ENGINEERING DIVERSE YEAST SPECIES FOR OPTIMAL SUGAR CONSUMPTION AND ENHANCED PRODUCT FORMATION

BY

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THESIS

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ABSTRACT

Yeasts are employed ubiquitously within industry and academia for basic studies in biology and production of value-added compounds. Although the term yeast generally reminds people of the common baker's yeast, *Saccharomyces cerevisiae,* yeasts are a diverse group of organisms with an extensive evolutionary history. This thesis focuses on two vastly differing yeasts: *Yarrowia lipolytica* and *S. cerevisiae*.

Y. lipolytica is an obligate aerobic oleaginous yeast best known for its capabilities at digesting alkanes and strong capacity for producing lipids. The species is commonly used as a model yeast for studying lipogenesis and has an extensive history in the academic literature. In industry, this yeast has been highlighted as having strong potential for production of a wide range of molecules. Recently, a genetically engineered strain of this yeast has been employed by DuPont in production of omega-3 fatty acids. Additionally, the species has been proposed for usage in environmental cleanup applications as well as bioconversion of industrial wastes, specifically glycerol from biodiesel production. While recent work has identified that this species possesses genes within the cellobiose and xylose metabolic pathways, most strains of species do not naturally possess the ability to digest lignocellulosic sugars. This thesis describes the creation of a cellobiose-consuming strain of *Y. lipolytica*. We investigate the necessary modifications to enable cellobiose utilization and physiologically characterizes a cellobiose-consuming strain. The strain is then used in a simultaneous saccharification and fermentation process to produce citric acid from cellulose.

The second half of this thesis focuses on *S. cerevisiae.* While this yeast is a hallmark of the phenomenon known as glucose repression, wherein glucose is

consumed before all other sugars, bypassing this effect and enabling simultaneous consumption of multiple sugars is desirable for robust fermentations and implementing continuous fermentation processes. This thesis demonstrates that slowing the rate of glucose consumption leads to simultaneous cofermentation of glucose and other sugars. Experiments are shown with glucose/xylose and glucose/galactose mixtures, however the mechanism described to explain this design indicates that other fermentable carbon sources may also be co-utilized in this fashion.

The final research chapter of this thesis investigates the consequences of glucose sensing through the membrane sensors Snf3 and Rgt2 on non-glucose sugar consumption and non-ethanol product formation. Fermentations are performed with strains devoid of these glucose sensors but capable of consuming the lignocellulosic sugars xylose and cellobiose. Later, the dual role of glucose sensing and import on product formation is investigated with sensor-deleted and inducible-hexokinase strains expressing a lactic acid dehydrogenase LdhA. Finally, deletion of sensors is found to increase yield of the amino acid intermediate 2-isopropylmalate.

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CHAPTER 1: THESIS INTRODUCTION

1.1 Introducing the cellobiose metabolic pathway into *Yarrowia lipolytica*

Yarrowia lipolytica is a promising production host for a wide range of molecules, but limited sugar consumption abilities prevent utilization of an abundant source of renewable feedstocks. In this study, a *Y. lipolytica* strain capable of utilizing cellobiose as a sole carbon source is created by using endogenous promoters to express the cellodextrin transporter *cdt-1* and intracellular β-glucosidase *gh1-1* from *Neurospora crassa*. The engineered strain was also capable of simultaneous co-consumption of glucose and cellobiose. Although cellobiose was consumed slower than glucose when engineered strains were cultured with excess nitrogen, culturing with limited nitrogen led to cellobiose consumption rates comparable to those of glucose. Under limited nitrogen conditions, the engineered strain produced citric acid as a major product and greater citric acid yields were observed from cellobiose (0.37g/g) than from glucose (0.28g/g). Culturing with a sole carbon source of either glucose or cellobiose induced additional differences on cell physiology and metabolism and a link is suggested to evasion of glucose-sensing mechanisms through intracellular creation and consumption of glucose. Ultimately, this cellobiose-utilization system is applied to produce citric acid from bioconversion of crystalline cellulose through simultaneous saccharification and fermentation (SSF).

1.2 Enabling simultaneous sugar utilization in *Saccharomyces cerevisiae*

Microorganisms commonly exhibit preferential glucose consumption and diauxic growth when cultured in mixtures of glucose and other fermentable carbon sources, hindering simultaneous utilization of mixed sugars. While various genetic perturbations have enabled simultaneous consumption of specific mixtures of sugars, a widely-applicable technique is desirable. This study demonstrates that reducing the rate of glucose consumption allows xylose and glucose to be simultaneously consumed. Additional investigations demonstrate that the optimal rate of glucose consumption depends on the absolute and relative extracellular ratio of glucose and xylose. Further experiments reveal that this technique also allows simultaneous consumption of glucose and galactose. These results demonstrate that sugar mixtures can be simultaneously consumed without modifications to the endogenous transport machinery. In the case of both glucose/xylose and glucose/galactose mixtures, tuning the rate of glucose phosphorylation allowed adjusting the relative sugar consumption rates as desired. This study contributes knowledge regarding the role of pathway flux in catabolite repression and presents a general design principle enabling simultaneous sugar utilization across a range of metabolic pathways without regard to competitive transport inhibition.

1.3 Investigations into glucose sensing in *Saccharomyces cerevisiae*

S. cerevisiae is commonly employed in biotechnological applications such as sugarcane and corn ethanol, making use of its highly optimized capabilities at consuming glucose and producing ethanol. However, growing efforts to use this yeast in bioconversion of lignocellulosic feedstocks and production of complex fuels and chemicals suggest that the glucose-to-ethanol optimization may be detrimental to these new initiatives. This study regards the membrane glucose sensors Snf3 and Rgt2, which detect glucose and optimize membrane sugar transport and growth. This study investigates the potential role of these sensors in production of lactic acid and consumption of the lignocellulosic sugars xylose and cellobiose. Experimental results indicate that the impact of removing these sensors may differ depending on the lignocellulosic sugar consumed and whether or not they require a heterologous transporter for uptake. Experiments are also performed to determine whether the presence of these sensors is detrimental to production of lactic acid. This report finds that sensor deletions lead to increased lactic acid yield from strains expressing lactic acid dehydrogenase LdhA, however this is merely a side-effect of a reduced glucose uptake rate rather than any direct link to membrane glucose sensing mechanisms. A second investigation finds that production of the amino acid intermediate 2-isopropylmalate can also be enhanced through sensor deletions, in an effect that cannot be explained solely by reduced uptake flux.

CHAPTER 2: DEVELOPMENT AND PHYSIOLOGICAL CHARACTERIZATION OF CELLOBIOSE-CONSUMING *YARROWIA LIPOLYTICA*

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2.1 Introduction

The decreasing supply of fossil fuels and growing awareness of environmental impacts of conventional energy and chemical production processes have motivated sustainable and environmentally-friendly production of fuels and chemicals from renewable feedstocks [1, 2]. Among microorganisms identified to have high potential for application in the renewable chemicals industry, *Yarrowia lipolytica* has been highlighted as a viable microbial platform for production of a broad range of target molecules, including proteins [3], organic acids [4-6], lipids [7-9], and specialized fatty acids [10-12]. The organism has been used and characterized for industrial applications [13, 14] and has been extensively studied as a model organism for a variety different cellular processes including lipogenesis [15, 16], protein secretion [17-19], peroxisome biogenesis [20], hydrophobic substrate utilization [21, 22], and fungal dimorphism [23, 24]. Interest in *Y. lipolytica* stemmed historically from its unusual ability to consume and convert then-inexpensive alkanes to organic acids [25]. However, the species has limited endogenous ability to digest sucrose, maltose, xylose, arabinose, galactose and cellobiose [26]. These limitations prevent usage of a large supply of renewable plant biomass in any *Y. lipolytica*-based processes until efficient and reliable lignocellulosic sugar utilization pathways are developed.

Cellulose, a substantial component of the biomass in lignocellulosic feedstocks [27], consists of a linear chain of glucose molecules joined by β(1→4) glycosidic bonds. This polymer is not water soluble, cannot be consumed by most strains of *Y. lipolytica,* and accordingly requires additional processing before it is suitable for bioconversion [28]. Cellulosic biomass is initially subjected to thermochemical pretreatment wherein

long cellulose chains are broken into smaller more enzymatically-accessible oligomeric components suitable for use in simultaneous saccharification and fermentation (SSF) [29-31]. The SSF process combines treated biomass, cellulolytic enzymes, and an industrial organism to simultaneously convert treated biomass to digestible sugars and the desired end product [32, 33]. Continuous sugar consumption by the production host maintains the equilibrium of cellulolytic reactions in favor of biomass degradation and end product formation. In the conventional SSF process, cellulase and β-glucosidase are used to degrade treated biomass to a digestible sugar. The SSF process can be economically improved by enabling cellular consumption of cellobiose, which allows the action of cellulases alone to be sufficient for production of a digestible sugar and eliminates the costs associated with supplementing β-glucosidase [34]. Although a few strains of cellobiose-consuming *Y. lipolytica* have been isolated [35], it is not a general feature of the species [26, 36, 37], and thus a widely applicable method for enabling cellobiose utilization in this species is necessary to establish an efficient and economic process of using *Y. lipolytica* for chemical production from cellulosic biomass.

The strategy to establish cellobiose assimilation and utilization capability in *Saccharomyces cerevisiae* has been demonstrated by expression of cellodextrin transporter and intracellular beta-glucosidase [38, 39]. The approach allows intracellular hydrolysis of cellodextrins and subsequent consumption of released glucose, which minimizes glucose repression [40]. In this study, a cellobiose utilization pathway is introduced to *Y. lipolytica* by expressing the cellodextrin transporter *cdt-1* (NCU00801) and the intracellular β-glucosidase *gh1-1* (NCU00130) from *Neurospora crassa* [38] under the control of strong endogenous promoters [41]. This approach

successfully enabled the consumption of cellobiose in engineered *Y. lipolytica*. Cultivation of the cellobiose-consuming strain on media with varied carbon sources and nitrogen content confirmed differential effects of carbon sources on growth, lipid body (LB) formation, and citric acid production. Practical applications of this type of cellular engineering are demonstrated by using this engineered strain to produce citric acid from an insoluble cellulose substrate in an improved SSF process.

2.2 Materials and Methods

2.2.1 Strains and Plasmids

The strains and plasmids used in this study are listed in Table 1. Strains were stored at -80°C in 20% vol/vol glycerol solutions. The *Yarrowia lipolytica* strain Po1f used in this study is a leucine and uracil auxotroph with deletions in the AXP and AEP protease-encoding genes [42].

2.2.2 Analytical Methods

Sugar and citrate concentrations were determined by high-performance liquid chromatography (Agilent 1200 series) with a refractive index detector using a Rezex ROA-Organic Acid H+ (8%) column eluted with 0.005N H_2SO_4 at 50°C and a flow rate of 0.6 mL/min. Biomass formation was quantified by measuring optical density (OD) at 600 nm using a UV-visible spectrophotometer (Biomate 5; Fisher, NY).

2.2.3 Genetic Techniques

Standard molecular cloning techniques were used in this study [43]. Po1f genomic DNA (gDNA) was purified with a YeaStar Genomic DNA Kit (Zymo Research Corporation; Irvine, CA, USA) following the manufacturer's instruction. The primers used in this study are listed in Table 2.

Plasmid pINT03-hrGFP is derived from pSL16-cen1-1(227) [44]. The CYC terminator was PCR amplified by primers 1 and 2 using p413GPD (ATCC ® 87354™) [45] as the template and introduced into pSL16-cen1-1(227) by Gibson assembly [46], resulting in plasmid pSL16-cen-cyc. The UAS1B-LEUM promoter was cut from

pUAS1B-LEUM [47] using the restriction enzymes BstBI and AscI and then ligated into the same site of plasmid pSL16-cen-cyc, resulting in plasmid pSL16-cen-UAS1B-LEUM-cyc. The fluorescent reporter protein hrGFP was PCR amplified by primers 3 and 4 from phrGFP-1 (Agilent), omitting ATG and harboring AscI restriction sites on both ends, and ligated into AscI-digested pSL16-cen-UAS1B-LEUM-cyc, resulting in plasmid pSL16-LEUM16-hrGFP.

To generate the integrative expression plasmid, the ORI and CEN sites were removed by following steps: pSL16-LEUM16-hrGFP was digested with NdeI and BstBI to remove cen1-1(227) and ligated with a LIP7 cassette harboring NdeI and BstBI site, resulting in plasmid pINT02-hrGFP. The LIP7 cassette was constructed with a two-step overlap extension PCR. In the first step, *Y. lipolytica* W29 genomic DNA were amplified using primers 5 and 6 for LIP7_U (upstream), and primers 7 and 8 for LIP7_D (downstream). In the second step, LIP7_U and LIP7_D fragments were fused together using primers 5 and 8. In this process, an AvrII restriction site was introduced in the middle of LIP7 in order to linearize the plasmid. The purpose of LIP7 fragment was to enable integration into the LIP7 locus by homologous recombination [41], however integration into the LIP7 locus was not performed in this study. Finally, pINT02-hrGFP was digested with NotI and recircularized to remove the NotI-ORI1001-NotI cassette, resulting in pINT03-hrGFP. For subsequent use in this study, pINT03-hrGFP was digested with AscI to remove the GFP reporter and inserted with the target gene expression cassette of interest or recircularized to form plasmid pINT03.

In order to enable efficient expression of *cdt-1* and *gh1-1,* new gene cassettes were created based on the promoters and terminators of the *Y. lipolytica* FBA1 and

TDH1 genes, previously observed to induce measurable heterologous gene expression [41]. The TDH1 promoter and terminator were cloned from Po1f genomic DNA using primer pairs 9,10 and 13,14, respectively, and the FBA1 promoter and terminator were cloned from Po1f genomic DNA using primer pairs 15,16 and 19,20, respectively. The β-glucosidase was cloned from the plasmid pRS425-gh1-1 using primers 11 and 12 and the cellodextrin transporter was cloned from pRS426-cdt-1 using primers 17 and 18. The TDH1p-gh1-1-TDH1t-FBA1p-cdt-1-FBA1t gene cassette was assembled using Gibson Assembly Master Mix (New England Biolabs; Ipswich, MA, USA) using the cloned fragments described above. To create plasmid pINT03-BGL, the 2.7kb TDH1p-gh1-1-TDH1t fragment was PCR amplified from the Gibson assembly reaction using primers 9 and 21, digested with AscI, and ligated into pINT03 at the AscI site. To create plasmid pINT03-BGL-CDT, the full 5.6kb gene cassette was PCR amplified from the Gibson assembly reaction using primers 9 and 20 followed by AscI digestion and ligation into pINT03.

Plasmids were linearized by digestion with AvrII and integrated into the Po1f genome using the lithium acetate transformation method described previously [48]. Transformants were selected on leucine deficient minimum media agar plates (20 g/L glucose, 0.69 g/L CSM-Leu, 1.7 g/L YNB, 5 g/L ammonium sulfate, 20 g/L agar).

2.2.4 Media Recipes and Culture Conditions

Seed media is comprised of 10 g/L Bacto yeast extract (BD; Franklin Lakes, NJ, USA), 20 g/L Bacto peptone (BD), and 20 g/L glucose (Sigma-Aldrich; St Louis, MO, USA). Excess nitrogen media is comprised of 1.7 g/L yeast nitrogen base (MP

Biomedicals; Santa Ana, CA, USA), 0.79 g/L complete supplement mixture (MP Biomedicals), 5 g/L ammonium sulfate (Sigma), and 200 mg/L uracil (Sigma), with a carbon source of 15 g/L cellobiose (Fluka, Sigma-Aldrich, USA) and/or 15 g/L glucose (Sigma). Excess nitrogen media was adjusted to an initial pH of 6.5 using 10M sodium hydroxide (Sigma). The components of limited nitrogen media are 1.7 g/L yeast nitrogen base (MP Biomedicals), 0.79 g/L complete supplement mixture (MP Biomedicals), 0.5 g/L ammonium sulfate (Sigma), 200 mg/L uracil (Sigma), 12 g/L dibasic potassium phosphate (Thermo-Fisher Scientific; NY, USA), and 12 g/L monobasic potassium phosphate (Thermo), with a carbon source of either 30 g/L cellobiose or glucose. SSF media is comprised of 1.7 g/L yeast nitrogen base (MP Biomedicals), 0.79 g/L complete supplement mixture (MP Biomedicals), 200 mg/L uracil (Sigma), 12 g/L dibasic potassium phosphate (Thermo-Fisher Scientific; NY, USA), and 12 g/L monobasic potassium phosphate (Thermo), with a carbon source of 40 g/L Avicel PH-101 (Sigma). Limited nitrogen and SSF media were adjusted to an initial pH of 6.5 using 2.5M sulfuric acid (Sigma).

Unless stated otherwise, all cultures in the fermentation experiments were initiated with fresh pre-cultured cells from seed media. Seed cultures were inoculated with cells from frozen stocks and grown for 24 hours prior to use (4 mL in Falcon tube, 30°C, 250 rpm). Excess nitrogen experiments were inoculated to an initial cell density equal to an OD of 0.05 and performed in 25 mL media in 125 mL shake flasks at 30°C and 250 rpm. Limited nitrogen experiments were inoculated to an initial cell density equal to an OD of 0.01 and carried out in 250 mL flasks at 30°C and 250 rpm in 50 mL media with either glucose or cellobiose as a carbon source. Experiments were

conducted in duplicate. Sugar consumption rates and citric acid yields displayed in Table 3 are calculated from the data collected at the initial and final data points.

2.2.5 Measurement of β-glucosidase activity

β-glucosidase activity assay was performed as described previously [34, 38]. Fresh cells from seed media were inoculated at OD 0.05 into 25 mL excess nitrogen media in a 125 mL shake flask. After 24 hours of growth at 30°C and 250 rpm, cells in exponential phase were harvested for use in the activity assay. Extracellular β-glucosidase activity was determined by centrifugation of the cell suspension and assaying the supernatant. Intracellular activity was determined by lysing cells with Y-PER solution (Fisher), centrifuging to remove cell debris, and assaying the supernatant. Activities were standardized by total protein content determined with a BCA Protein Assay Kit (Pierce; Rockford, IL, USA). Enzyme assay results are displayed as the mean of two technical replicates with the standard deviations indicated by error bars.

2.2.6 Visualization of Lipid Bodies

Lipid visualization was performed by staining described previously [8]. Fresh cells from seed media were inoculated at OD 0.05 in excess nitrogen media with either glucose or cellobiose and grown at 30°C and 250rpm. After 50 hours, cells were spun down and resuspended at OD 25. Lipids were stained by adding Nile red (Sigma) 1 mg/mL solution in acetone to the cell suspension (1/10 vol/vol) and incubating for 60 minutes at room temperature. Cells were then washed with saline and resuspended to OD 25 in 50mM potassium phosphate buffer. Cells were immobilized by mixing with

low melting-point agarose (Fisher) and placed on a Fluorodish (World Precision Instruments; Sarasota, FL, USA) for viewing. Stained cells were viewed with an Andor Technology Revolution System spinning disk confocal microscope (Andor Technology; Belfast, UK).

