03%) and treated with trypsin (0.5 μg/μL, Promega) overnight at 37 °C. The digested peptides were acidified with formic acid (5%), spun at max speed for 30 min and the supernatant was stored at −80 °C until further use. The resulting tryptic peptides were loaded onto inline filters and peptides were chromatographically separated a C18 nanospray column. Peptides were analyzed by an LTQ-XL. The resulting data were analyzed against the *S. stiptis* and *S. cerevisiae* tryptic proteomes using Integrated Proteomics Pipeline. ms2 spectra data were searched using the SEQUEST algorithm (Version 3.0) (Eng et al., 1994). SEQUEST searches allowed for oxidation of methionine residues (16 Da), static modification of cysteine residues (57 Da-due to alkylation), no enzyme specificity and a mass tolerance set to ±1.5 Da for precursor mass and ±0.5 Da for product ion masses. The resulting ms2 spectra matches were assembled and filtered using DTASelect (version 2.0.27) (Cociorva and Yates, 2006). A quadratic discriminant analysis was used to achieve a maximum peptide false positive rate of 1% as previously described (Cociorva et al., 2007; Tabb et al., 2002).

3. Results

3.1. Construction and enrichment of combinatorial pathway expression libraries

To determine the optimal expression profile of enzymes involved in xylose utilization in the BY4741 laboratory *S. cerevisiae* strain, we chose to employ a combinatorial approach to simultaneously modulate expression of each pathway enzyme using a set of five previously characterized constitutive promoters (Lee et al., 2013). These promoters sample evenly-spaced transcriptional strengths over approximately three orders of magnitude of expression space and include *pTDH3*, the strongest constitutive promoter in *S. cerevisiae* (Fig. S1) (Kuroda et al., 1994). In contrast to a promoter mutagenesis-based approach, our library is not limited by the availability of multiple high-strength promoters, which allows each gene to sample the highest possible expression. Additionally, these promoters enable rapid genotyping of library members using the previously described TRAC method (Lee et al., 2013).

For the eight *S. stipitis* genes, libraries of all possible promoter-gene combinations were constructed using Golden Gate assembly (Engler et al., 2009) (Fig. 1B, Supplementary results). Following transformation into yeast, we employed a selection for growth on xylose to enrich for strains with superior xylose utilization, as shown in Fig. 1C and described in the supplementary results. Serial enrichments were performed until the population converged to a consensus combination of promoters, which we term "enrichment profiles." These enrichment profiles allowed us to track global expression trends within the library culture. Holistic understanding of promoter enrichment for each gene is useful because, based on the promoter driving a given protein, we can predict relative expression of that protein between strains where different promoters are present. Although absolute abundances will differ from protein to protein, the relative rank-ordering of expression from this characterized promoter set remains consistent irrespective of the coding sequence (Lee et al., 2013).

3.2. Aerobic enrichments and strain characterizations

Combinatorial expression optimization of pathway enzymes has been shown to be dependent on the strain background (Du et al., 2012). A corollary hypothesis is that optimal enzyme expression is dependent on which genes are expressed and purposefully varied within a strain. Thus, we sought to investigate the dependence of enrichment profiles on which heterologous enzymes are included during the optimization. Our initial experiments were performed under aerobic conditions to better compare findings to previous combinatorial expression engineering efforts for xylose utilization (Du et al., 2012; Lu and Jeffries, 2007). Similar to these previous experiments, we tested a library of only the three xylose utilization enzymes (i.e., XR, XDH, and XK), which we refer to as the "partial pathway," as well as a second library, expanded to include additional *S. stipitis* copies of the pentose pathway enzymes (i.e., RKI, RPE, TKL, and TAL) and the glycolytic enzyme PYK, which we term the "full pathway."

