Development and Validation of Yeast Biosensors for Signaling Pathways

Master Thesis

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Abstract

Efficient bioconversion of the pentose xylose is vital for the development of competent lignocellulose biorefineries. Although many proper attempts of metabolic engineering for recombinant xylose utilization have been made in *Saccharomyces cerevisiae*, growth on xylose is still far from being optimal. Previous findings point out a more complex explanation to this feature involving interactions between xylose and signaling pathways. To assess this question we have developed and validated a panel of GFP yeast biosensor strains for the characterization of three signaling pathways (Snf3/Rgt2, Snf1/Mig1, cAMP/PKA) when subjected to different xylose conditions. Interestingly, the presence of 50 g/L xylose alone did not trigger any effect on these signaling pathways, however, when having a mixed condition of 50 g/L xylose and 5 g/L glucose higher induction patterns were observed than in glucose 5 g/L for the low affinity hexose transporters (HXT2 and HXT4) and the SUC2 gene. Together these results support the hypothesis that xylose alone produces a true starvation response in non-engineered W303 *S. cerevisiae* strains instead of a non-fermentable carbon response. Furthermore, sufficient information has been acquired to propose that mixed conditions of glucose and xylose produce a more profound effect in signaling that should be investigated further.

Keywords: Xylose, yeast, signaling, Snf3/Rgt2, Snf1/Mig1, cAMP/PKA, flow cytometry, high-throughput, GFP

Popular Science Summary

Baker's yeast *Saccharomyces cerevisiae* has for years been the ideal species for expression and production of numerous chemicals, such as bioethanol, bioplastics and genetically engineered proteins. Nowadays, interest to implement this species in the lignocellulose (main component of plant matter) biorefinery industry is clear, due to its known robustness and capacity to grow in environments that are toxic to many other microorganisms. Importantly, lignocellulose is a complex carbohydrate matrix, commonly degraded by enzymes to produce a solution rich in sugars (monosaccharides), being the hexose glucose and the pentose xylose the two major components. Although great efforts have been made to genetically modify *S. cerevisiae* to uptake and metabolize xylose, its growth on this pentose is still less efficient than on glucose, and thus not economically feasible for the industrial production scale.

Many advanced technological techniques and tools have been used to produce strains with higher capabilities in terms of xylose transport and growth. However, knowledge about how this yeast senses the presence of xylose is still mostly unknown. Being able to understand the role that xylose has outside and inside the cells could open the door for the identification of new targets for genetic manipulation that would aid the development of a tailor-made strain for efficient growth on xylose.

This Master Thesis project is part of a current research project at Applied Microbiology, which aims to unveil the effect of xylose in yeast physiology. For this purpose a panel of biosensors strains have been developed and validated to massively screen the effect that different extracellular xylose conditions have on three different intracellular mechanisms. These mechanisms are involved in sugar sensing, metabolism and stress response. The biosensor strains were constructed by isolating regulatory regions (promoters) from genes affected by these mechanisms and the green fluorescent protein gene. By measuring the different levels of green light emitted by the cells exposed to different xylose conditions valuable data was acquired, much of which would have been a complex challenge with traditional techniques.

Our findings suggest that *S. cerevisiae* is unable to sense the presence of xylose in the surroundings. However, interesting patterns of induction were observed in the hexose-sensing pathway when xylose and glucose were both present in the growth medium. This could imply that hexose transporters induced by glucose could have a transient level of transportation for xylose, and therefore, the signaling mechanism could be affected by intracellular xylose concentrations. Further experiments are still necessary to be certain that intracellular xylose is able to affect any physiological traits in *S. cerevisiae*.

List of Abbreviations

ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
FI	Fluorescence Intensity
GFP	Green Fluorescent Protein
HPLC	High Performance Liquid Chromatography
KHPh	Potassium Hydrogen Phthalate
mRNA	Messenger Ribonucleic Acid
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
yEGFP	Yeast Enhanced Green Fluorescent Protein
YNB	Yeast Nitrogen base

Contents

1. Introduction

1.1. Lignocellulose Biorefineries

Considering that currently only 28% of the total tree biomass is utilized for the production of sawn timber (ITTO, 2005), the main component to manufacture any wooden article, leaves the door open for the development and improvement of new technologies that could take advantage of this unused material. In this sense, the substantial amounts of lignocellulosic waste produced by the wooding industry give biorefineries a unique opportunity to use this material for the production of more valuable goods, such as: second generation bioethanol or polyhydroxybutyrate and polyhydroxyalkanoates (commonly known as bioplastics).

In addition, lignocellulose is a complex matrix made of principally hexose and pentose sugars (mainly glucose and xylose) and aromatic compounds (Liguori and Faraco, 2016). This makes the task of genetically engineering an organism to co-consume these sugars and to be able to withstand the inhibitory properties of the aromatic compounds a challenge. In addition, only few known organisms are capable of naturally degrading lignocellulose making metabolic engineering an important strategy to incorporate exogenous and enhance specific traits. This challenge implies an immense technical hurdle that must be overcome in order to efficiently use lignocellulosic biomass for sustainable bioconversion into other compounds.