2.3 Results

2.3.1 Effect of *cdt-1* and *gh1-1* expression on cellobiose consumption ability

Three *Y. lipolytica* strains were constructed to investigate the genetic engineering required to enable cellobiose consumption: a strain producing an intracellular β-glucosidase (Po1f-B), a strain producing both an intracellular β-glucosidase and a cellodextrin transporter (Po1f-BC), and a negative control strain containing an empty vector (Po1f-pINT03). Initial investigations of sugar consumption ability were performed in excess nitrogen media with either glucose or cellobiose as the sole carbon source. As expected, the control strain Po1f-pINT03 was unable to consume cellobiose (Figure 1A). The strain Po1f-B expressing only β-glucosidase consumed 0.68 g/L of cellobiose over 51 hours, grew marginally to a final OD of 0.78, and exhibited a specific cellobiose uptake rate of 1.32 g cellobiose / g cell / h. The strain Po1f-BC grew to an OD of 11.2 and consumed 15.25 g/L over the same time period with a specific uptake rate of 2.36 g cellobiose / g cell / h, indicating that expression of a cellobiose transporter is essential for efficient cell growth and intracellular cellobiose utilization (Figure 1A). Only Po1f-BC was able to effectively assimilate carbon and accumulate biomass in the medium containing cellobiose while Po1f-B and Po1f-pINT03 did not show significant growth (Figure 1B). When cultured in glucose as a control condition, all three strains exhibited nearly identical glucose uptake rates and biomass accumulation (Figures 1C and 1D). Noticeably, the strain Po1f-BC cultured on cellobiose consumed sugar slower and accumulated more biomass than when cultured on glucose. The strain Po1f-BC grew to final cell densities equal to an OD of 10.6 (cellobiose) and 8 (glucose), with specific

sugar consumption rates of 2.36 g cellobiose / g cell / h and 3.00 g glucose / g cell / h, respectively (Table 3).

Intracellular β-glucosidase activity assays were performed for all three strains in glucose cultures. Cells were lysed to release cell contents and the lysate was assayed. As expected, no β-glucosidase activity was observed in the empty vector strain Po1f-pINT03 and intracellular β-glucosidase activity was detected in both Po1f-B and Po1f-BC (Figure 2). Po1f-BC exhibited an almost 3-fold increase in intracellular activity per microgram of protein as compared to Po1f-B. This difference in intracellular β-glucosidase activity between the two strains expressing *gh1-1* may be due to different location of chromosomal integration, as *Y. lipolytica* integrative transformation tends to occur randomly within the genome through non-homologous end-joining [12, 49]. The ability for Po1f-B to inefficiently assimilate small amounts of cellobiose could be due to unintended secretion of β-glucosidase. To investigate this possibility, the three strains were cultured in excess nitrogen media with cellobiose and assayed for β-glucosidase activity in the media. However, no detectable extracellular β-glucosidase activity was observed in any culture assayed.

To investigate mixed-sugar consumption ability and the possible presence of any glucose repression mechanisms on cellobiose consumption, Po1f-pINT03 and Po1f-BC were each cultured in excess nitrogen media with a mixture of cellobiose and glucose as carbon sources. While Po1f-pINT03 consumed only the glucose and finished sugar uptake within 42 hours, Po1f-BC was able to digest both sugars simultaneously and finish consumption within about 72 hours (Figures 3A and 3B). Interestingly, simultaneous co-consumption of cellobiose and glucose improved the total specific

sugar consumption rate of Po1f-BC (2.96 g sugar / g cell / h) to a similar rate to a control strain consuming only glucose (3.07 g sugar / g cell / h), although Po1f-BC consumed each individual sugar at a slower rate than Po1f-pINT03 (Table 3, Figure 3C). Po1f-BC also accumulated slightly more biomass per gram of sugar consumed (Figure 3D).

2.3.2 Citric acid production by cellobiose-consuming strain Po1f-BC

Strain Po1f-BC was cultured with a carbon source of either glucose or cellobiose in limited nitrogen media, known to induce citric acid production in *Y. lipolytica* [50]. Citric acid production was observed earlier in cellobiose cultures (48h) than glucose cultures (60h) and total yield from cellobiose (0.37 g citrate/g cellobiose) exceeded that of glucose (0.28 g citrate/g glucose) (Figures 4A and 4B, Table 3). Although the specific sugar uptake rate of both cultures was similar when calculated over the entire course of the fermentation (1.90 g glucose / g Po1f-BC / h vs. 1.94 g cellobiose / g Po1f-BC / h), there was a significant difference at the 48h time point (2.34 g glucose / g Po1f-BC / h vs. 2.92 g cellobiose / g Po1f-BC / h) corresponding with the earlier observation of citrate production in cells cultured on cellobiose.

2.3.3 Lipid Body Formation

The results on sugar consumption rates and citric acid yields indicate that the carbon sources, cellobiose versus glucose, might cause some difference in the cell metabolism of the *Y. lipolytica* strains. To investigate the potential effects on lipid body (LB) formation, Po1f-pINT03, Po1f-B, and Po1f-BC were grown to early stationary

phase, stained with Nile red, and viewed under a spinning-disk confocal microscope. In conditions of both excess and limited nitrogen all three strains developed LBs when grown on glucose and were used a reference for normal LB formation (Figures 5A-C, G-I). When cultured with cellobiose, no LBs formed in Po1f-pINT03 (Figures 5D, J), however small LBs were observed in some Po1f-B cells (Figures 5E, K). Po1f-BC cultured in cellobiose formed LBs and confirmed the potential to convert cellobiose to lipids (Figures 5F, L). Effects on internal metabolism were indicated by smaller LB production in cells cultured with cellobiose as a sole carbon source. Cells grown in conditions of excess nitrogen (Figures 5A-F) showed a similar pattern of LB formation as cells grown in limited nitrogen (Figures 5G-L): strains Po1f-pINT03 and Po1f-B were unable to consume cellobiose and did not show any significant formation of LBs while strain Po1f-BC was able to form lipid bodies from cellobiose as a sole carbon source. However, some differences were observed depending on culturing with excess or limited nitrogen. With glucose as a sole carbon source, strains cultured on limited nitrogen (Figures 5G-I) showed larger LB formation than strains cultured with excess nitrogen (Figures A-C). Additionally, while culturing strain Po1f-BC on cellobiose showed reduced LB formation as compared to glucose regardless of the presence of nitrogen, this difference was far less significant when the strain was cultured with limited nitrogen (Figures I, L) as compared to excess nitrogen (Figures C, F).

2.3.4 Citrate Production by Simultaneous Saccharification and Fermentation

In the standard SSF process, insoluble cellulose is digested by supplemented cellulases into cellodextrin oligomers, which are then further broken down into digestible

glucose monomers by supplemented β-glucosidase. The ability for Po1f-BC to directly consume cellobiose should enable it to perform SSF without supplementing β-glucosidase [34, 38] and its simultaneous co-consumption abilities may aid in driving the enzymatic digestion of solid cellulose to oligomeric components. In order to investigate these possible benefits, an SSF experiment to produce citrate from microcrystalline cellulose is performed. Strains Po1f-pINT03 and Po1f-BC were precultured in glucose and cellobiose, respectively, and allowed to naturally reach a state of citric acid production. At this point, cells were harvested and inoculated into SSF media at a cell density of 1 g/L dry cell weight. All flasks were supplemented with endoglucanase, and one set of Po1f-pINT03 replicates was supplemented with β-glucosidase. After 240 hours, cultures were harvested and citrate concentration was determined (Figure 6). As expected, Po1f-pINT03 supplemented only with endoglucanase showed the worst performance with only 2.8 g/L citrate production and Po1f-pINT03 supplemented with both endoglucanase and β-glucosidase exhibited modest improvement to about 3.7 g/L citrate production. Without supplementing any β-glucosidase, the cellobiose-consuming strain Po1f-BC displayed the highest citrate production at about 5.1 g/L, suggesting that engineering cells for intracellular cellobiose consumption may be a viable method to reduce enzyme costs in bioconversion of cellulosic feedstocks by *Yarrowia lipolytica*.

2.4 Discussion

The development of sustainable processes for microbial bioconversion of renewable resources to energy or commercially valuable chemicals is a promising method to reduce reliance on fossil fuels and curb the environmental effects of conventional industrial chemical production [51, 52]. Lignocellulosic biomass has high potential for use in industrial bioprocesses due to its high security of supply and lesser impact on food prices than food crop-based feedstocks [53-55]. While *Y. lipolytica* has been recognized for its strong production potential [10, 56], limited sugar consumption capabilities prevent use of many crop-based feedstocks [35, 36]. This work is a step towards enabling bioconversion of lignocellulosic biomass using *Y. lipolytica* by enabling consumption of cellobiose. Through chromosomal expression of heterologous cellodextrin transporter *cdt-1* and intracellular β-glucosidase *gh1-1*, a cellobiose-consuming strain of *Y. lipolytica* (Po1f-BC) was created that achieved comparable cellobiose consumption rates to endogenously-consumable glucose.

It was determined that efficient cellobiose consumption depended on expression of a cellodextrin transporter by comparing with an inefficient cellobiose-consuming strain (Po1f-B) expressing only an intracellular β-glucosidase. The ability for this strain to consume a small amount of cellobiose over a period of days indicates that the β-glucosidase is coming into contact with some amount of substrate. One possible mechanism of this interaction is due to some level of unintended protein secretion; however, no extracellular β-glucosidase activity was detected in any strain. This result coupled with the fact that directing heterologous protein secretion requires inclusion of a signal peptide [19, 42] suggests that unintentional protein secretion is not the root cause

of the inefficient cellobiose consuming ability of strain Po1f-B. A possible explanation may be that a small amount of cellobiose is able cross the *Y. lipolytica* membrane through some unidentified transporter, but further study will be needed for confirmation.

Cellobiose consumption has been established in *S. cerevisiae* as a practical technique to improve bioconversion of cellulose through SSF and decrease process costs associated with cellulolytic enzymes required to degrade biomass into consumable sugars [34, 38]. These benefits were revealed to also exist in *Y. lipolytica* through the results on citric acid production from crystalline cellulose: the control strain supplemented with β-glucosidase and cellulase produced less citric acid as compared to the engineered cellobiose-consuming strain supplemented only with cellulase. Although the SSF experiments in this study were carried out at biologically-permissive conditions of 30°C with pH buffered at 6.5, it is often advantageous to carry out SSF at high temperature and low pH to promote the depolymerization of cellulosic biomass [33]. Consequently, further work is necessary to optimize SSF conditions and find a balance between rapid biomass degradation and efficient production of target molecules.

Interestingly, growth on glucose/cellobiose mixtures resulted in simultaneous co-consumption of both sugars. In *S. cerevisiae,* glucose repression systems result in preferential consumption of glucose before utilization of other sugars [57, 58]. In contrast, strong glucose repression was not observed here, supporting previous results that *Y. lipolytica* has a weaker glucose repression system relative to *S. cerevisiae* [59] and suggesting that further engineering of *Y. lipolytica* may enable simultaneous sugar

utilization [40, 60] without the considerable difficulty encountered in attempts to bypass strong carbon catabolite repression systems in other production hosts [61].

Glucose repression has a wide range of metabolic and regulatory effects on many organisms and can impact their use in bioconversion processes. For example, glucose repression in *S. cerevisiae* prevents simultaneous utilization of two sugars and evasion of glucose sensing is required for cofermentation of sugars [40, 60, 62]. In *Y. lipolytica* glucose repression hinders efficient production of lipase [63] and modifications to carbon catabolite repression are required to enable efficient lipase production from glucose [64, 65]. Intracellular cellobiose degradation has proven to be useful as an effective way to evade glucose-sensing mechanisms in *S. cerevisiae* [40, 66] and several results within this work suggest that the technique may be applied to *Y. lipolytica.* First, cells cultured with excess nitrogen on cellobiose display a deficient sugar consumption rate that can be restored through co-consumption of a glucose and cellobiose mixture. Further, culturing on cellobiose with excess nitrogen results in smaller lipid bodies and increased biomass accumulation. Finally, in limited-nitrogen cultures greater citric acid yields were observed from cellobiose (0.37 g citric acid/g cellobiose) than from glucose (0.28 g citric acid/g glucose). These results in combination suggest that cellobiose-consuming cells bypass some carbon-sensing system that induces optimal cell growth and regulates the transition to lipid and organic acid production phases. Such a system has been identified in the regulatory protein Snf1, but the mechanism responsible for its induction is currently unknown [67]. Improving cellobiose consumption in growth-oriented media through evolutionary engineering or genetic investigations may be able to lead to identification of mutations

resulting in constitutively active carbon-sensing regulatory pathways. Accordingly, this system of cellobiose consumption could be used as a tool to further elucidate the mechanisms of carbon sensing in this popular production host.

Yarrowia lipolytica is generally considered to have variable ability in cellobiose consumption [26] and there are conflicting reports on the ability for the species to use sucrose [68-70], arabinose, and xylose [26, 71-73]. Studies on the same strain of the species have yielded inconsistent results on sugar consumption abilities [71, 73], confusing the situation even further and emphasizing the need for more work in this area to establish a consensus on some of the more controversial issues regarding the substrate utilization capabilities of *Y. lipolytica*. This study investigated the challenge of enabling consumption of cellobiose, a sugar that cannot be used by most strains of *Y. lipolytica.* By taking a strain devoid of cellobiose consumption ability and determining the genetic modifications necessary to enable efficient cellobiose utilization, the barriers preventing use of cellulosic feedstocks in *Y. lipolytica*-based bioprocesses have been reduced.

2.5 Tables

Table 1 – Strains and Plasmids

Plasmids and strains	Relevant Features	Reference or source
Strains		
Po1f	MatA, Ura-, Leu-	[42]
Po1f-pINT03	Po1f / pINT03	This study
Po1f-B	Po1f / pINT03-BGL	This study
Po1f-BC	Po1f / pINT03-BGL-CDT	This study
Plasmids		
pSL16-cen1-1(227)	*Y. lipolytica* plasmid, CEN-1, ORI1001, Amp, LEU2	[44]
p413GPD	p413GPD containing CYC1 terminator	ATCC 87354 [45]
pSL16-cen-cyc	pSL16-cen1-1(227) / MCS / CYC1 terminator.	This study
pUAS1B-LEUM	pUC19 backbone / UAS1B-LEUM promoter	[47]
pSL16-cen-UAS1B-LEUM-cyc	pSL16-cen-cyc/UAS1B-LEUM	This study
pSL16-LEUM16-hrGFP	pSL16-cen-UAS1B-LEUM-cyc / hrGFP.	This study
pINT02-hrGFP	pSL16-LEUM16-hrGFP / CEN-1 replaced with lip7	This study
pINT03-hrGFP	pINT02-hrGFP / ORI1001 removed	This study
pINT03	pINT03-hrGFP / GFP removed	This study
pINT03-BGL	pINT03 / TDH1p-gh1-1-TDH1t	This study
pINT03-BGL-CDT	pINT03 / TDH1p-gh1-1-TDH1t-FBA1p-cdt-1-FBA1t	This study
pRS426-cdt-1	pRS426 containing *N. crassa* cellodextrin transporter NCU00801	[38]
pRS425-gh1-1	pRS425 containing *N. crassa* β-glucosidase NCU00130	[38]

Table 2 – Synthetic Oligonucleotides

Primer	Sequence
1	AGTCGACCTGCAGGCATGCAAGCTTCGAAGGCGCGCCCGGGCTCGAGTCATGTAATTAGT
2	CTATGACCATGATTACGCCAAGCTGCAAATTAAAGCCTTCGAGC
3	AAGGCGCGCCGTGAGCAAGCAGATCCTGAA
4	AAGGCGCGCCCTATTACACCCACTCGTGCA
5	AAACATATGGTCAGCTTTGGAGCTCGAATCAAGGACTT
6	TGCCAGGTCTCGGTCCTAGGGAAAGCCTTGGAGAAGCCGTCATGGATCTTGCACT
7	TCTCCAAGGCTTTCCCTAGGACCGAGACCTGGCACAACATCGGAGACCTGCTGGA
8	AAATTCGAATTAGTTGGAGAGCTCGAGACCCTCGGCGATAAACT
9	GTACGTACGGCGCGCCAAGCGAAATGAAAAATGAGTAGGAA
10	AAATCCTTAGGAAGAGACATTTGTTGATGTGTGTTTAATTCAAGAATGAATATAGAG
11	AATTAAACACACATCAACAAATGTCTCTTCCTAAGGATTTC
12	CGCTCTTGATCTTCGGATAGTTAGTCCTTCTTGATCAAAGAG
13	CTTTGATCAAGAAGGACTAACTATCCGAAGATCAAGAGC
14	GTACTGCGTACACTGTTGTTATATAATATTACGTAATCGACTCGC
15	TCGATTACGTAATATTATATAACAACAGTGTACGCAGTAC
16	TGGGAGCCGTGAGACGACATTGTGTGATGTGTAGTTTAGATTTC
17	TCTAAACTACACATCACACAATGTCGTCTCACGGCTCC
18	TAACATCATCTTTTATACATAACCTAAGCAACGATAGCTTCGG
19	CCGAAGCTATCGTTGCTTAGGTTATGTATAAAAGATGATGTTAGAATG
20	GTACGTACGGCGCGCCAAAGAGCCGGGACCG
21	GTACGTACGGCGCGCCATATAATATTACGTAATCGACTCGCGGTATTTTAA

Table 3 – Sugar consumption rates and citric acid yields of strains cultured in this study.