3.2.1. Expression optimization of the partial pathway results in a local optima

We hypothesized that including or omitting the downstream pathway enzymes in our library would alter the optimal expression levels of the upstream xylose utilization genes. Thus, we enriched both the partial and full versions of the pathway under aerobic conditions and compared the resulting enrichment profiles. Indeed, we found the enrichment profile of the promoters for XR, XDH and XK to be considerably different between the full and partial libraries (Fig. 2A and B). Strains expressing the partial pathway strongly enrich for the medium-strength *pRPL18B* promoter driving expression of XR, higher (*pTDH3* or *pTEF1*) expression of XDH, and variable XK expression (Fig. 2A). Such expression enrichment is consistent with previous optimizations in micro-aerobic conditions (Kim et al., 2012; Du et al., 2012; Karhumaa et al., 2007). However, when the full pathway is expressed, XR is enriched exclusively for the stronger *pTEF1* or *pTDH3* promoters, while XDH and XK are driven by somewhat lower strength promoters (moderate to high). These results for the full pathway are more consistent with what would be expected from the

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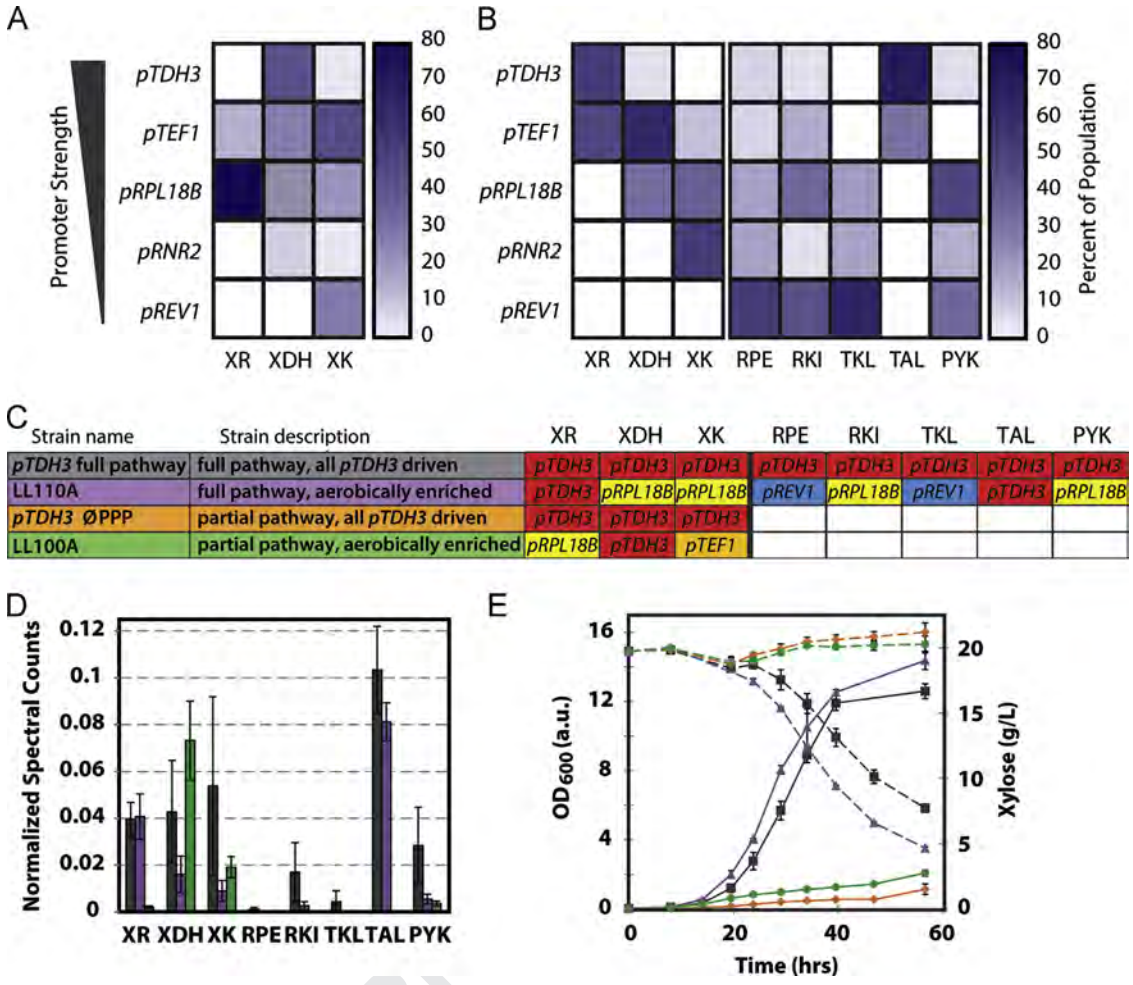

Fig. 2. Inclusion of the PPP enzymes produces superior growth and xylose consumption rates with optimal expression being not all genes driven by *pTDH3*. (A) Heatmap representation of the enrichment profile generated from screening 32 colonies isolated from an aerobically enriched promoter library regulating the first three enzymes in the xylose metabolic pathway. Heatmap colors represent the percentage of colonies with a given promoter regulating the corresponding gene. (B) Enrichment profile generated from screening 48 colonies isolated from an aerobically enriched promoter library regulating the eight enzymes. (C) Genotype and description of reference strains and enriched genotypes identified from the library enrichments. (D) Shotgun proteomic data indicating relative protein abundance in most strains indicated in (C) when grown aerobically to mid-log phase on xylose. Protein spectral counts were normalized to total endogenous spectral counts. There is no statistical significance between total endogenous protein expression between samples. (E) Aerobic growth curve for yeast strains indicated in (C): OD_{600} (——); Xylose (– – –). Error bars represent SD of biological triplicates.

in vitro catalytic efficiencies of the pathway, where XR is predicted to be the limiting enzyme (Table S3). These drastic differences in expression profiles between partial and full pathways are intriguing as they suggest that the optimal expression profile is highly dependent on the other enzymes that are also varied.

To experimentally validate the predicted lower XR expression in the partial pathway compared to the full pathway, we chose to regenerate strains with two highly enriched genotypes, LL100A and LL110A, by specifically assembling those plasmids and transforming them into the original strain background (Fig. 2C). These two strains were grown on xylose and analyzed by shotgun proteomics to determine relative protein abundances (Fig. 2D). This technique has the advantage of directly measuring relative protein abundance rather than mRNA levels, an attractive feature since measuring mRNA amounts would miss any potential translational effects on final protein concentration. This is particularly useful as one might suspect that as the number of overexpressed enzymes increases, there is a possibility of saturating shared cellular machinery. Comparison of XR and XDH expression between LL110A and LL100A shows the expected change in XR and XDH peptide abundance, indicating a major reduction in XR expression and increase in XDH expression in LL100A (Fig. 2D). From these findings, we conclude that optimization of the xylose utilization enzymes alone results in a local utilization optimum of low XR expression, whereas under conditions where portions of the PPP are also overexpressed, far more XR is required to reach a higher, more global optimum.

3.2.2. *Transaldolase activity is limiting for aerobic xylose consumption*

By simultaneously titrating multiple genes' expression, we hoped to identify those genes whose activities were limiting growth on xylose. When we enriched the full pathway library, we observed weaker promoters driving most of the PPP genes, predominantly the lowest strength *pREV1*, suggesting that either these enzymatic activities are not limiting growth on xylose or their overexpression is detrimental to the cell. Only TAL enriched for the strongest promoter, *pTDH3*, suggesting that its activity is limiting. Other rational and inverse metabolic engineering efforts in various yeast backgrounds along with in vitro enzyme kinetics have also identified transaldolase activity to be significantly limiting for aerobic and microaerobic xylose utilization (Table S3) (Jeppsson et al., 2002; Walfridsson et al., 1995; Jin et al., 2005).

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Considering these studies, we were able to corroborate TAL's role as a limiting component for xylose utilization in the presence of oxygen.

3.2.3. *Aerobic expression optimization yields small improvements in xylose utilization*

Since most studies conventionally use high-strength promoters in a mostly arbitrary manner, we were curious how the enriched strains compared to a naïve overexpression strain in their ability to utilize xylose. Again, we used strains LL110A and LL100A, as well as two strains that simply use our strongest promoter to drive all genes ("*pTDH3* full pathway" and "*pTDH3* ØPPP") (Fig. 2C). These latter two strains mimic the arbitrarily high expression of each gene that would be naively done as opposed to balanced expression (Zhou et al., 2012; Matsushika et al., 2009b; Scalcinati et al., 2012; Johansson et al., 2002b). As expected, the strains expressing only XR, XDH, and XK grow poorly on xylose compared to the strains expressing extra copies of the PPP (Scalcinati et al., 2012; Jin et al., 2005; Walfridsson et al., 1995; Karhumaa et al., 2007). Strain LL110A grows at a maximum growth rate of $0.25 \pm 0.01\ h^{-1}$, making it the fastest aerobically growing strain in 2% xylose reported in literature to date (Fig. 2D) (Runquist et al., 2010; Bengtsson et al., 2008). Despite this, both LL110A and LL100A show only modest improvements in growth and xylose consumption compared to their respective all *pTDH3*-driven reference strain (Fig. 2E). This is surprising as we expected a flux imbalance or protein burden in the *pTDH3* full pathway strain to more drastically reduce growth and consumption of xylose. By comparing proteomic data between LL110A and *pTDH3* full pathway, we observe no change in expression of genes regulated by *pTDH3* in both strains (e.g., XR and TAL), but for all other genes expressed with lower strength promoters we observe the expected decreases in expression in the enriched strain (Fig. 2D). Having verified reductions in protein expression, we theorize that aerobic xylose consumption is primarily determined by XR and TAL expression.