R_{Sugar} (g / g / h): Specific sugar consumption rate (grams consumed sugar / grams dry cell weight / hour); $Y_{Citrate}$ (g / g): Citrate yield (grams citrate / grams consumed sugar)

Strain	Carbon Source	Nitrogen content	R_{Sugar} (g / g / h)	$Y_{citrate}$ (g / g)
Po1f-pINT03	Glucose	Excess	3.12	N/A
Po1f-B	Glucose	Excess	3.01	N/A
Po1f-BC	Glucose	Excess	3.00	N/A
Po1f-pINT03	Cellobiose	Excess	N/A	N/A
Po1f-B	Cellobiose	Excess	1.32	N/A
Po1f-BC	Cellobiose	Excess	2.36	N/A
Po1f-pINT03	Glucose + Cellobiose	Excess	3.07	N/A
Po1f-BC	Glucose + Cellobiose	Excess	2.96	N/A
Po1f-BC	Glucose	Limited	1.90	0.28 ± 0.002
Po1f-BC	Cellobiose	Limited	1.94	0.37 ± 0.002

2.6 Figures

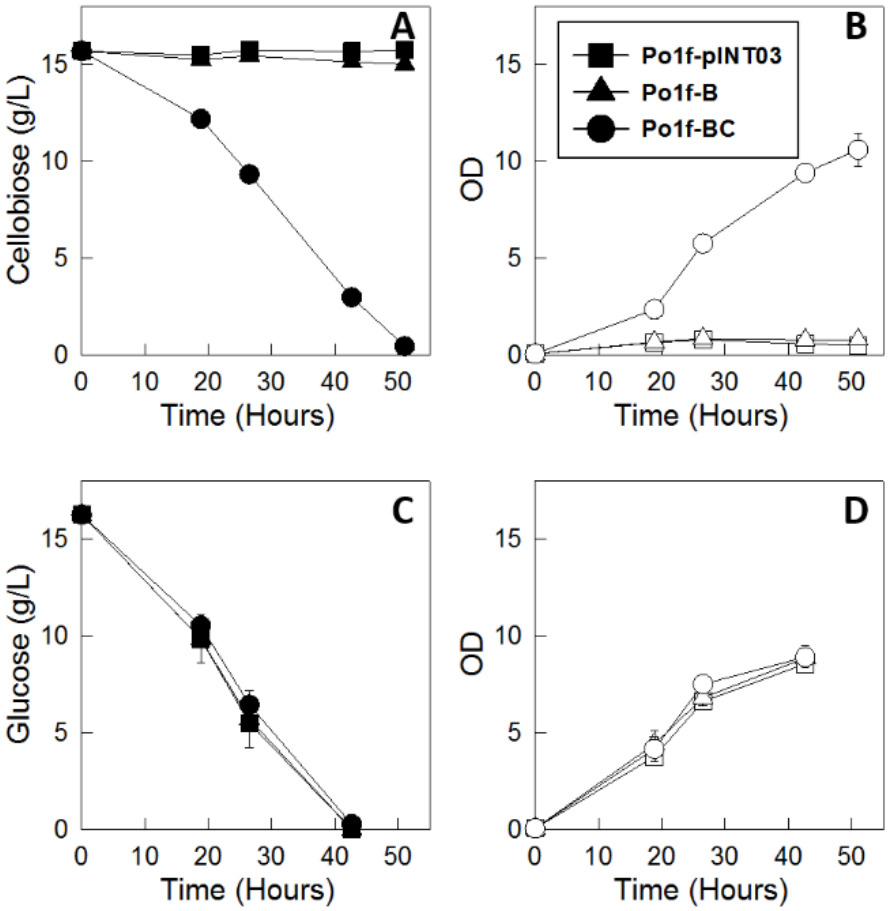

Figure 1: Growth investigations of control and engineered cells.

Profiles of sugar consumption (solid symbols) and biomass accumulation (hollow symbols) of cells cultured in excess nitrogen media with cellobiose (top) and glucose (bottom). Cultures were inoculated to an initial cell density equal to an OD of 0.05 and incubated at 30°C and 250 rpm. The strains profiled are Po1f-pINT03 (square), Po1f-B (triangle), and Po1f-BC (circle). Results are the mean of duplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size. Consumption rates displayed in Table 3 are calculated from the 43 hour data point.

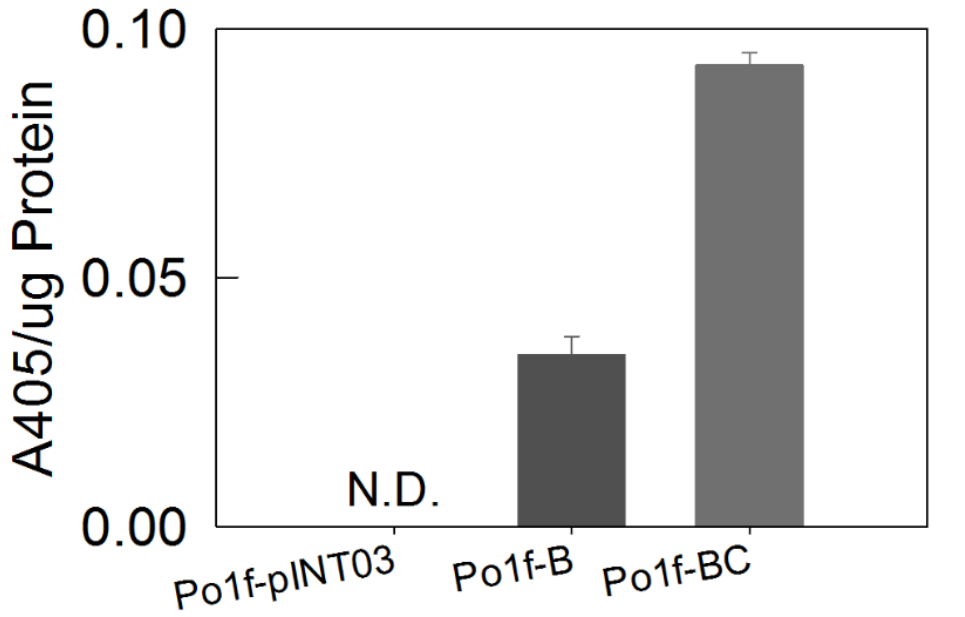

Figure 2: β-glucosidase activity from control and engineered strains.

β-glucosidase activity is shown as the absorption at 405nm divided by total protein content. Cells were inoculated at OD 0.05 and harvested after 24 hours of growth in excess nitrogen media with cellobiose at 30°C and 250 rpm. Results are the mean of duplicate experiments and error bars indicate standard deviations. N.D. indicates no detectable level of enzyme activity.

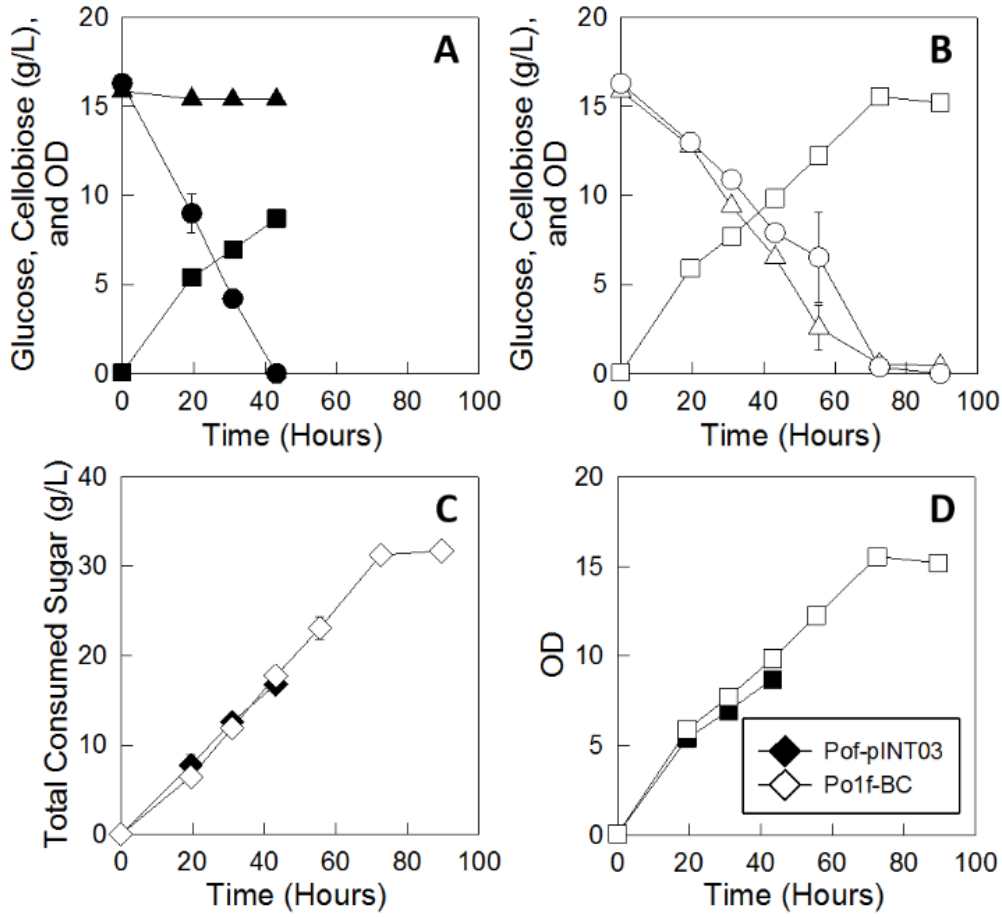

Figure 3: Mixed-sugar utilization by wild-type and engineered cells.

Growth profiles of Po1f-pINT03 (A, solid symbols) and Po1f-BGL-CDT (B, hollow symbols) in excess nitrogen media with glucose and cellobiose as carbon sources. Data points indicate glucose (circle) and cellobiose (triangle) concentrations, OD (square), and total consumed sugar (diamond). Cultures were inoculated to an initial cell density equal to an OD of 0.05 and incubated at 30°C and 250 rpm. Results are the mean of duplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size. Consumption rates displayed in Table 3 are calculated from the 43 hour data point.

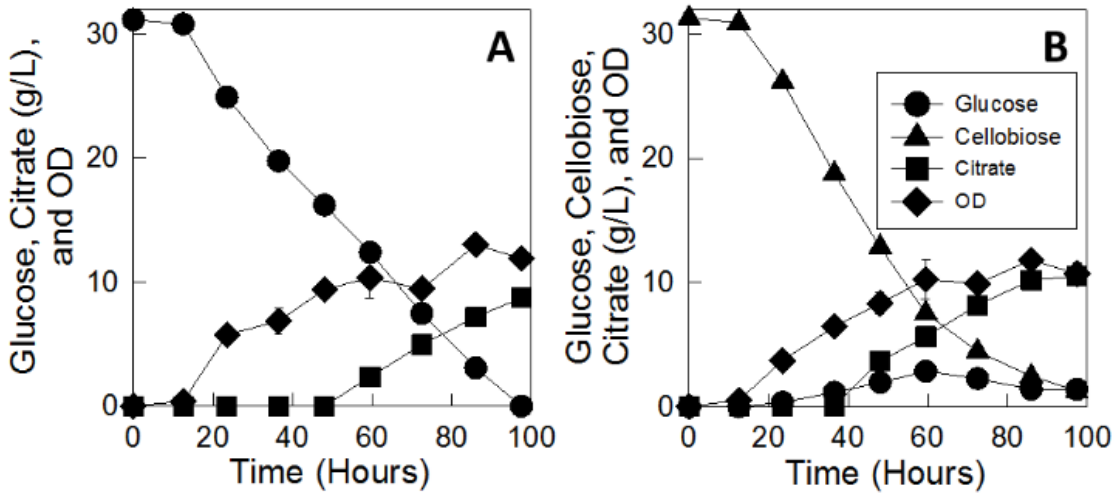

Figure 4: Citric acid production from glucose and cellobiose.

Cellobiose-consuming strain Po1f-BC cultured in glucose (4A) and cellobiose (4B). Data points represent the concentrations of glucose (circle), cellobiose (triangle), citrate (square) and OD600 (diamond). Cultures were inoculated to OD 0.01 and grown in 250 mL flasks with 50 mL limited nitrogen media at 30°C and 250 rpm. Results are the mean of duplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size. Sugar consumption rates and citric acid yields displayed in Table 3 are calculated from the 98 hour data point.

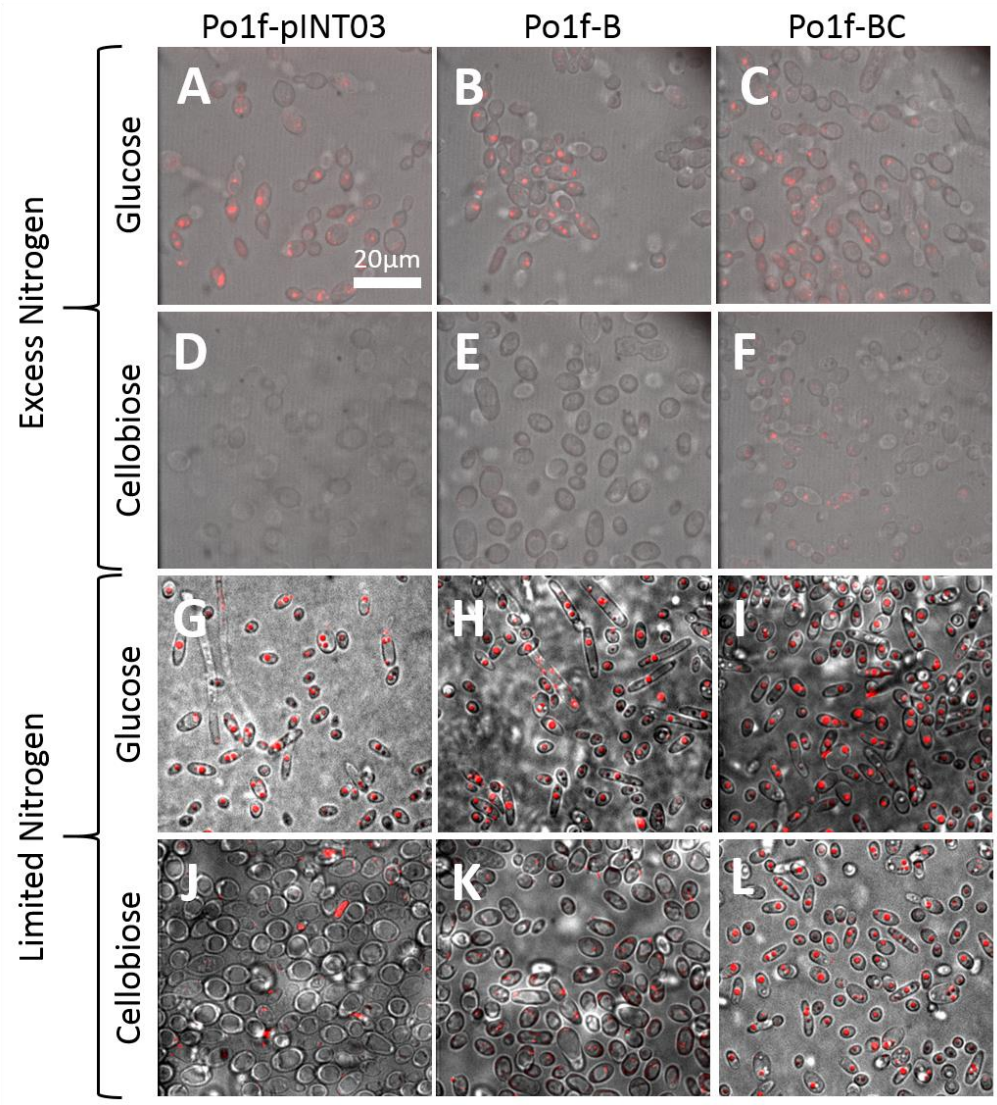

Figure 5: Lipid body formation in engineered cells.

Yarrowia lipolytica cells grown at 30°C and 250 rpm for 50 hours in excess nitrogen media with either glucose (Figures A-C) or cellobiose (Figures D-F) as a sole carbon source. Images are of strains Po1f-pINT03 (Figures A, D), Po1f-B (Figures B, E), and Po1f-BC (Figures C, F). Lipid bodies were stained with Nile red fluorescent dye and viewed under a spinning-disk confocal microscope. The scale bar indicates 100 µm.

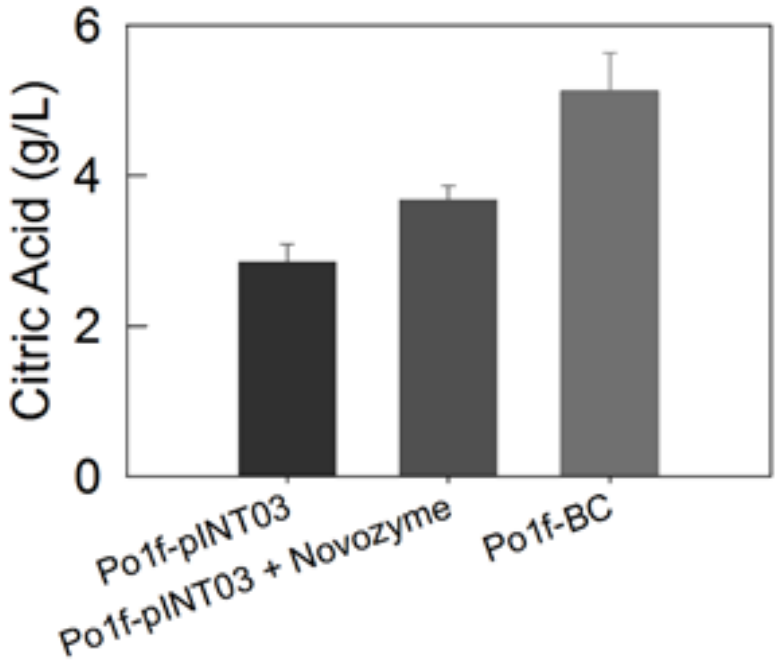

Figure 6: Citrate production from microcrystalline cellulose.

Bar height represents citrate titer after 240 hours of SSF culture. Cultures were initiated with 1 g/L of fresh cells pre-cultured for 72 hours in limited nitrogen media with the appropriate carbon source. Results are displayed as the mean of two biological replicates with the standard deviation indicated by error bars.

CHAPTER 3: CONTROLLING GLUCOSE CONSUMPTION ALLOWS SIMULTANEOUS SUGAR UTILIZATION BY *S. CEREVISIAE*

Some content in this chapter is currently in preparation for a manuscript to be submitted for peer-review. Haiqing Xu and myself will be listed as equally-contributing first-authors on this publication, followed by Guochang Zhang, Anasthasia Lesmana, Eun Joong Oh, Heejin Kim, Ching-Sung Tsai, Yong-Su Jin, and Soo-Rin Kim

3.1 Introduction

The threat of global climate change and environmental concerns about traditional chemical and fossil fuels industries have motivated efforts into renewable microbial production of biofuels and chemicals [74]. Among many potential microbes capable of industrial bioconversion, the brewer's yeast *Saccharomyces cerevisiae* has been heavily used in these pursuits due to its established presence as a model organism [75], multitude of available genetic tools [76-79], and capability for producing and tolerating high ethanol concentrations [80].

Common feedstocks for renewable microbial bioconversion can be broadly broken into two categories: terrestrial [81] and marine [82]. Terrestrial lignocellulosic biomass is primarily comprised of glucose, xylose, and arabinose [83] whereas marine biomass, depending on the type, contains mixtures of either glucose and galactose or glucose, mannitol, and 4-deoxy-L-erythro-5-hexoseulose urinate [82, 84]. Regardless of the variety, a common theme in all of these renewable carbon sources is the presence of mixed sugars. Thus, simultaneous consumption of mixed sugar hydrolysates is desirable for robust fermentations and enabling processes such as continuous fermentations [83, 85].