3.3. *Anaerobic enrichments and strain characterizations*

A major advantage of the described combinatorial expression strategy is that we can readily select under different conditions to characterize how enrichment profiles change depending on the growth environment. Accordingly, we grew our libraries anaerobically because, given the enormous fermentation volumes that are required for large-scale biofuel production, the process would need to be fully anaerobic.

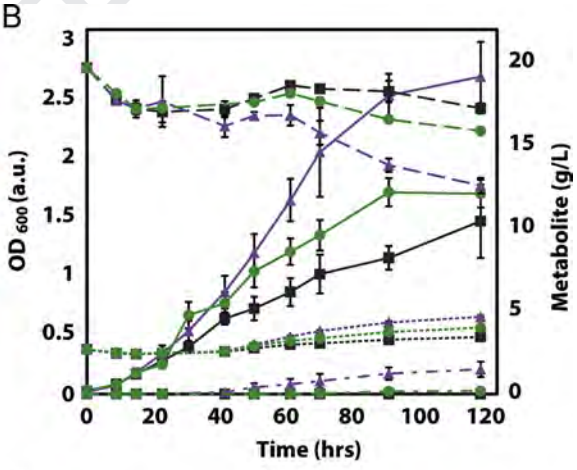
3.3.1. *Inclusion of the PPP only improves anaerobic xylose utilization upon expression optimization*

Unlike the aerobic libraries, the enrichment profiles for XR, XDH and XK for the anaerobically enriched partial (XR, XDH, and XK only) and full (XR, XDH, XK, RPE, RKI, TKL, TAL, and PYK) pathways were notably similar with XR enriching for the strongest

A

Strain name	*pTDH3* full pathway	LL110B	LL111A
Description	all *pTDH3* driven	aerobically enriched	anaerobically enriched
XR	*pTDH3*	*pTEF1*	*pTDH3*
XDH	*pTDH3*	*pTEF1*	*pRPL18B*
XK	*pTDH3*	*pTEF1*	*pRPL18B*
RPE	*pTDH3*	*pTDH3*	*pREV1*
RKI	*pTDH3*	*pTEF1*	*pREV1*
TKL	*pTDH3*	*pRNR2*	*pRPL18B*
TAL	*pTDH3*	*pTDH3*	*pTDH3*
PYK	*pTDH3*	*pRPL18B*	*pRNR2*

B



C

Strain name	*pTDH3* ØPPP	LL100A	LL101A
Description	all *pTDH3* driven	aerobically enriched	anaerobically enriched
XR	*pTDH3*	*pRPL18B*	*pTDH3*
XDH	*pTDH3*	*pTDH3*	*pTEF1*
XK	*pTDH3*	*pTEF1*	*pRPL18B*
RPE			
RKI			
TKL			
TAL			
PYK			

D

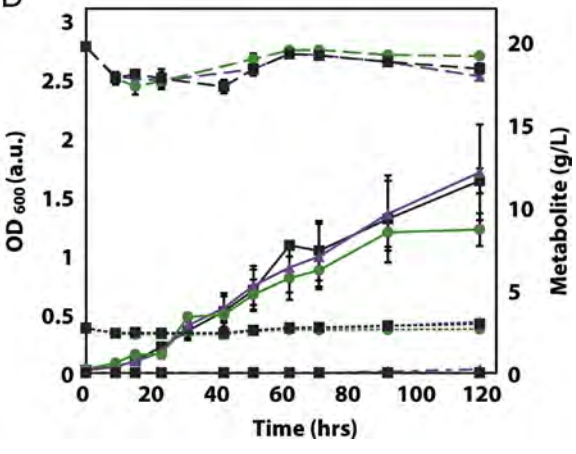


Fig. 3. Selection conditions are important for strain optimization. (A) Genotype and description of partial pathway reference strains and enriched strains identified from aerobic and anaerobic library enrichments. (B) Anaerobic fermentations in SX media supplemented with 0.01 g/L ergosterol, 0.43 g/L Tween 80 and 2.8 g/L ethanol for yeast strains indicated in (A): OD_{600} (——); Xylose (– – –); Ethanol (• • • • • •); Xylitol (– · – · –). (C) genotype and description of full pathway reference strains and enriched strains identified from aerobic and anaerobic library enrichments. (D) Anaerobic fermentations as described in (B) for yeast strains indicated in (C). Error bars indicate the SD of biological triplicates.

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promoter and the promoter rank ordering following XR ≥ XDH ≥ XK (Fig. S2). Anaerobically enriched strains were regenerated and characterized in comparison to the *pTDH3* reference strains to determine the effects of expression optimization (Fig. 3). Unlike in aerobic conditions, where only small differences were observed between enriched and reference strains, the full pathway LL111A significantly outperforms all other strains in biomass accumulation, xylose consumption, and ethanol production (Fig. 3B). Both of the *pTDH3* reference strains and the enriched partial strain LL101A all perform comparably, with the *pTDH3* full pathway consuming slightly more xylose. Interestingly, inclusion of the downstream enzymes with naïve *pTDH3* expression results in minimal to no improvement in growth and xylose consumption compared to overexpressing only the partial pathway.

3.3.2. *Expression optimization is sensitive to oxygenation conditions*

Comparing the aerobic and anaerobic full pathways, the enrichment profiles were similar between conditions with many of the strains having comparable genotypes (Figs. S2 and S3). However, some of the individual strain genotypes varied between the aerobic and anaerobic libraries, such as strains LL111A with LL110B (Fig. 3A). We examined the anaerobic performance of strains enriched both aerobically and anaerobically to ascertain the extent to which selection conditions result in strain specialization (Fig. 3). Despite the similarity of the consensus enrichment profiles between the full aerobic and anaerobic libraries, the two aerobically enriched strains LL110B and LL100A, underperform compared to their anaerobically enriched counterparts, LL111A and LL101A, under anaerobic growth conditions (Fig. 3B and D). Therefore, it is important during strain optimization to not only use the correct genetic context (i.e., full vs. partial pathway), but also to enrich under the target conditions because high-performance strains may not translate well from one condition to another.

3.4. *Expression optimization with a mutant, cofactor-balanced xylitol dehydrogenase*

The net cofactor imbalance in the fungal xylose pathway caused by the NADPH preference of XR and the strict NAD^+ requirement for XDH has long been implicated as a limitation in achieving high ethanol yields during xylose fermentation using these enzymes (Petschacher and Nidetzky, 2008; Hahn-Hägerdal et al., 2007). Numerous attempts to address this problem have included engineering these enzymes to have switched cofactor usage (Watanabe et al., 2005; Khoury et al., 2009; Bengtsson et al., 2009; Jeppsson et al., 2006). Despite their promise, reports on the effectiveness of these mutant enzymes in improving xylose consumption have been mixed (Watanabe et al., 2007; Bera et al., 2011; Cai et al., 2012). One limitation when assessing mutated pathways has been the inescapable complication of altered enzyme activity, which changes simultaneously with the cofactor affinities upon mutation of XR or XDH (Krahulec et al., 2010). Consequently, fermentation differences observed between cofactor balanced and imbalanced pathways could be attributed to either switched cofactor preference or altered balance of enzymatic activities. With our ability to optimize expression of the entire xylose utilization pathway, we can better separate the role of cofactor balancing from altering catalytic activity levels to address this long-standing question.