Attempts to enable efficient simultaneous sugar utilization have been ongoing for nearly two decades. Initial approaches for simultaneous glucose and xylose consumption involved co-cultures of *S. cerevisiae* with xylose-fermenting yeasts such as *Pichia stipitis* and *Candida shehatae* [86-89]. The next notable development was the creation of a strain that hydrolyzed glucose oligomers intracellularly [90] and was able to simultaneously consume mixtures of cellobiose and xylose [40, 91]. Eventually, xylose transport in the presence of glucose was mathematically determined to be greatly

reduced during glucose/xylose co-cultures [92] and later experimentally verified as an overall limiting factor in xylose utilization from mixed sugars [93]. This determination of the molecular mechanism behind glucose repression of xylose utilization has motivated significant recent work into engineered transporters with increased xylose specificity [94-99]. While efficient consumption of xylose in the presence of glucose is now feasible, glucose is nonetheless consumed more rapidly.

Glucose repression of galactose metabolic genes occurs in response to a certain extracellular ratio of the two sugars [100]. However, controlling sugar ratios in hydrolysates is generally not a viable option and methods to enable simultaneous utilization are necessary. Modifying the glucose repression pathway through deletion of *HXK2* [101] or the transcription factors *GAL80* and *MIG1* [102] allows simultaneous consumption, however, in both these cases consumption of glucose outpaces that of galactose.

From these two examples a common thread emerges: glucose repression on consumption of alternative carbon sources can be bypassed, but glucose consumption is consistently faster. In this study, this problem is tackled through an approach which in principle can be applicable across multiple fermentable carbon sources repressed by glucose. This report concludes that simultaneous utilization of glucose and other fermentable carbon sources can be realized by modulating the consumption rate of glucose.

3.2 Materials and Methods

3.2.1 Strains and Culture Conditions

All plasmids and strains used in this study are listed in Tables 4 and 5, respectively. Preculture was performed in 5 mL of YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) aerobically at 30°C for 36 hours with 40 g/L of the appropriate carbon source (i.e. fermentations in mixtures of glucose and xylose were precultured in xylose while fermentations mixtures of glucose and galactose were precultured in galactose). For experiments using doxycycline induction of hexokinases, hexokinase expression was induced with the same amount of doxycycline during preculture as used in the main culture. The initial cell densities for fermentation experiments were adjusted to an OD of 1. Fermentation experiments were performed in 125 mL flasks containing 25 mL medium at 30°C and 100 rpm. Anaerobic fermentations were carried out in 25 mL media in 125 mL anaerobic flasks.

3.2.2 Genetic Manipulations and Strain Construction

Standard molecular cloning techniques were employed in this study [103] and the pRS4xx series of yeast shuttle vectors was used for gene expression [104, 105]. CRISPR/Cas9 genome engineering was used as previously described [77, 106]. For gRNA construction, gBlocks were synthesized from IDT and cloned into the pRS42H plasmid. The quadruple auxotrophic SR8 was created with CRISPR/Cas9 genome engineering as previously described [107]. The sequence for gRNAs used in this study are listed in Table 6 and primers used for creation of CRISPR donor DNA are listed in Table 7.

For doxycycline-regulated control of gene expression, the rtTA(S2) variant [108] was employed under control of the yeast *MYO2* promoter [103]. The rtTA(S2) variant attached to the yeast *CYC1* terminator was synthesized from IDT and cloned into the pRS406 plasmid using SpeI and NotI restriction enzymes. The *MYO2* promoter was then amplified from yeast genomic DNA and cloned into the plasmid upstream of the rtTA(S2)-CYC1t cassette to create plasmid pRS403-rtTA. The doxycycline-inducible expression plasmids were created by cloning the pTetO7-GFP-CYC1t expression cassette from pFA6a-kanMX-tetO-pCYC1-GFP plasmid, which was a gift from Michael Nick Boddy (Addgene Plasmid #41025) [109], into pRS403. The *GFP* gene was then removed and replaced with a multi-cloning site to create plasmid pRS403-TetO7p, into which *HXK1* and *HXK2* were cloned to create plasmids pRS403-TetO7p-HXK1 and pRS403-TetO7p-HXK2, respectively.

3.2.3 Analytic techniques

High-performance liquid chromatography (HPLC) (Agilent, Santa Clara, CA) with the Rezex RCM- Monosaccharide Ca+2 (8%) column (Phenomenex Inc., Torrance, CA) was used to measure sugar concentrations while ethanol concentrations were quantified by the Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA). Biomass was quantified by measuring optical density (OD) at 600nm with a UV-visible spectrophotometer (Biomate 5, Fisher, NY).

3.3 Results

3.3.1 Glucose partially inhibits consumption of xylose

When cultured in pure glucose, SR8 rapidly grows, consumes glucose, and produces ethanol (Figure 7A). In contrast, SR8 grown on xylose grows much more slowly and exhibits a corresponding decrease in xylose consumption ability (Figure 7B). Growth on a mixture of glucose and xylose (Figure 7C) displays hallmarks of glucose repression; growth is rapid and glucose is consumed normally, however xylose consumption only reaches normal levels after glucose is depleted.

Although relatively to see in Figure 7, glucose repression of xylose utilization is most clearly revealed in a strain with a few further modifications. SR8 was therefore modified to contain deletions in *HXK1, HXK2,* and *GLK1.* This strain is able to consume xylose normally however it is unable to catalyze the first step in glycolysis: glucose phosphorylation. As such, without interference of a glucose pathway the effects of glucose on xylose consumption can be more directly examined. This strain was cultured anaerobically in xylose with 0, 5, 10, 20, and 40 g/L of glucose and growth (Figure 8A), xylose consumption (Figure 8B), and ethanol production (Figure 8C) were measured. In each case, there is a clear reduction in growth, xylose consumption, and ethanol production as glucose concentrations are increased. Further, the specific xylose consumption rate is repressed as extracellular glucose concentrations increase (Figure 8D). Nonetheless, this repression is partial and, even at the highest tested levels of extracellular glucose, the xylose consumption rate was still roughly 25% of the pure-sugar xylose consumption rate.

Since glucose repression on xylose consumption is only partial, it can be hypothesized that simultaneous utilization of these sugars may be achieved by reducing glucose consumption rates down to the rates of xylose consumption observed in Figure 8D. In this case, the rates of consumption of both sugars would be equalized, leading to perfect co-consumption.

3.3.2 Reducing glucose consumption rate allows simultaneous glucose and xylose utilization

In order to reduce the glucose consumption rate to levels observed in Figure 8D, a system of controlling glucose consumption rates using an external inducer was employed. In this case, varying the level of inducer would allow varying the rate of glucose consumption. The yeast Tet system [108, 110] was therefore used in this study. Starting with a quadruple auxotroph (Δ*his*Δ*leu*Δ*trp*Δ*ura*) version of the xylose-fermenting yeast SR8, glucose utilization was first disabled by deleting the three endogenous hexokinases *glk1, hxk1,* and *hxk2* to yield strain SR8Δ3. The synthetic doxycycline-controlled transactivator rtTA was introduced to the URA3 locus of SR8Δ3 followed by integration of the *hxk2* gene at the HIS3 locus under control of the rtTA-modulated tetO7 promoter to create the SR8Δ3iH2 strain (Figure 9D).

SR8Δ3iH2 was first cultured in pure glucose to confirm it exhibits a glucose consumption rate dependent upon extracellular doxycycline (Figure 10) and then cultured in a mixture of glucose and xylose with various amounts of inducer. In validation of the proposed model, nearly-equivalent glucose and xylose consumption rates were observed when SR8Δ3iH2 was induced with 4-6 µg/mL doxycycline (Figure

9B,E), while less doxycycline resulted in a xylose consumption rate exceeding that of glucose and more doxycycline resulted in the opposite (Figure 9A,C,E, Figure 11). While the specific xylose consumption rate stayed largely constant regardless of hexokinase induction, the glucose consumption rate was readily modulated, demonstrating that the glucose/xylose consumption rate ratio can be precisely tuned by modulating hexokinase activity.

Although the SR8Δ3iH2 strain consumed both glucose and xylose at equivalent rates when cultured in media with equivalent amounts of the two sugars, changing extracellular sugar concentrations impacts net transport efficiency and ultimately relative sugar consumption rates. Furthermore, most natural sources of glucose and xylose do not contain equal ratios of the sugars. To investigate the effects of varied extracellular sugar concentrations on co-fermentation ability the SR8Δ3iH2 strain was cultured in a variety of glucose and xylose concentrations with 4 µg/mL doxycycline (Figure 9F, Figure 12). Presumably due to the presence of highly-specific glucose transporters, the glucose consumption rate stayed relatively constant across all tested sugar concentrations. However, in agreement with previous reports of competitive transport inhibition [94], the xylose consumption rate was significantly affected by changing extracellular glucose and xylose concentrations. With hexokinase induction by 4 µg/mL doxycycline, only certain combinations of sugar concentrations resulted in equivalent consumption rates for both sugars.

3.3.3 Reducing glucose consumption rate allows simultaneous glucose and galactose utilization

The idea of balancing fluxes to enable simultaneous sugar utilization is not specific to glucose and xylose and could conceivably be generalized to other sugars repressed by glucose, such as galactose. As SR8 was originally isolated from an evolution to enhance xylose consumption, and hence has numerous genetic alterations, the original parent strain D452-2 was used for investigating the potential for simultaneous glucose and galactose utilization through modulating glucose consumption rates. *HXK1* and *HXK2* were thus deleted and *GLK1* was replaced with an r*tTA* expression cassette in D452-2. Then, inducible *HXK1* (D452Δ3iH1) and *HXK2* (D452-2Δ3iH2) strains (Figure 13D) were constructed to investigate whether hexokinase choice affected co-fermentation capabilities.

In strain D452-2, galactose consumption was repressed while glucose was rapidly consumed within 12 hours (Figure 13A). This period of preferential glucose consumption was followed by a prolonged phase of galactose consumption. In the case of the engineered strains, modulating either *HXK1* (Figure 13B, 14) or *HXK2* (Figure 13C, 15) allowed simultaneous utilization of both glucose and galactose. Interestingly, despite previous reports that deletion of *HXK2* enabled co-fermentation of glucose and galactose [101, 111], simultaneous sugar consumption was observed even at maximum *HXK2* induction (Figure 15). Regardless of modulating either *HXK1* or *HXK2* expression, the relative amounts of consumed sugars were controlled by adjusting the addition of doxycycline (Figure 13E, F).

3.4 Discussion

This work demonstrates that multiple carbon sources can be consumed simultaneously by controlling the rate of glucose consumption. Specifically, mixtures of glucose/xylose and glucose/galactose can be co-consumed by reducing the consumption rate of glucose to levels comparable to xylose and galactose. Considering that xylose is metabolized by the pentose phosphate pathway while galactose utilization occurs through the Leloir pathway [112], this is the first report of a robust co-fermentation design effective throughout a range of metabolic pathways. This broad result indicates that this strategy could be generalized to include co-consumption of glucose and other industrially-relevant carbon sources such as arabinose [113, 114] and 4-deoxy-L-erythro-5-hexoseulose urinate [84].

Previous work to enable simultaneous glucose and xylose utilization has primarily focused on engineering xylose-specific transporters [94, 96, 97, 115]. However, this work demonstrates that transporter engineering is not an essential prerequisite for simultaneous glucose and xylose consumption. Rather, the endogenous transport machinery has a remarkable capacity for sustaining simultaneous glucose/xylose utilization. As such, reducing the great disparity in glucose and xylose consumption rates is an additional and complementary design for enabling simultaneous co-fermentation of the two sugars. While transporter engineering is limited to enhancing the consumption rate of xylose in the presence of glucose, in most cases glucose is consumed much faster than xylose irrespective of the expression of optimally engineered xylose-specific transporters. In continuous cultures, managing relative consumption rates will be important as current implementations have large

disparities in glucose and xylose consumption rates and steady-state concentrations [116].

However, although the endogenous transport machinery was capable of sustaining simultaneous glucose/xylose consumption, the more rapid consumption of glucose/galactose mixtures is likely due to a combination of factors. First, galactose is consumed faster than xylose in the strain SR8, indicating a stronger metabolic potential. Second, similar kinetic properties of Gal2p for transporting glucose and galactose [117] leads to a lesser impact of competitive transport inhibition as compared to glucose and xylose. As such, expressing high-affinity xylose transporters and further improvement of the xylose metabolic rate should allow faster simultaneous glucose and xylose utilization while still maintaining comparable consumption rates during the fermentation.

Galactose metabolic genes are repressed by glucose when nuclear Hxk2p interacts with Mig1p to form a repressor complex [118, 119]. While previous reports have shown that deletion of *HXK2* allows co-fermentation of glucose and galactose mixtures [101, 111], this work shows that modulating expression of either *HXK1* or *HXK2* enabled simultaneous utilization of the two carbon sources while providing control over relative sugar consumption rates. Although this study did not recreate glucose repression on galactose consumption by modulating *HXK2* expression, this strain consumed glucose slower than wild-type even when maximally induced. This indicates that *HXK2* expression was still below natural levels and may have been insufficient to exert repressive effects on *GAL* gene expression. Of note, a recent study engineered a yeast strain capable of production of 2,3 butanediol from simultaneous consumption of glucose and galactose [120], although the consumption rates were overall quite slow as

compared to an wild-type control. While that study was unable to completely explain the molecular mechanism behind simultaneous utilization, these results indicate that a reduced glucose consumption rate alleviates the effects of competitive transport inhibition and the subsequent downstream repression effects on *GAL* gene expression [100].

Although this study employed doxycycline induction to explore a wide range of hexokinase expression, this design may be problematic in an industrial setting in terms of long-term stability and the usage of antibiotics. However, the recent development of CRISPR-based transcription factors [121, 122] and synthetic xylose-sensing proteins [123] opens the door for direct control of hexokinase expression in response to glucose and xylose without need for an antibiotic inducer. Further, as this general design principle allows simultaneous utilization by implementing an intracellular metabolic bottleneck that counteracts the effects of competitive transport inhibition, other metabolic bottlenecks within glycolysis may lead to similar effects.

As this study was primarily a proof-of-concept regarding a biological design for managing simultaneous sugar utilization in *Saccharomyces cerevisiae*, this work aimed only to produce the natural by-product of yeast: ethanol. However, recent work has demonstrated that modulating glycolytic flux can enhance the production of non-ethanol compounds [124]. As the industry focusing on creating high-value compounds using engineered microbes continues to expand [125], this design may be particularly useful in the conversion of mixed-sugar hydrolysates to a variety of non-ethanol products.

3.5 Tables

Table 4 – Plasmids

Plasmid	Description	Reference
pRS41N-Cas9	A single-copy plasmid containing Cas9 and a nourseothricin marker	[107]
pRS42K -gRNA-*GLK1*	A multi-copy plasmid containing a guide RNA for *GLK1* and a geneticin marker	This study
pRS42H -gRNA-*HXK2*	A multi-copy plasmid containing a guide RNA for *HXK2* and a hygromycin B marker	This study
pRS42K -gRNA-*HXK1*	A multi-copy plasmid containing a guide RNA for *HXK1* and a geneticin marker	This study
pRS406-rtTA	Integrative plasmid pRS406 with pMYO2-*rtTA(S2)*-tCYC1 expression cassette	This study
pRS403-TetO7p	Integrative plasmid pRS403 with a multicloning site between the TetO7 promoter and *ADH1* terminator	This study
pRS403-TetO7p-*HXK1*	Integrative plasmid pRS403 with TetO7p-*HXK1*-ADH1t expression cassette	This study
pRS403-TetO7p-*HXK2*	Integrative plasmid pRS403 with TetO7p-*HXK2*-ADH1t expression cassette	This study

Table 5 – Strains

Strain	Description	Reference
SR8	Xylose-fermenting *Saccharomyces cerevisiae* strain	[126]
SR8 pRS41N-Cas9	SR8 with Cas9 protein expressing plasmid	This study
SR8-4xAuxotroph	SR8 *leu2 his3 ura3 trp1*	This study
SR8Δ3	SR8-4xAuxotroph with deletions in *HXK1 HXK2* and *GLK1*	This study
SR8Δ3iH2	SR8Δ3 with pRS406-rtTA and pRS403-TetO7p-*HXK2*	This study
D452-2	*MATα leu2 his3 ura3*	[127]
D452-2+403	D452-2 with pRS403	This study
D452-2Δ3i	D452-2 with deletions in *HXK1, HXK2,* and *glk1*Δ::MYO2p-*rtTA(S2)*-CYC1t	This study
D452-2Δ3iH1	D452-2Δ3i with pRS403-TetO7p-*HXK1*	This study
D452-2Δ3iH2	D452-2Δ3i with pRS403-TetO7p-*HXK2*	This study

Table 6 – gRNA Sequences

Part	Sequence	Length (bp)
SNR52 promoter	TCTTTGAAAAGATAATGTATGATTATGCTTTCACTCATATTTATACAGAAACTTGATGTT TTCTTTCGAGTATATACAAGGTGATTACATGTACGTTTGAAGTACAACTCTAGATTTTG TAGTGCCCTCTTGGGCTAGCGGTAAAGGTGCGCATTTTTTCACACCCTACAATGTTCT GTTCAAAAGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGGC GTTCGAAACTTCTCCGCAGTGAAAGATAAATGATC	269
GLK1 target	AACAGAACATATACGGAAAT	20
HXK2 target	TCAAAGCAACAACTTCAA	20
HXK1 target	TAGTTTATACTTGGATTGAG	20
Structural crRNA	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAA GTGGCACCGAGTCGGTGGTGC	79
SUP4 terminator	TTTTTTTGTTTTTTATGTCT	20

Table 7 – Primers

Name	Sequence	Description
JIN3707	ccccatcagtgcccaactcagcttccgtaaaccacaacaccaccactaatacaactctatcatacacaagTAAGGCGAGCTCATACCGTC	Δ*GLK1*_Cas9_F
JIN3708	tatatatataaaggagagaagatggtaagtacggtgggatacgtacacaaaccaaaaaaatgtaaaaagaGACGGTATGAGCTCGCCTTA	Δ*GLK1*_Cas9_R
JIN3709	taccaattagacatgctgcttgc	Δ*GLK1*_Confirm_F
JIN3710	GACGGTATGAGCTCGCCTTA	Δ*GLK1*_Confirm_R
JIN3762	actcaattagaattcttttcttttaatcaaactcacccaaacaactcaattagaatactgaaaaaataagGTGTAACTCAGATGAGCTAC	Δ*HXK1*_Cas9_F
JIN3763	ggcatcactcataagaataataatattaagggagggaaaaacacatttatatttcattacatttttttcaGTAGCTCATCTGAGTTACAC	Δ*HXK1*_Cas9_R
JIN3764	GCCAGATCTCAGTATAGCAG	Δ*HXK1*_Confirm_F
JIN3765	GTAGCTCATCTGAGTTACAC	Δ*HXK1*_Confirm_R
JIN3767	ttcgctttttctttgaaaaggttgtaggaatataattctccacacataataagtacgctaattaaataaaACGACCGACGTACGATTCAA	Δ*HXK2*_Cas9_F
JIN3768	tagaaaacatgttcacataagtagaaaaagggcaccttcttgttgttcaaacttaatttacaaattaagtTTGAATCGTACGTCGGTCGT	Δ*HXK2*_Cas9_R
JIN3769	GCTCCAGAGCTCCACATTG	Δ*HXK2*_Confirm_F
JIN3770	TTGAATCGTACGTCGGTCGT	Δ*HXK2*_Confirm_R
JIN5143	AAATTTTAGACGCGGCGCTTGCACCCCGCATTATAAGTGGTGctcaagcaaggttttcag	Δ*GLK1::rtTA*_F
JIN5144	TACCGGTACCGAAAATACATCCGATGACCGGCTCCGAGgaattccacttaatgtatcaac	Δ*GLK1::rtTA*_R
JIN5145	GTGTCACTAGGTGCAATTGCC	Δ*GLK1::rtTA*_Confirm_F
JIN5146	CGACAGCACTTCGGATGA	Δ*GLK1::rtTA*_Confirm_R