3.4.1. *Expression optimized anaerobic cofactor balancing improves growth, but not fermentation yields*

We assembled alternative XR-XDH-XK promoter library plasmids where xylitol dehydrogenase was substituted with the ARSdR mutant XDH developed by Watanabe et al. (2005), which almost exclusively uses $NADP^+$ instead of NAD^+. Mutant libraries were anaerobically enriched and genotyped as described previously. The expression profiles for mutant libraries show a somewhat higher mutant XDH expression levels than the native XDH

A

Strain name	Strain description	XR	XDH	XK	RPE	RKI	TKL	TAL	PYK
pTDH3 wtXDH	full pathway, all *pTDH3* driven	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*
pTDH3 mutXDH	full pathway, all *pTDH3* driven	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*	*pTDH3*
LL111A wtXDH	full pathway, anaerobically enriched	*pTDH3*	*pRPL18B*	*pRPL18B*	*pREV1*	*pREV1*	*pRPL18B*	*pTDH3*	*pRNR2*
LL211A mutXDH	full pathway, anaerobically enriched	*pTEF1*	*pTEF1*	*pRNR2*	*pREV1*	*pTEF1*	*pRPL18B*	*pRPL18B*	*pRNR2*

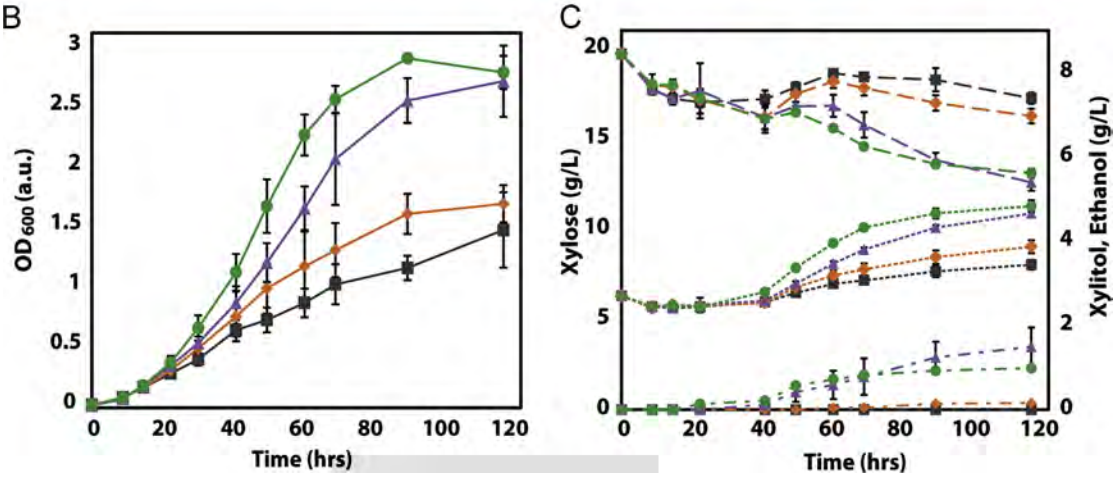

Fig. 4. Differences in xylose fermentation due to mutating XDH for the full pathway are reduced with expression optimization. (A) Genotype and description of reference strains and enriched strains identified from anaerobic library. (B) Anaerobic fermentations in SX media supplemented with 0.01 g/L ergosterol, 0.43 g/L Tween 80 and 2.8 g/L ethanol for yeast strains indicated in (A): OD_{600} (——) (C) extracellular metabolites for the fermentation shown in (B). Xylose (– – –); Ethanol (·····); Xylitol (–·–·–). Error bars indicate the SD of biological triplicates.

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and slightly lower TAL and PYK expression levels (Fig. S4). Highly-enriched genotypes from both mutant and wild-type XDH libraries along with *pTDH3* reference strains were chosen for comparison to assess the differences resulting from mutating XDH (Fig. 4A).

Comparing the *pTDH3* mutXDH and *pTDH3* wtXDH strains verified previous work with the mutant XDH: the mutant pathway shows slightly improved growth (Fig. 4B) and a 47% increase in xylose utilization and ethanol titer compared to the wild-type pathway (Fig. 4C) (Watanabe et al., 2007; Matsushika et al., 2009c). These fermentation differences could be the result of changes in both catalytic activity and cofactor preference. Surprisingly, this increase in utilization upon mutating XDH was reduced between the expression optimized strains. Strains LL111A and LL211A consume nearly identical amounts of xylose, and concurrently produce nearly the same amounts of xylitol and ethanol (Fig. 4C).