3.6 Figures

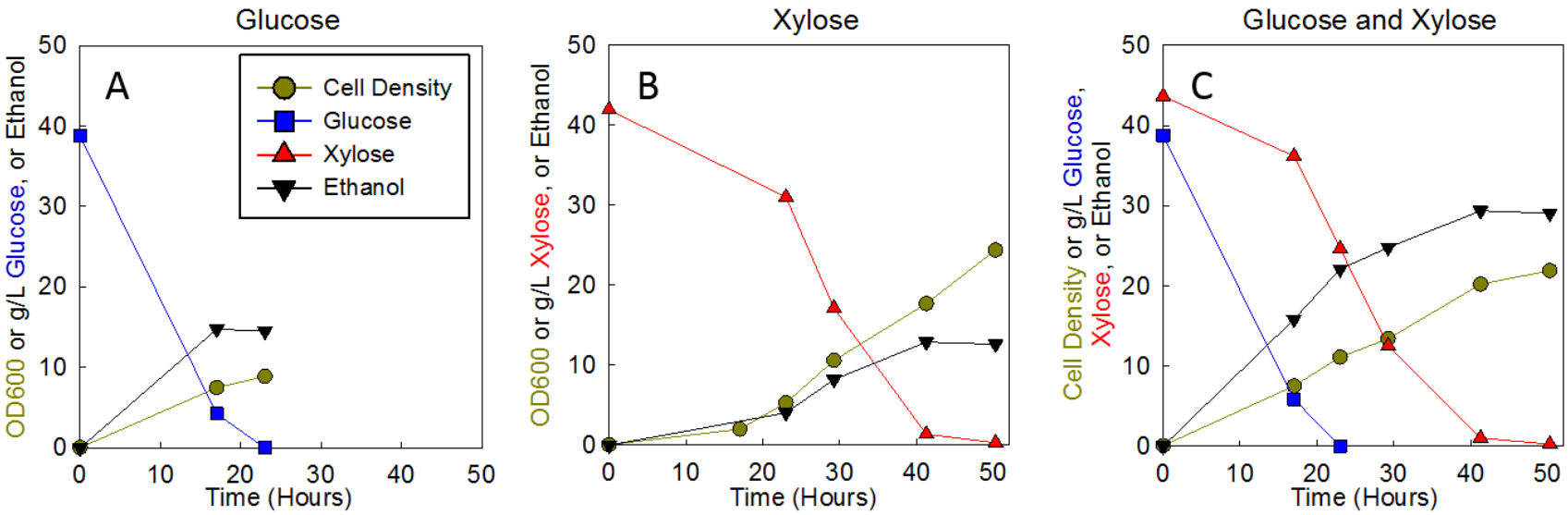

Figure 7: SR8 cultured in pure and mixed sugars.

Fermentation profiles of SR8 cultured on pure (A) glucose, (B) xylose, and (C) a mixture of glucose and xylose. Fermentations were carried out in oxygen-limited conditions in 25 mL media in 125 mL flasks with an initial OD of 0.05. Data points indicate OD600 (yellow circles) and glucose (blue squares), xylose (red upward triangles), and ethanol (black downward triangles) concentrations.

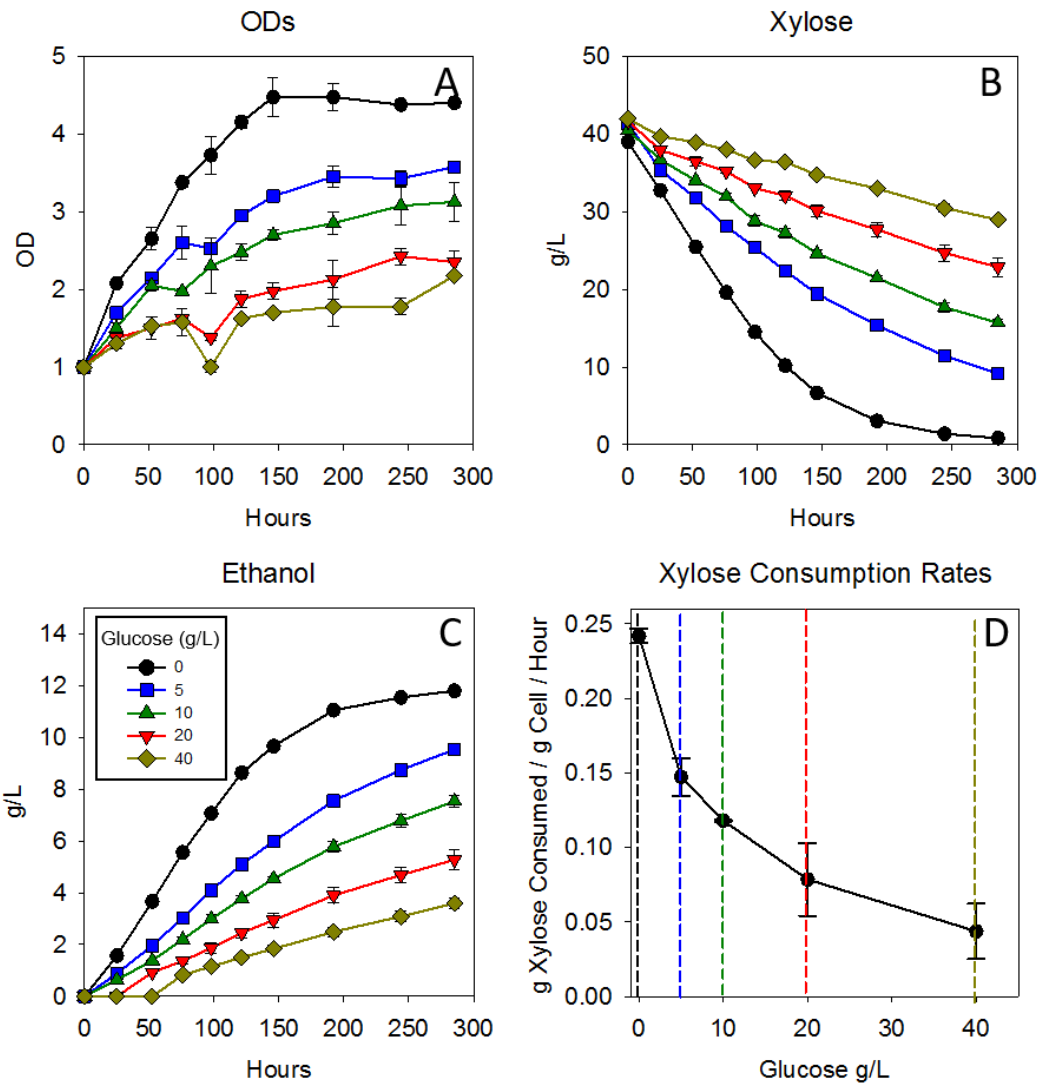

Figure 8: SR8Δ*hxk*^{0} cultured anaerobically in xylose with various amounts of glucose.

The fermentation was carried out in 25 mL media in anaerobic flasks purged with nitrogen. (A) Cell growth measured by optical density (OD) at 600 nm. Measurements of (B) xylose and (E) ethanol concentrations as determined by HPLC analysis with extracellular glucose at 0, 5, 10, 20 and 40 g/L indicated by black ricles, blue squares, green upward triangles, red downward triangles, and yellow diamonds, respectively. (D) Specific xylose uptake rates in mid-exponential phase across a range of extracellular glucose concentrations.

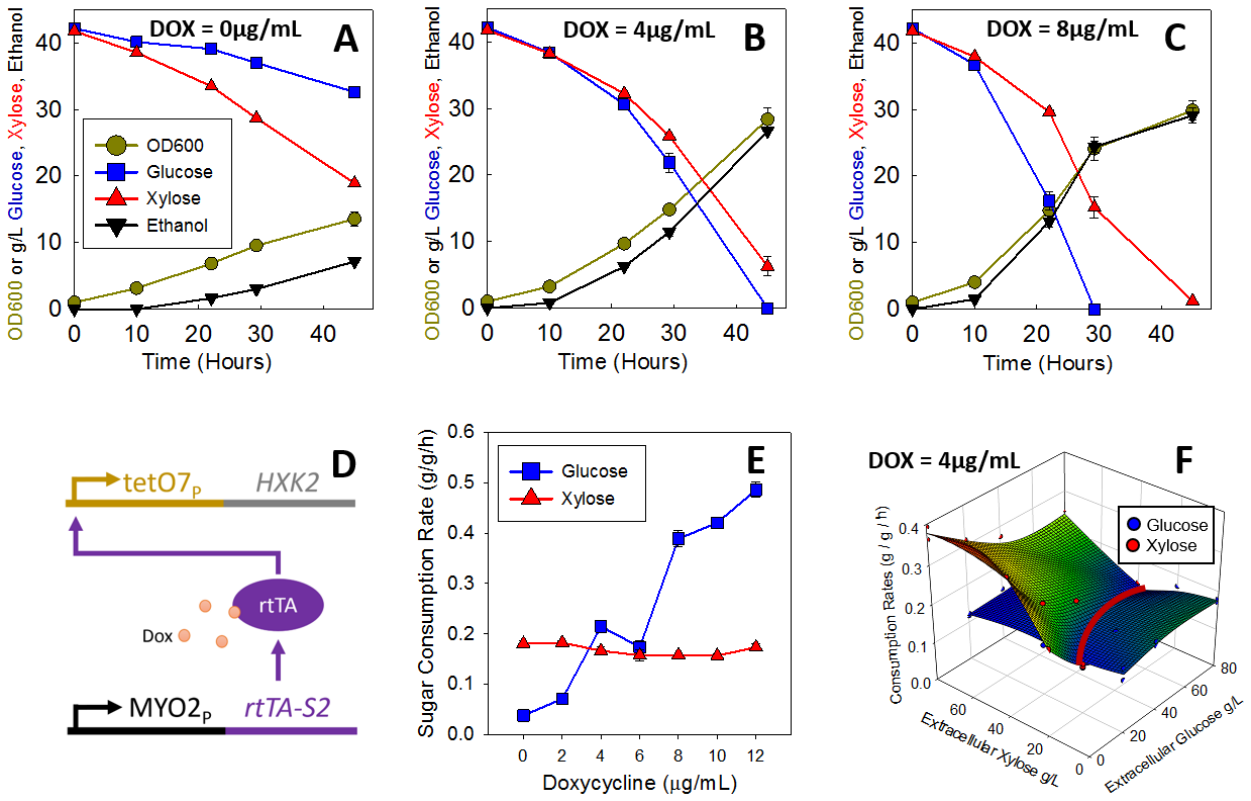

Figure 9: Modulating glucose consumption rate allows simultaneous glucose and xylose utilization.

(A-C) Fermentation profiles of SR8 with inducible-HXK2 with doxycycline concentrations of 0 (A), 4 (B), and 8 (C) µg/mL. Data points indicate OD600 (yellow circles) and glucose (blue squares), xylose (red upward triangles), and ethanol (black downward triangles) concentrations. (D) Scheme for controlling hexokinase production. The doxycycline-controlled transactivator rtTA-S2 was placed under the control of the constitutive MYO2 promoter in a hexokinase-null strain background. An *HXK2* expression cassette is then reintroduced with the rtTA-regulated tetO7 promoter. (E) Comparison of glucose (blue squares) and xylose (red triangles) consumption rates during mixed-sugar fermentations with varying amounts of doxycycline. (F) Effects of changing extracellular sugar concentration on sugar cofermentation ability with constant induction of hexokinase at a doxycycline concentration of 4 µg/mL. The red line indicates the intersection between the two planes, where sugar consumption rates are equal. Two-dimensional slices of this graph are displayed in Figure 12.

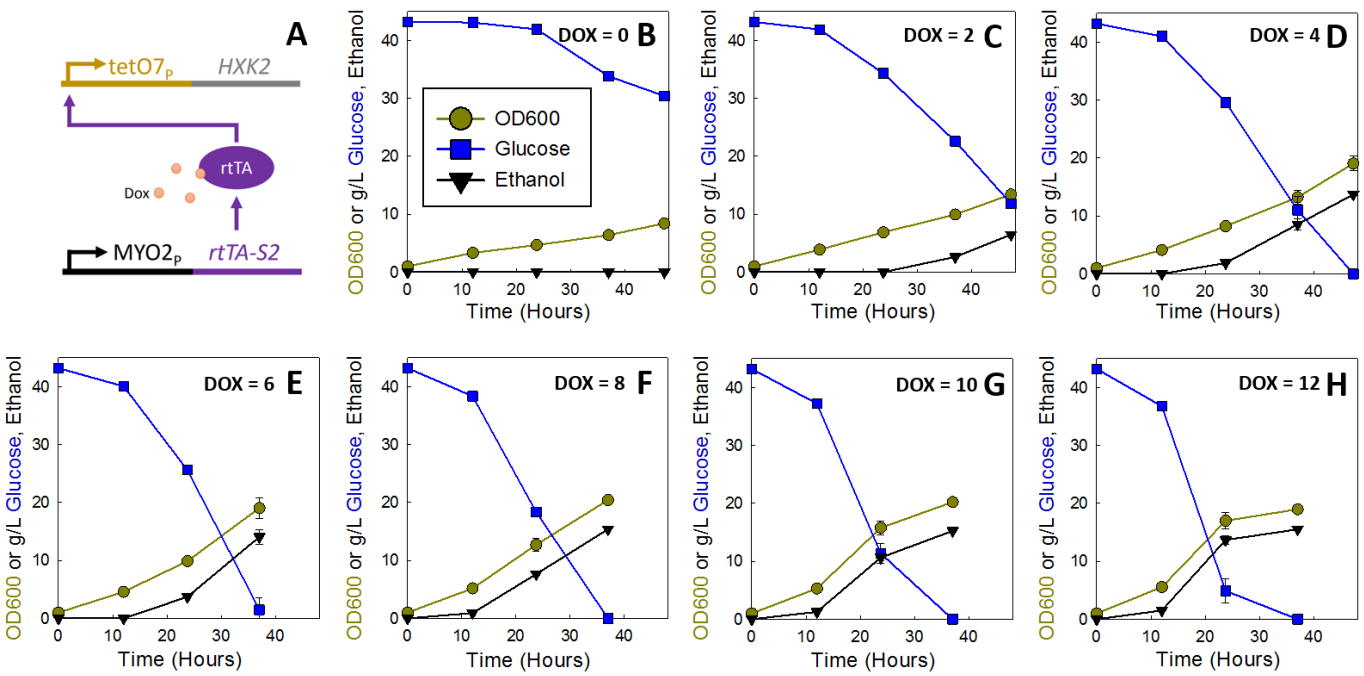

Figure 10: Controlling glucose consumption rate through the external inducer doxycycline.

(A) Scheme for control over *HXK2* expression using the external inducer doxycycline. The synthetic transactivator rtTA-S2 activates expression of *hxk2* when bound to doxycycline. (B-H) Pure glucose fermentations of controllable *HXK2* SR8. SR8Δ*hxk*^{0} with inducible *HXK2* (B-H) was cultured with various levels of doxycycline at (B) 0, (C) 2, (D) 4, (E) 6, (F) 8, (G) 10, and (H) 12 µg/mL. Fermentations were performed with 25mL YP media in 125mL flasks at an initial OD of 1. Data points are the result of duplicate experiments with standard deviations indicated by error bars. Data points are: OD (yellow circle), glucose (blue square), and ethanol (black downward triangle).

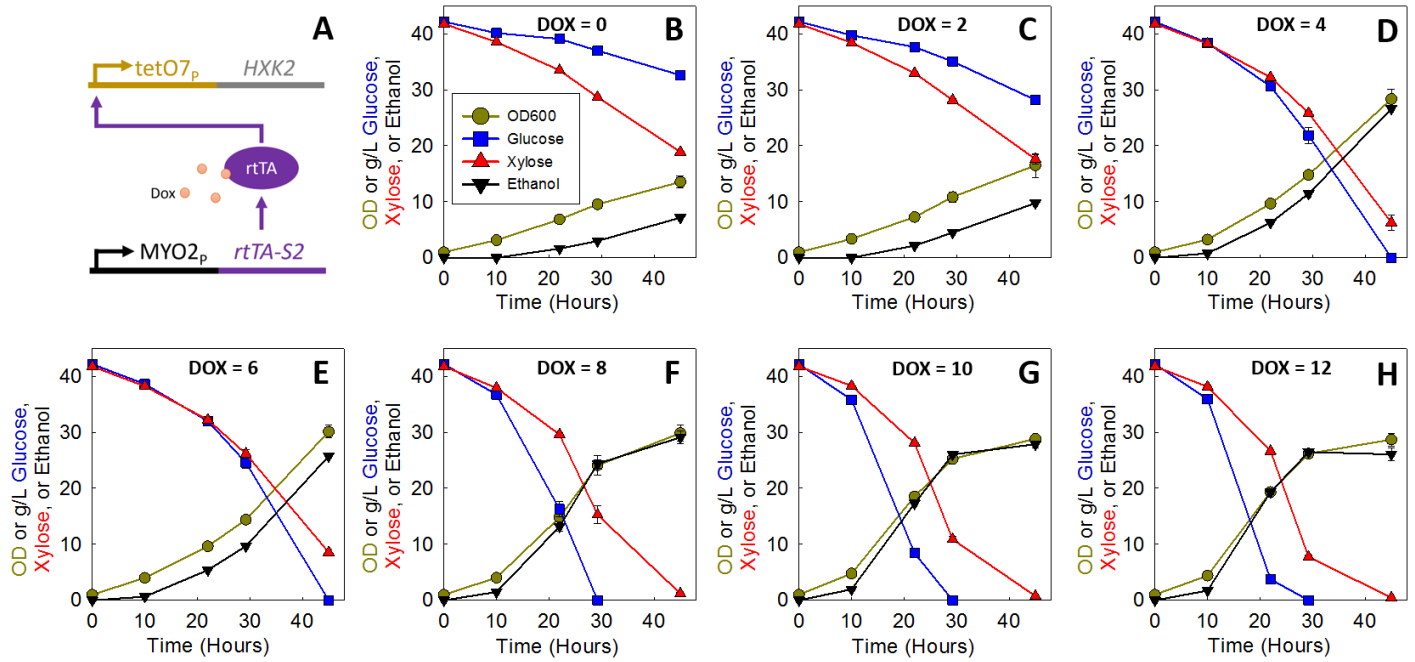

Figure 11: Cofermentation of glucose and xylose by controlling glucose consumption rate.

(A) Scheme for control over *HXK2* expression using the external inducer doxycycline. The synthetic transactivator rtTA-S2 activates expression of *hxk2* when bound to doxycycline. (B-H) Controllable *HXK2* SR8 cultured in a mixture of glucose and xylose. SR8Δ*hxk*^{0} with inducible *HXK2* (B-H) was cultured with various levels of doxycycline at (B) 0, (C) 2, (D) 4, (E) 6, (F) 8, (G) 10, and (H) 12 µg/mL. Fermentations were performed with 25 mL YP media in 125 mL flasks at an initial OD of 1. Data points are the result of duplicate experiments with standard deviations indicated by error bars. Data points are: OD (yellow circle), glucose (blue square), xylose (red triangle), and ethanol (black downward triangle).

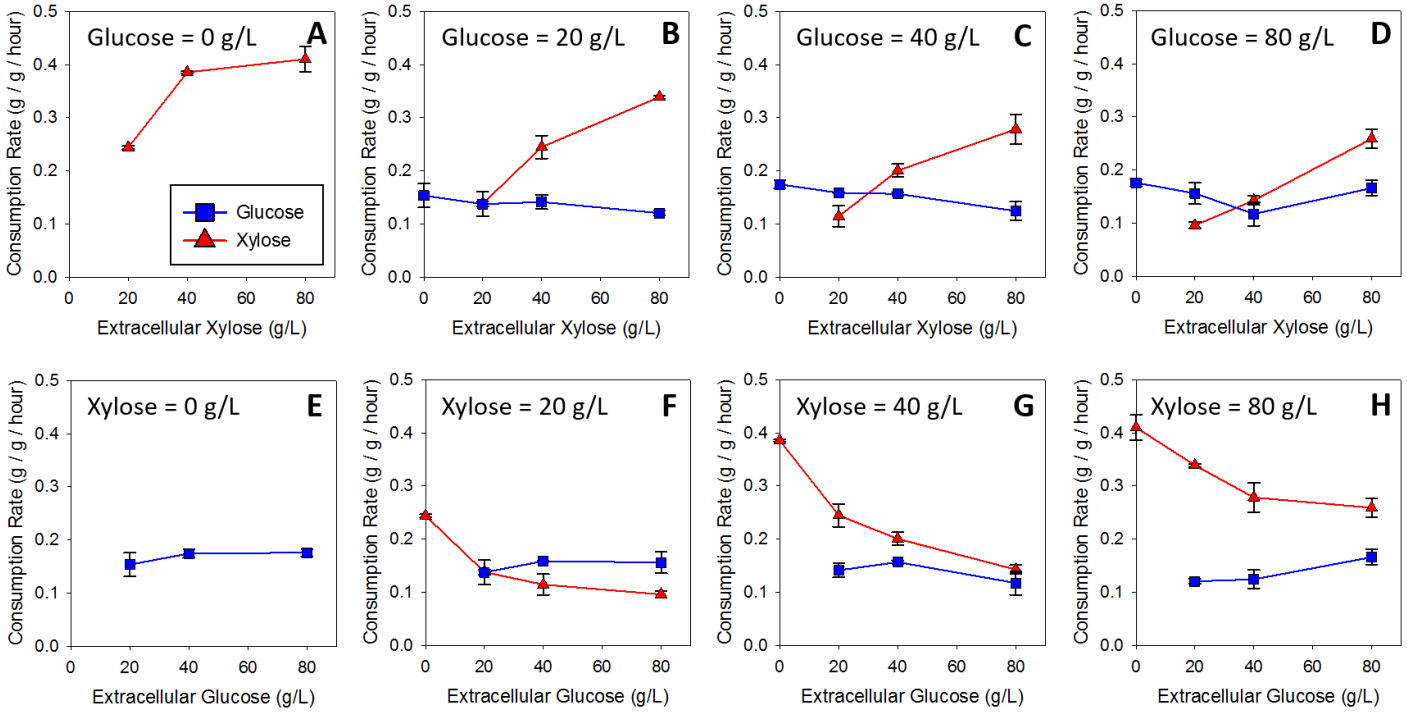

Figure 12: Impact of extracellular sugar ratios on cofermentation ability.

(A-H) Measurements of sugar consumption rates across different sugar ratios. Glucose and xylose consumption rates are indicated by blue squares and red triangles, respectively. (A) Pure xylose and (E) pure glucose conditions are pictured along with (B-D) constant glucose concentrations with varied xylose and (F-H) constant xylose concentration with varied glucose. Experiments were conducted in duplicate with errors represented by error bars, which are not visible when the standard error is smaller than the size of the data point. The three-dimensional compilation of all the above figures is shown in Figure 9F.

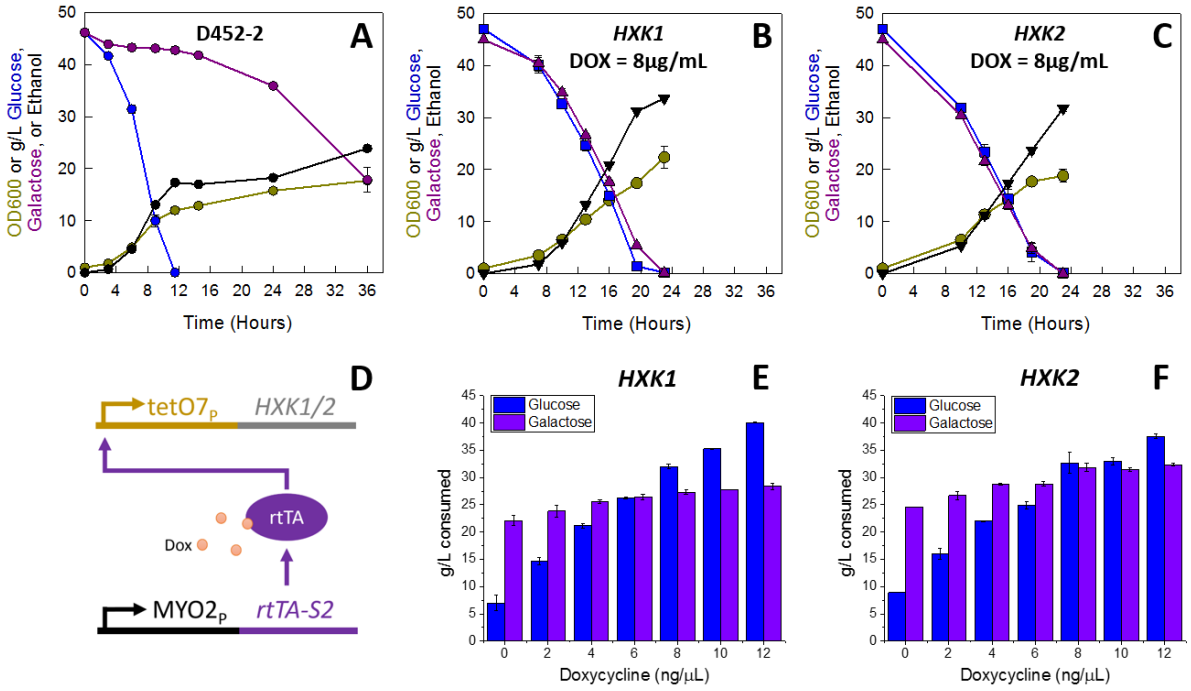

Figure 13: Modulating glucose consumption rate allows simultaneous glucose and galactose utilization.

(A-C) Fermentation profiles of control strain D452-2 + pRS403 (A), inducible *HXK1* D452-2 (B), and inducible *HXK2* D452-2 (C) in mixtures of glucose and galactose. (D) Scheme for controlling hexokinase production. The doxycycline-activated transcription factor rtTA-S2 was placed under the control of the constitutive MYO2 promoter in a hexokinase-null strain background. Expression of a single hexokinase is controlled by the rtTA-regulated tetO7 promoter. (E-F) Total consumed sugars after 16 hours when cultured with varied amounts of doxycycline in inducible *HXK1* (E) and *HXK2* (F) strains. Full fermentation profiles are provided in Figures 14 and 15.

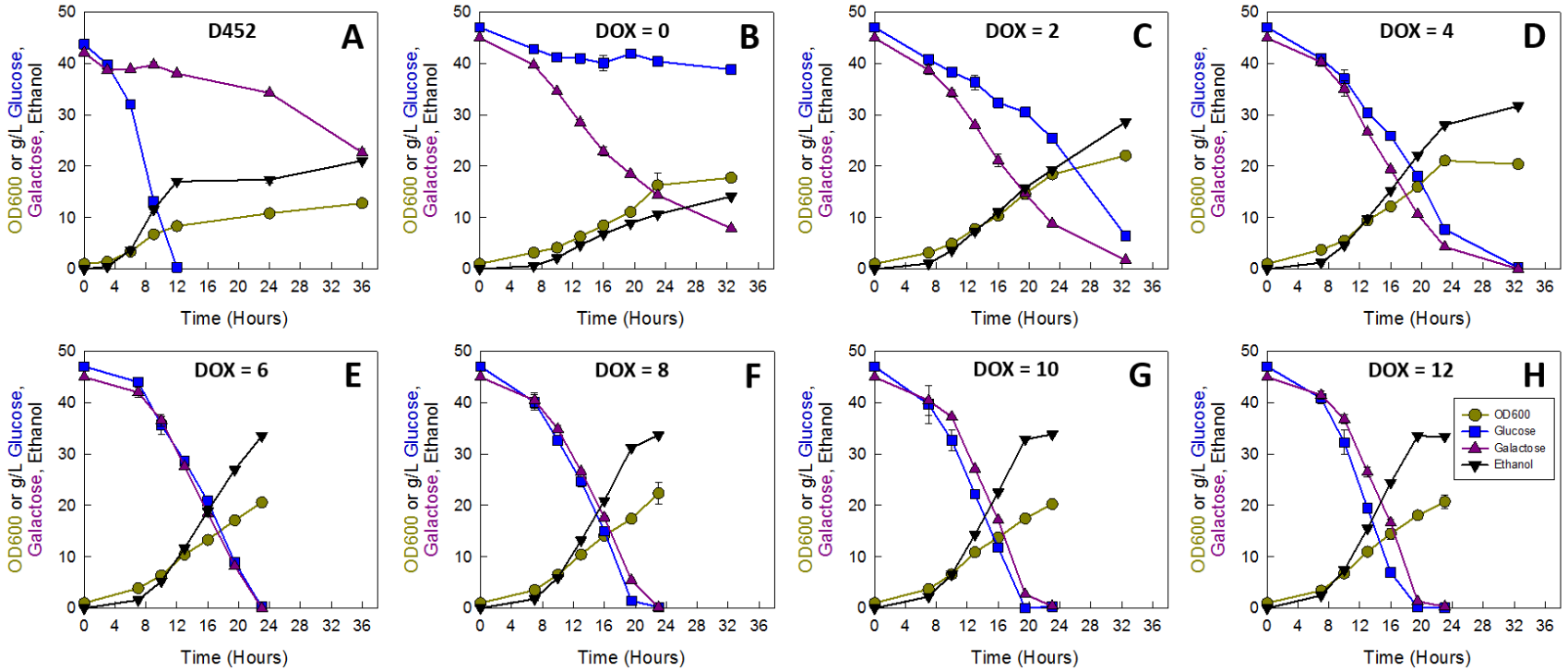

Figure 14: Modulating *HXK1* expression enables simultaneous utilization of glucose and galactose.

Fermentation profiles of (A) parent D452-2 pRS403 and (B-H) D452-2Δhxk^0 with inducible *HXK1* cultured in a mixture of glucose and galactose. The inducible *HXK1* strain (B-H) was cultured with various levels of doxycycline at (B) 0, (C) 2, (D) 4, (E) 6, (F) 8, (G) 10, and (H) 12 µg/mL. Fermentations were performed with 25mL YP media in 125mL flasks at an initial OD of 1. Data points are the result of duplicate experiments with standard deviations indicated by error bars. Data points are: OD (yellow circle), glucose (blue square), galactose (purple triangle), and ethanol (black downward triangle).

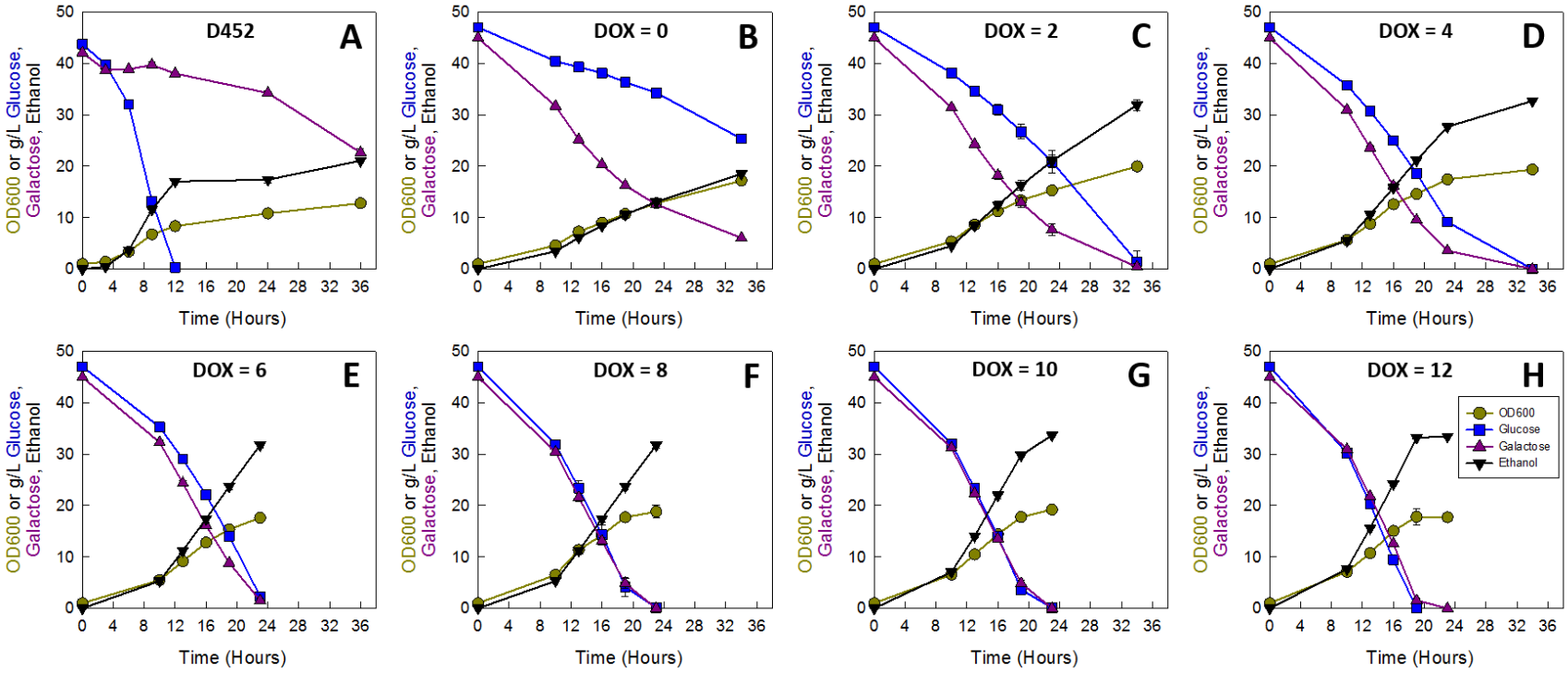

Figure 15: Modulating *HXK2* expression enables simultaneous utilization of glucose and galactose.

Fermentation profiles of (A) parent D452-2 pRS403 and (B-H) D452-2Δhxk^0 with inducible *HXK2* cultured in a mixture of glucose and galactose. The inducible *HXK2* strain (B-H) was cultured with various levels of doxycycline at (B) 0, (C) 2, (D) 4, (E) 6, (F) 8, (G) 10, and (H) 12 µg/mL. Fermentations were performed with 25 mL YP media in 125 mL flasks at an initial OD of 1. Data points are the result of duplicate experiments with standard deviations indicated by error bars. Data points are: OD (yellow circle), glucose (blue square), galactose (purple triangle), and ethanol (black downward triangle).

CHAPTER 4: THE ROLE OF GLUCOSE SENSING IN CONSUMING NON-GLUCOSE SUGARS AND PRODUCING NON-ETHANOL COMPOUNDS

4.1 Introduction

The model yeast *Saccharomyces cerevisiae* is superbly optimized for consumption of glucose and production of ethanol [26]. However, there are growing efforts to use this yeast in bioconversion of lignocellulosic sugars and other non-traditional feedstocks [55, 84]. Even further, *S. cerevisiae* is increasingly being used in the production of compounds other than ethanol [120, 128-134]. It is therefore important to understand the mechanisms that enable this yeast to be an optimal converter of glucose to ethanol and, if necessary, remove them when the feedstock is not glucose and the target product is not ethanol.

This study first focuses on the membrane sensors Snf3 and Rgt2. Snf3 is a high-affinity glucose sensor optimized for the detection of low quantities of glucose [135]. On the other hand, Rgt2 is a low-affinity sensor intended for the detection of high concentrations of glucose [136]. When bound to glucose, these sensors activate the membrane-bound casein kinase Yck1, which phosphorylates and triggers degradation of the *HXT* repressors Mth1 and Std1 [137, 138]. As pictured in Figure 16A, This signaling cascade ultimately relieves *HXT* repression [139] and allows yeast to optimize membrane transport and growth in response to a wide range of glucose concentrations [140]. Because these sensors optimize transporter expression for growth on glucose, it is possible that their activity may be detrimental for efficient consumption of lignocellulosic sugars. Even further, this optimization of glucose transport may prevent simultaneous sugar utilization. This report begins by investigating the impact of sensor deletions in strains SR8 and EJ4, optimized for consuming xylose and cellobiose, respectively.

The second half of this study is regarding optimizing glucose consumption for the production of non-ethanol compounds. Previous reports have found that lactic acid yield from xylose or cellobiose is significantly higher than from glucose [141, 142], although a specific mechanism explaining this effect has yet to be identified. By investigating the role of glucose sensing through the membrane sensors Snf3 and Rgt2 and the contributions of metabolic flux, this report is able to partially explain these previous findings.

4.2 Materials and Methods

4.2.1 Media Recipes and Culture Conditions

All strains and plasmids used in this study are listed in Tables 8 and 9, respectively. Preculture was performed in 5 mL of YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) aerobically at 30°C for 36 hours with 40 g/L of glucose as a carbon source. Fermentation experiments were performed in 125 mL flasks containing 25 mL YP medium at 30°C and 100 rpm, with initial carbon sources indicated in fermentation profiles. For transporter expression analysis, strains were precultured in xylose and then inoculated in triplicate into a plate reader with different glucose concentrations at an initial OD equal to 0.1. Plates were then incubated for 3 hours at 30°C and 300 rpm and fluorescence was measured.