4. Discussion

Lignocellulosic fermentation promises a sustainable route for converting enormous plant biomass resources into biofuels. Xylose utilization is a critical step toward that goal, but it is difficult to understand how a multiple heterologous enzyme, high-flux pathway can be integrated into the metabolism of the host cell. Although there have been numerous studies inspecting various aspects of the xylose utilization pathway in *S. cerevisiae*, comparing the findings from these reports (Matsushika et al., 2009a) is complicated by the different strain backgrounds and growth conditions used. Therefore, it is imperative that the pathway is examined as a whole in order to find the optimal balance of catalytic activities for a given host and growth condition. Accordingly, we employed a recently described combinatorial expression strategy (Lee et al., 2013) to simultaneously vary the expression levels of the full *S. stipitis* xylose utilization and pentose phosphate pathways, which allowed us to better understand the importance of both intrinsic (genotypic) and extrinsic (growth conditions) variables for xylose utilization in our engineered yeast. One major advantage of this particular approach is that every gene can access the maximal range of transcriptional strength irrespective of the pathway size. Other strategies that rely on promoter mutagenesis are limited by a lack of unique, high-strength promoters that can be used for each gene. While repeated use of promoter sequences in a single construct may result in gene loss through homologous recombination, we have not observed frequent instances of recombination in direct tests (Lee et al., 2013) or difficulties in either cloning or repeated cycles of growth of our all-*pTDH3* strains (up to eight genes driven by the same promoter sequence). With our experimental design, we easily changed whether the partial or full pathway was expressed and which version of XDH was included. We also tested both aerobic and anaerobic growth conditions. This unbiased, systematic survey allowed us to glean some insight into the aggregate of prior research, which at times is unclear.

Genotyping many colonies from a library allowed us to determine convergent enzyme expression patterns, for example, bottleneck enzymes were expected to enrich for the highest-strength promoters. By comparing the enrichment profiles in different genetic contexts, we revealed interplay between the upstream xylose utilization pathway and the downstream pentose phosphate pathway. Depending on the number of enzymes included in the library, we observed markedly different enrichment profiles and dramatically different growth capabilities, showing that omission of genes in a pathway library can select for local optima that fail to perform as well as the optima achieved with co-expression and optimization of more enzymes. For example, under aerobic conditions, our full pathway libraries enriched for strong promoters driving XR, but the partial pathway libraries almost exclusively enriched for the medium-strength *pRPL18B* promoter (Fig. 2A and B); the full pathway strains also grow substantially faster (Fig. 2E). Our hypothesis for the cause of these enrichment differences is that for our strain background, enzymatic activity downstream of the first three enzymes is limiting, likely in the form of transaldolase activity based on the strong enrichment for *pTDH3* expression of TAL in the full pathway, along with previous metabolic engineering efforts by others (Jin et al., 2005; Johansson and Hahn-Hägerdal, 2002). Under this hypothesis, excess expression of upstream pathway enzymes beyond endogenous TAL activity may cause depletion of NADPH and accumulation of NADH (Hector et al., 2011), and depletion of ATP (Jin et al., 2003). Two possible solutions, both of which were enriched in our library selections, are either to reduce upstream expression or increase downstream capacity.

During their expression optimization of the first three enzymes, Zhao and coworkers observed that lower XR expression relative to XDH improved their laboratory strain's microaerobic fermentation while a high XR:XDH ratio was best for their industrial strain (Du et al., 2012). Our aerobic enrichment expression profiles of XR, XDH, and XK between the partial and full pathways (Fig. 2A and B) mirror their observed trends between the lab and industrial strains, respectively, suggesting that their laboratory strain may be limited in downstream expression and that a contributing factor to the better performance of their industrial strains could be increased PPP activity. This hypothesis could also explain the observation by Sedlak and coworkers that overexpression of the PPP resulted in almost no improvement in their industrial strain, as these activities may not be limiting in this strain background (Bera et al., 2011).