4.2.2 Genetic Techniques

Standard restriction digestion and molecular cloning techniques were employed for plasmid creation [143]. For doxycycline induction experiments, the rtTA(S2) variant [108] was synthesized from IDT as a gBlock. To create SR8 and EJ4 strains expressing a single copy of LdhA, CRISPR genome editing was used to integrate a pPGK1-*LdhA*-tPGK1 expression cassette into the PBN1-SBP1 intergenic region at the PAM site TTTATTTTCAATATTATACAAGG. To measure transporter expression in response to glucose, six different strains were derived from SR8 with replacement of HXT1-6 with a GFP-terminator-KanMX cassette. Transformants were then selected on plates with G418 and confirmed through colony PCR.

4.2.3 Analytic methods

Sugar and ethanol concentrations were quantified by the Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA). Biomass was quantified by measuring optical density (OD) at 600nm with a UV-visible spectrophotometer (Biomate 5, Fisher, NY). GFP Fluorescence was quantified in a 96-well Corning Costar black plate using a Biotek Synergy Multi-Mode 96-well plate reader.

4.3 Results

Yeast glucose sensors allow cells to adjust their membrane transporter composition to optimally transport glucose through a signal cascade (Figure 16A). This results in a changing transporter transcription profile in response to different extracellular glucose concentrations (Figure 16B). In agreement with previous studies, high-affinity transporters HXT6 and HXT2 are most highly expressed in low glucose concentrations while expression of the high-affinity HXT1 continuously increases with higher glucose concentrations.

In the expectation that this glucose transport optimization may not favor efficient consumption of xylose, the glucose sensors were deleted from SR8 and fermentations were conducted. In glucose, SR8 rapidly consumes glucose and produces ethanol (Figure 17A). With a deletion of sensors, there is an extended lag time followed by rapid consumption of glucose along with comparatively lower ethanol production and higher cell growth (Figure 17D). This trend continues with fermentations in xylose; deletion of sensors results in an extended lag time, a decreased rate of sugar uptake, and reduced ethanol production (Figure 17E) as compared to a control strain with sensors (Figure 17B). Although the initial hypothesis was that optimal glucose transport may decrease efficiency of simultaneous sugar utilization, deletion of the glucose sensors further inhibited xylose uptake while glucose was present in the medium (Figure 17F) as compared to the parental control (Figure 17C).

The impact of sensor deletions on cellobiose fermentations was investigated next. In EJ4—a derivative strain of SR8 evolved for cellobiose fermentation—results on glucose were very similar as in SR8. EJ4 consumed glucose rapidly (Figure 18A) while

EJ4A displayed an increase in lag time and cell growth (Figure 18D). Cellobiose fermentation results, however, were quite different than those observed with SR8 on xylose. EJ4A actually consumed cellobiose slightly faster (Figure 18E) than EJ4 (Figure 18B) although the results were fairly similar between the two strains. In a mixture of glucose and cellobiose, the results were very much a combination of the pure sugar fermentations: EJ4 consumed glucose rapidly followed by extended period of cellobiose consumption (Figure 18C), while EJ4A displayed an extended lag period before glucose consumption followed by cellobiose consumption similar to the parental strain (Figure 18F).

While the removal of sensors did not have any outstanding beneficial effects for consumption of either xylose or cellobiose, it is possible that they may aid in the superb ability for yeast to convert sugars to ethanol. LdhA was thus introduced into strain D452 and the sensor-deleted D452A, yielding D452L and D452AL strains. These strains were then cultured in YP medium on glucose buffered with $CaCO_3$. D452L yielded mostly ethanol with a small amount of lactic acid (Figure 19A) while D452AL produced almost equal amounts of both products (Figure 19B). Overall, the yield of lactic acid was higher in D452AL than from D452L (Figure 19C). However, this result cannot be definitively tied to glucose sensors because glucose was consumed slower while cell growth was higher, leading to a lower specific glucose consumption rate. It is thus necessary to investigate how glucose uptake effects lactic acid production.

To explore the effects of changing glucose consumption rates on lactic acid production, the hexokinases were deleted in D452 and a doxycycline-activated transactivator rtTA and lactic acid production pathway were introduced. Then, *HXK1* or

HXK2 were introduced under the rtTA-controlled TetO7 promoter to create strains D452Δ*hxk*^{0}-iH1L and D452Δ*hxk*^{0}-iH2L. These two strains were then cultured in YP medium on glucose buffered with $CaCO_3$ and induced with varied amounts of doxycycline. There was a clear link between glucose uptake rate and the yield during mid-exponential phase and final titer of ethanol and lactic acid (Figure 20A,B). Moreover, D452AL fit perfectly on the trend observed, indicating that the increased lactic acid yield observed in a sensor-deleted strain is only due to the reduced glucose consumption rate rather than anything directly to do with membrane glucose sensors.

While these results indicate that membrane glucose signaling through Snf3 and Rgt2 are not inherently linked to enhancing production of lactic acid, this data can be used to investigate whether increased lactic acid yields from cellobiose and xylose result from reduced sugar uptake rates. An LdhA expression cassette was therefore introduced into SR8 and EJ4 and these strains were cultured in xylose and cellobiose, respectively. As shown in Figure 21, the yields of lactic acid and ethanol during mid-exponential phase by EJ4 cultured on cellobiose fit perfectly on the trend observed with the D452-derivatives cultured on glucose. However, the yield of lactic acid from xylose during mid-exponential phase in SR8 was near the maximum theoretically possible (Figure 21).

Although the effect of enhanced lactic acid production from glucose in sensor-deleted strains was found to be solely related to reduced flux, other compounds may still be enhanced by removing sensors. The production of 2-isopropylmalate (2-IPM) was thus studied as it is an intermediate of amino acid biosynthesis and requires acetyl-CoA. It can thus be hypothesized that the increased biomass formation observed in

sensor-negative strains may aid production of this compound. To investigate this further, the transcription factor *URE2* was deleted in D452 and D452A. Previously, Heejin Kim and Jingjing Liu found that *URE2* deletion aids in production of 2-IPM (unpublished data). Indeed, a higher accumulation of 2-IPM is observed from D452A (Figure 22B) as opposed to D452 (Figure 22A) when both strains contain a deletion in *URE2.*

However, similar to the results with lactic acid production, a decrease in glucose uptake rate is observed and additional experiments must be performed to investigate whether this is the sole cause behind the increased 2-IPM yield. As such, *URE2* was deleted in D452 iH1 and the strain was cultured on glucose with varied levels of hexokinase induction. In this case, however, a clear trend between 2-IPM yield and glucose uptake was not observed (Figure 22C). These results suggest that deletion of glucose sensing enhances 2-IPM yield through a mechanism beyond a decreased glucose uptake rate.

4.4 Discussion

There are several potential explanations for why sensor deletions affected xylose and cellobiose differently. First, xylose uptake relies on endogenous transport machinery while uptake of cellobiose is mediated by cellodextrin transporters from *Neurospora crassa* [90]. As such, sensor deletions may greatly affect the expression of transporters essential for xylose uptake while the expression of transporters essential for cellobiose uptake are expressed regardless. Moreover, sensor deletion led to a marginal increase in efficiency of cellobiose utilization. As sensors have been implicated in causing vacuolar degradation of transporters [144], this may be due to a decrease in endocytosis of CDT-1.

Xylose uptake in the presence of glucose was even more inhibited in SR8A than in SR8. Presumably, this indicates that low-affinity glucose transporters were not expressed. As higher affinity transporters would lead to increased preference for glucose over xylose, an increased expression of HXT2, 6, and 7 would explain the results observed in Figure 17F.

This study determined that while deletion of glucose sensors increases the yield of lactic acid from glucose in strains expressing an LdhA, this was merely a side-effect of a decreased glucose consumption rate rather than anything inherently to do with membrane glucose sensing through Snf3 and Rgt2. Further, this report demonstrated that a decreased flux explained the increased lactic acid from strains cultured on cellobiose [142]. However, lactic acid production from xylose could not be explained by reduced flux. Bolstering this result is the fact that the cellobiose-consuming strain EJ4 is a derivative of SR8, confirming that none of the mutations in SR8 leading to

enhanced xylose consumption, such as Pho13, are responsible for the increased lactic acid yield. This implies that some other mechanism during xylose fermentation leads to enhanced lactic acid yield. Gpr1, another membrane glucose sensor not investigated in this study, may play a role in this effect.

4.5 Tables

Table 8 – Strains

Strain	Description	Reference
SR8	Xylose fermenting *Saccharomyces cerevisiae*	[126]
SR8 4xAuxotroph	SR8 Δ*his* Δ*leu* Δ*ura* Δ*trp*	This study
SR8A	SR8 Δ*snf3* Δ*rgt2*	This study
SR8L	SR8 with pPGK1-LdhA-tPGK1 inserted in PBN1-SBP1 intergenic region	This study
EJ4	Cellobiose and xylose fermenting *Saccharomyces cerevisiae*	[145]
EJ4A	EJ4 Δ*snf3* Δ*rgt2*	This study
EJ4L	EJ4 with pPGK1-LdhA-tPGK1 inserted in PBN1-SBP1 intergenic region	This study
D452	Δ*his* Δ*leu* Δ*ura*	[127]
D452L	D452 with pRS405-LdhA	This study
D452 AL	D452 Δ*snf3* Δ*rgt2* with pRS405-LdhA	This study
D452Δ*hxk*^{0}	D452 Δ*hxk1* Δ*hxk2* Δ*glk1*::pMYO2-rtTA(s2)	Chapter 3
D452Δ*hxk*^{0}-iH1L	D452 Δ*hxk1* Δ*hxk2* Δ*glk1*::pMYO2-rtTA(s2) with pRS403-iHXK1 and pRS405-LdhA	This study
D452Δ*hxk*^{0}-iH2L	D452 Δ*hxk1* Δ*hxk2* Δ*glk1*::pMYO2-rtTA(s2) with pRS403-iHXK2 and pRS405-LdhA	This study
D452Δ*ure2*	D452 Δ*ure2*::KanMX with pRS403	This study
D452AΔ*ure2*	D452 Δ*snf3* Δ*rgt2* Δ*ure2*::KanMX with pRS403	This study
D452 iH1 Δ*ure2*	D452 Δ*ure2*::KanMX Δ*hxk1* Δ*hxk2* Δ*glk1*::pMYO2-rtTA(S2) with pRS403-tetO7p-*HXK1*	This study

Table 9 – Plasmids

Plasmid	Description	Reference
pRS405	*LEU* integrative plasmid	[78]
pRS405-LdhA	pRS405 with pPGK1-LdhA-tPGK1	This study
pRS403	*HIS* integrative plasmid	[78]
pRS403-TetO7-HXK1	pRS403 with pTetO7-*HXK1* expression cassette	This study
pRS403-TetO7-HXK2	pRS403 with pTetO7-*HXK2* expression cassette	This study
pRS42H-gRNA-PBN1/SBP1	Hygromycin episomal plasmid with gRNA targeting PBN1/SBP1 intergenic region	This study

4.6 Figures

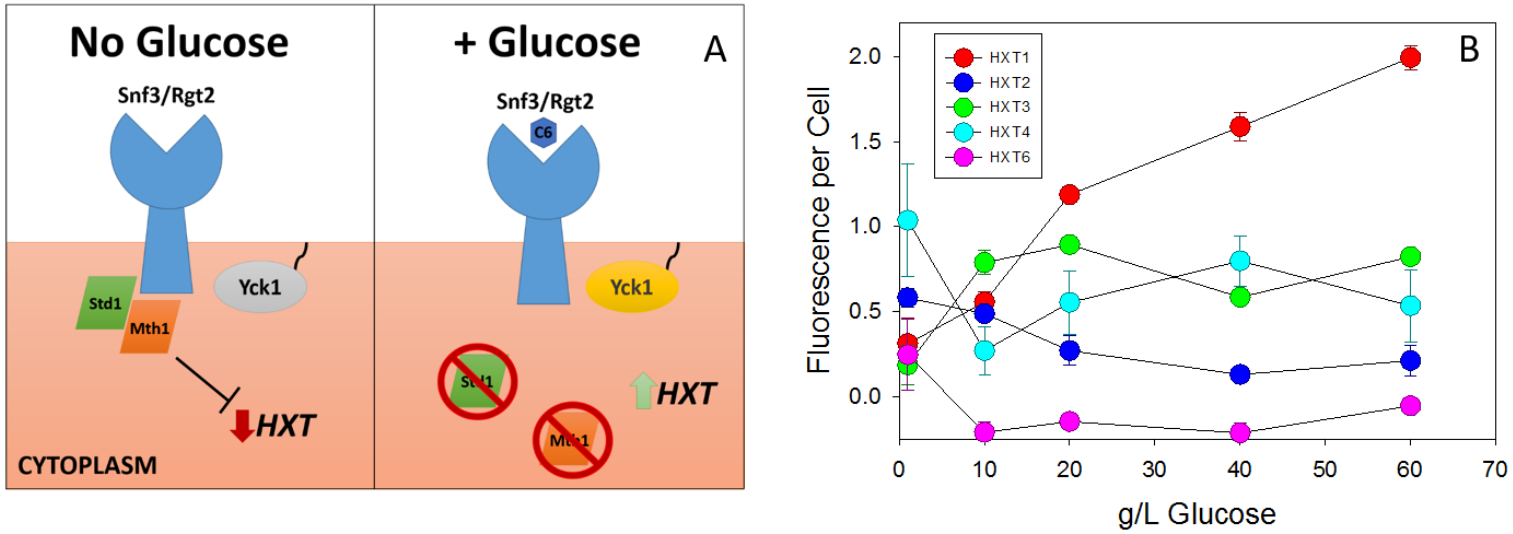

Figure 16: Glucose sensing determines membrane transport expression.

(A) Diagram of the mechanism of glucose sensors controlling transporter expression. Without glucose, the repressors Std1 and Mth1 accumulate, causing repression of the low-affinity glucose transporters such as HXT1. When glucose binds the low-affinity sensor Rgt2 or the high-affinity sensor Snf3, the membrane-bound protein kinase Yck1 phoshorylates Std1 and Mth1, triggering their degradation and releasing the repression on *HXT* genes. (B) Transporter expression in SR8 as a function of extracellular glucose concentration. Expression was monitored by measuring Gfp fluorescence in a strain where the endogenous *HXT* was deleted and replaced with *GFP* gene.

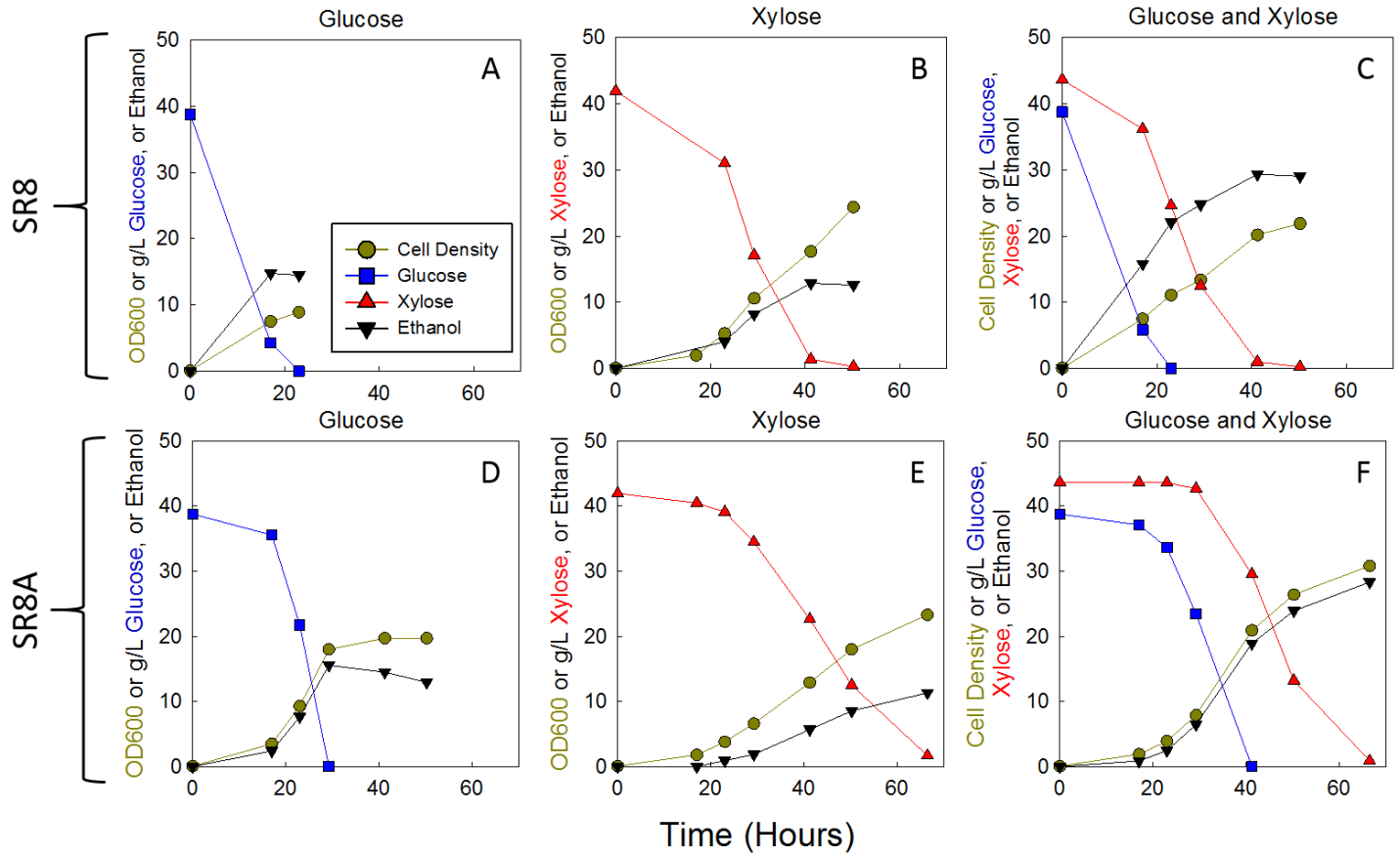

Figure 17: Deletion of glucose sensors reduces efficiency of glucose and xylose consumption.

Fermentation profiles of (A-C) SR8 and (B-D) SR8A cultured in (A,D) glucose, (B,E) xylose, and (C,F) a mixture of glucose and xylose. Fermentations were inoculated to an initial cell density equal to an OD of 0.05 and cultured in 25 mL media in 125 mL flasks. Data points are glucose (blue squares), xylose (red upward triangles), OD (yellow circles), and ethanol (black downward triangles).

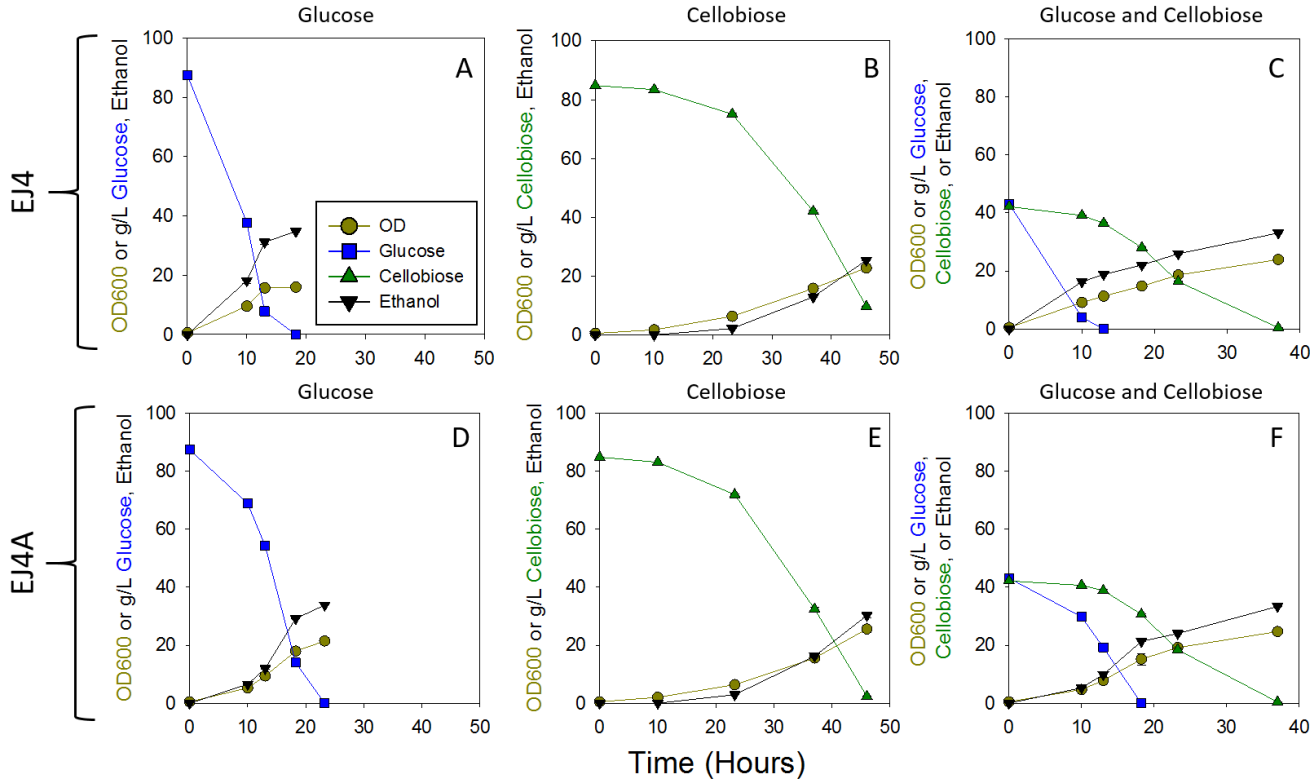

Figure 18: Deletion of glucose sensors has a comparatively smaller effect on cellobiose utilization.

Fermentation profiles of (A-C) EJ4 and (B-D) EJ4A cultured in (A,D) glucose, (B,E) cellobiose, and (C,F) a mixture of glucose and cellobiose. Fermentations were inoculated to an initial cell density equal to an OD of 0.5 and cultured in 25 mL media in 125 mL flasks. Data points are glucose (blue squares), cellobiose (green upward triangles), OD (yellow circles), and ethanol (black downward triangles). Experiments were conducted in duplicate with errors represented by error bars, which are not visible when the standard error is smaller than the size of the data point.

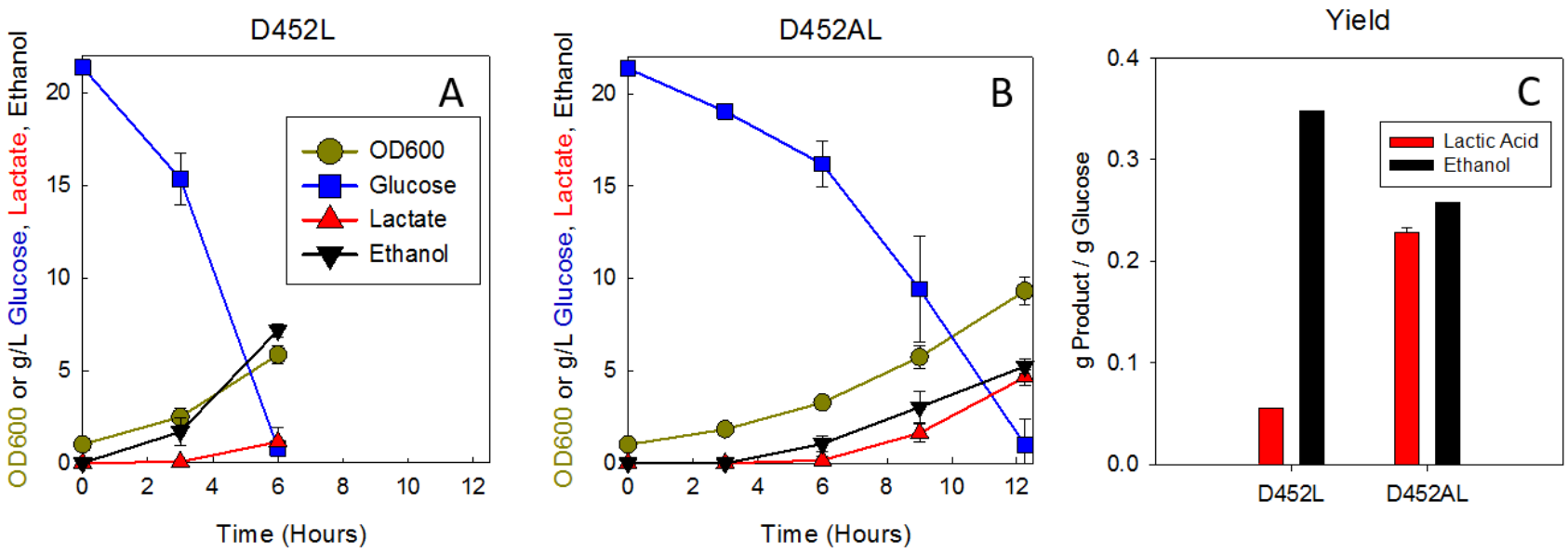

Figure 19: Sensor-negative yeast expressing LdhA shows an increase in lactic acid yield

Fermentation profiles of (A) D452L and (B) D452AL in YP medium with glucose as a sole carbon source. Fermentations were inoculated to an initial cell density equal to an OD of 1 and cultured in 25 mL media in 125 mL flasks with 10 g/L $CaCO_3$. Data points are OD (yellow circles), glucose (blue squares), lactic acid (red upward triangles), and ethanol (black downward triangles). Experiments were conducted in duplicate with errors represented by error bars, which are not visible when the standard error is smaller than the size of the data point.

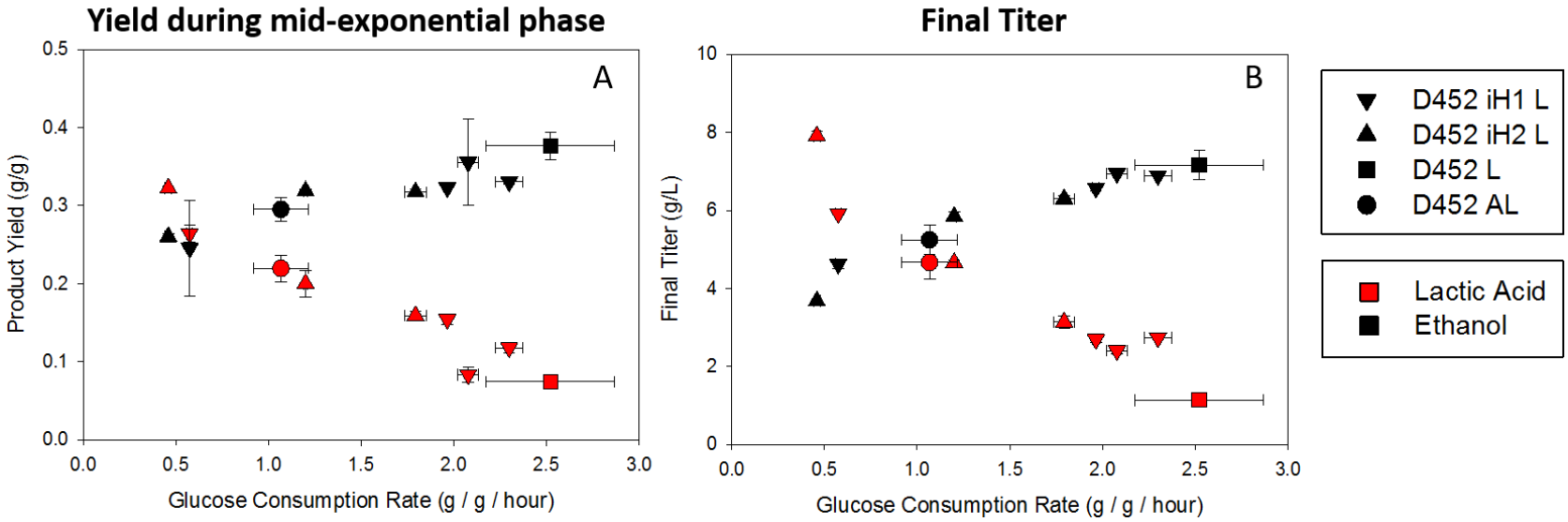

Figure 20: Reduced uptake flux leads to increased lactic acid production.

(A) Yield and (B) titer of lactic acid (red) and ethanol (black) in strains D452 L iH1 (downward triangles) and D452 L iH2 (upward triangles) with various amounts of doxycycline. Also graphed are strains D452 L (squares) and D452 AL (circles). Strains were cultured in 25 mL YP media in 125 mL flasks with 10 g/L $CaCO_3$ at an initial OD equal to 1. Data points are the average of duplicate experiments with standard deviations indicated by error bars, which are not visible when smaller than the data point.

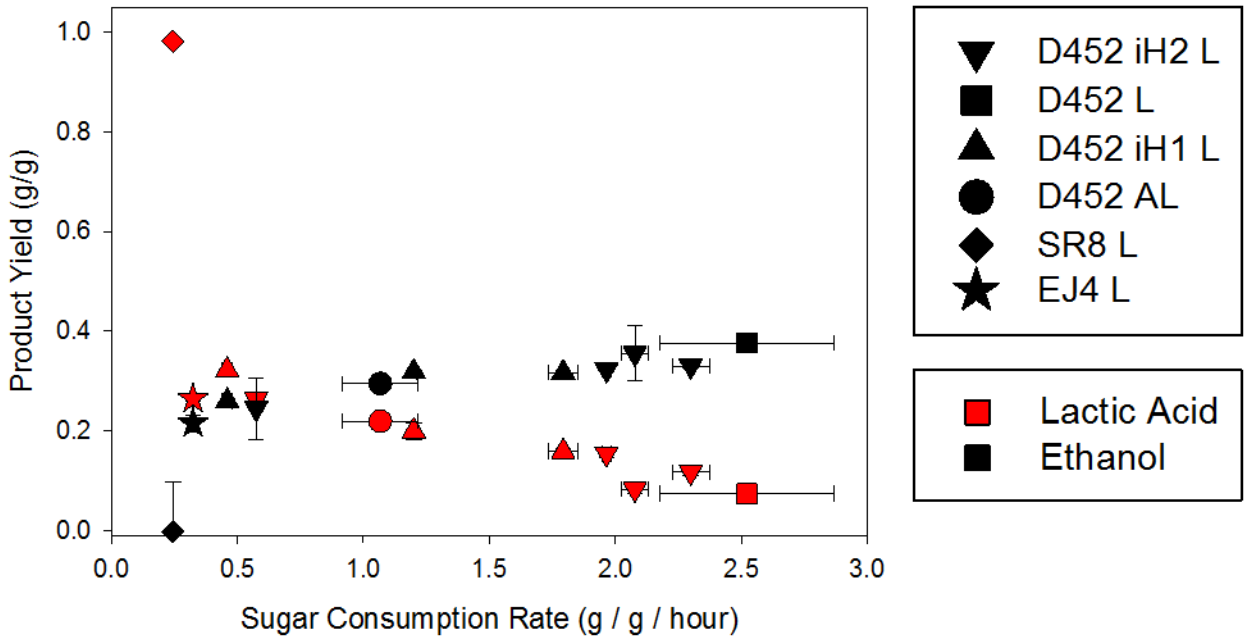

Figure 21: Decreased flux explains increased lactic acid production from cellobiose, but not from xylose.

SR8 L (diamond) cultured with xylose and EJ4 L (stars) cultured with cellobiose plotted on the same graph as Figure 4A. Strains were cultured in 25 mL YP media in 125 mL flasks with 10 g/L $CaCO_3$ at an initial OD equal to 1. Data points are the average of duplicate experiments with standard deviations indicated by error bars, which are not visible when smaller than the data point.

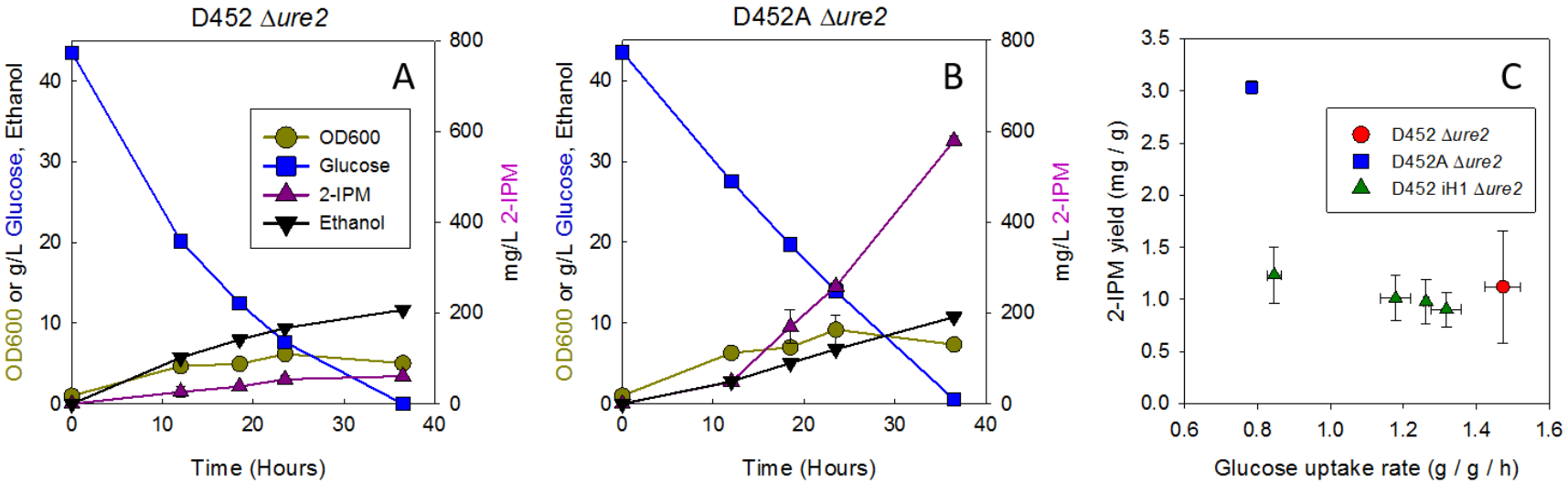

Figure 22: 2-IPM production is enhanced through deletion of glucose sensors.

D452 Δ*ure2* (A) and D452A Δ*ure2* (B) cultured in minimal media with glucose as a sole carbon source. Strains were cultured in 25 mL SC media in 125 mL flasks at an initial OD equal to 1. Data points are the average of duplicate experiments with standard deviations indicated by error bars, which are not visible when smaller than the data point. (C) Yield of 2-IPM plotted against glucose uptake rate during the first 12 hours of fermentation. Strains are D452 Δ*ure2* (red circle), D452A Δ*ure2* (blue square), and D452 iH1 Δ*ure2* with *hxk1* induced with varied levels of doxycycline (green triangle).

CHAPTER 5: CONCLUSIONS AND FUTURE OUTLOOK

The studies in this thesis tackled a diverse range of issues important to biotechnological use of yeasts. First, experiments were performed to analyze the modifications necessary to allow *Yarrowia lipolytica* to consume cellulose. Then, investigations were undertaken achieve an understanding of genetic modifications necessary to enable simultaneous sugar utilization and enhance production of non-ethanol compounds in *Saccharomyces cerevisiae*.

While the work contained within this thesis on developing *Y. lipolytica* capable of consuming cellulosic sugars was the first report published on this subject, recent work has provided additional information to this problem. One group identified that the *Y. lipolytica* genome contains endogenous cellodextrin transporters and beta-glucosidases [146]. Through overexpression of these genes using natural high-expression *Y. lipolytica* promoters, they were able to develop cellobiose-consuming strains without the need for any heterologous genes. Another group found that some small amounts of cellobiose-degrading ability can be developed in *Y. lipolytica* simply through sequential culturing of this strain on cellobiose [147]. Even further, within the *Y. lipolytica* genome they identified all the genes necessary to metabolize xylose as well as several putative xylose-specific transporters. Again, they found that merely sequential subcultures granted this yeast some small ability to degrade xylose. These results suggest that some strains of *Y. lipolytica* may have endogenous capabilities to digest cellulosic sugars and the species as a whole may have lost the ability to consume these sugars relatively recently. Thus, to ease the regulatory process involved with introducing new GM organisms to industrial applications, these newer more recent approaches may be

superior to transgenics. However, more work in this area will be necessary as the rates of consumption exhibited by strains developed with these newer strategies have not yet reached the consumption rates shown within this thesis. As such, if *Y. lipolytica* happened to lose the ability to consume these sugars long ago in its evolutionary history, the enzymes coded within its genome may not be highly functional in metabolizing these lignocellulosic sugars. If this is the case, the strategies employed within this thesis, i.e. transgenic expression of metabolic enzymes from organisms with strong ability to consume these sugars, may be superior in terms of bioprocess productivity and efficiency.

The latter part of this thesis hypothesized that membrane glucose sensors optimize uptake of glucose and may be detrimental to simultaneous sugar utilization. While this work demonstrated that sensor deletions in fact decrease efficiency of xylose utilization in both pure sugars and glucose/xylose mixtures, this result led into an identification that reduced glycolytic flux leads to enhanced lactic acid yields from glucose. This result is very similar to recent reports that isobutanol production can be enhanced in yeast by reducing the rate of glucose phosphorylation [124]. This result coupled with the findings in Chapter 3 of this thesis, namely that sugars can be simultaneous consumed by reducing the rate of glucose consumption, suggests that there is untapped potential in simultaneous consumption of lignocellulosic sugars for production of a large number of fuels and chemicals.

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