The discrepancies in the literature regarding the possible benefit from overexpression of the PPP for xylose fermentation could also be caused by variability in strain construction (Bera et al., 2011; Johansson and Hahn-Hägerdal, 2002; Kuyper et al., 2005; Karhumaa et al., 2005). As an example, the combinatorial optimization of *S. cerevisiae* TKL1, TAL1, and PYK1 by Lu and Jeffries found optimal xylose consumption when endogenous TAL1 was recombinantly expressed with a weaker promoter rather than a stronger one, while we observed higher TAL expression during our enrichments, particularly under aerobic conditions (Lu and Jeffries, 2007). This discrepancy could be the result of expressing different versions of TAL—*S. stipitis* in our study and *S. cerevisiae* in the Lu study. Indeed, toxicity associated with *S. cerevisiae* TAL1 overexpression has been reported (Jin et al., 2005). As another example within our study, benefits of heterologous expression of downstream genes under anaerobic conditions are only evident upon expression optimization. In fact, strains overexpressing the PPP at arbitrary high levels show no improved performance over the partial pathway (Fig. 3). Similar effects may be present in a subset of these other studies. Particularly under anaerobic conditions, there is likely a trade-off between high expression of requisite enzymes for xylose utilization and an expression burden to the cell, which would be more apparent upon further stresses from PPP overexpression.

Repeated use of a promoter may decrease transcription from this promoter due to saturation of cellular expression machinery. Of particular concern would be saturation of the strongest promoter, *pTDH3*, which also regulates the expression of the glycolytic enzyme, *GAPDH*. Indeed, shotgun proteomics revealed a 1.4-fold decrease ($p < 0.05$) in GAPDH protein between the *pTDH3* reference strain with eight copies of *pTDH3* and optimized strains containing only a couple copies. This GAPDH reduction appears to have minimal impact aerobically as the *pTDH3* full pathway grows similarly to LL110A (Fig. 2E). Thus, reduced GAPDH has either

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a minor contribution to differences in the *pTDH3* reference strains and optimized strains or its effects are condition dependent. Whatever the cause, arbitrarily high expression of a large number of pathway enzymes can produce a multitude of potential problems that can be solved, or at least largely alleviated, by combinatorial expression engineering, where complications such as these will be selected against by having non-limiting enzymes expressed with a weaker promoter.

Another interesting aspect of xylose utilization is the asymmetric cofactor usage, which has led to the hypothesis that resolving this imbalance would improve fermentation performance (Kotter and Ciriacy, 1993; Krahulec et al., 2010; Petschacher and Nidetzky, 2008). However, in mutating XDH to switch its cofactor preference (Watanabe et al., 2005), its activity is unavoidably also altered, thereby convoluting the contributions from cofactor balancing and potential activity balancing with other pathway enzymes. By incorporating this mutant into our library, we were able to empirically optimize the balance of enzymatic activities to investigate the importance of cofactor balance. Surprisingly, the mutant XDH only slightly improved fermentative yields in expression-optimized strains (Fig. 4B), certainly not as dramatic as would be expected if cofactor imbalances were a major flux impediment in our engineered system. The mutant XDH strains do show a modest improvement in growth, which implies cofactor balancing of this pathway aids in biomass accumulation, perhaps due to the role of NADPH in anabolic processes (Blank et al., 2005).

Finally, as expected, changing external variables led to differing expression profiles. Here we optimized under both aerobic and anaerobic conditions. Some of the aerobically enriched strains, such as LL110B, had notably inferior anaerobic fermentation performances (Fig. 3). The overall use of stronger promoters in LL110B may be affecting this strain's performance in an oxygen-dependent manner due to a flux imbalance or metabolic stress, such as XK toxicity (Jin et al., 2003). These findings, together with the observation that expression optimization under aerobic conditions had a fairly small impact on strain growth over arbitrarily high expression of all pathway genes, suggest that expression balancing is more critical under the more stringent anaerobic conditions.

In conclusion, we applied combinatorial expression engineering for the optimization of the full *S. stipitis* xylose utilization pathway in the favored production host, *S. cerevisiae*. We observed, in both aerobic and anaerobic conditions, dramatically improved performance of strains with the full pathway optimized over complementing *S. cerevisiae* with only the minimal, requisite activities. Expression optimization was also used to separate the effects of activity balancing from cofactor balancing and effects from altered oxygenation. Based on our findings, it would be prudent to include more metabolic genes – perhaps from glycolysis, gluconeogenesis, and/or the oxidative PPP – in further studies of this pathway. Additionally, this described strategy can be employed toward other pathways with outputs amenable to high-throughput screening or selection.

Uncited reference

Q2 Flanagan and Waites (1992).

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.06.002.

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