Baker's yeast *Saccharomyces cerevisiae* offers an unparalleled platform to develop this technology. Being the subject of studies for decades has given well-documented details about its biology and a set of genetic tools in order to manipulate and introduce different exogenous genes with great accuracy (Cubillos, 2016). Furthermore, its characterized robustness against growth inhibitors has promoted the implementation of this microorganism in many industrial processes that involve the use of mixed feedstock and strong chemicals. In recent years the development of metabolic engineering approaches has enabled *S. cerevisiae* to grow on pentoses, however, this growth is still less efficient than growth on glucose as a carbon source (Jeffries and Jin, 2004). In addition, *S. cerevisiae* has endogenous genes for xylose metabolism (Toivari *et al*, 2004), presumably remnants of an archaic xylose-metabolizing predecessor. However, these genes are not properly regulated by a signaling mechanism for xylose metabolism, making this yeast unable to utilize xylose by any endogenous trait (Konishi *et al*, 2015). Nevertheless, this characteristic suggests the possibility of a vestige mechanism for sensing this pentose, which will be addressed in the current investigation.

1.2. Signaling Pathways in *S. cerevisiae*

Being able to construct an optimal engineered organism for metabolism of lignocellulose has been the dream of scientists and industry for many decades, many

efforts have been made in order to design and introduce exogenous metabolic pathways into yeast for xylose metabolism (Sanchez and Karhumaa, 2015). Although these efforts have helped understand better the complex dynamics that exist regarding gene regulation and signaling pathways, there are still many crucial interactions that remain unknown. In this sense, it is of great importance to develop methods to screen for gene activity in a high-throughput approach, to be able to assess which conditions or elements are interfering with these pathways and how they affect xylose uptake and metabolism in *S. cerevisiae*.

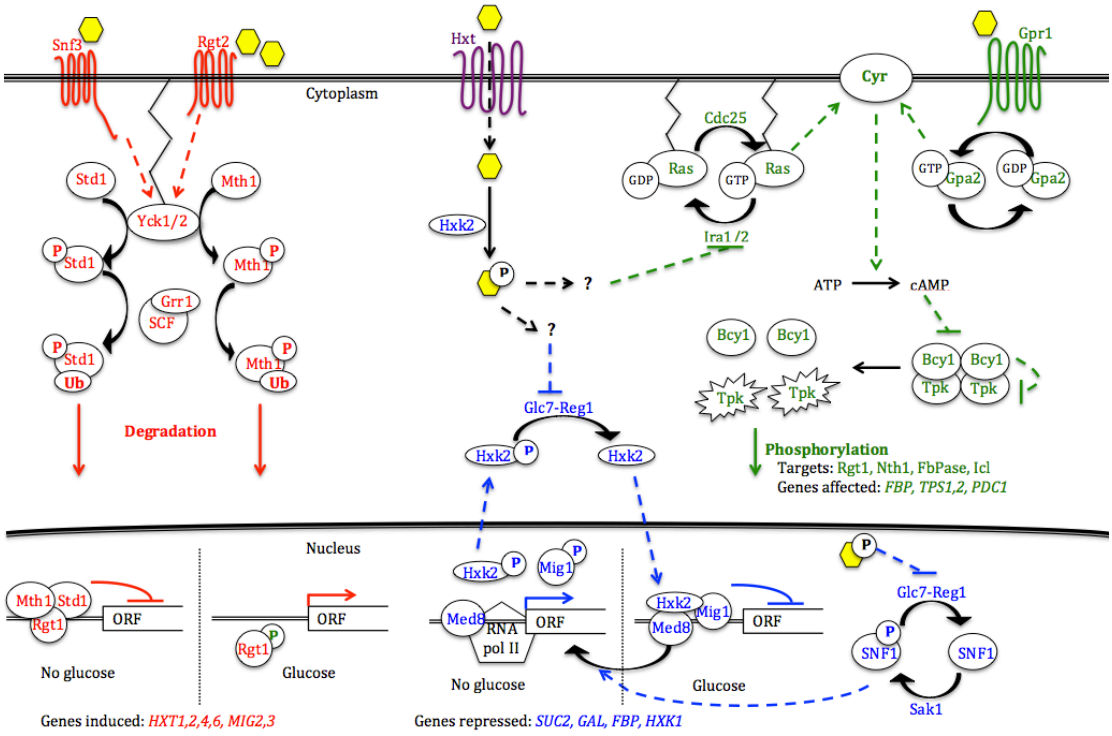

Figure 1. Schematic representation of the three signaling pathways investigated. Red: Snf3/Rgt2 pathway, Blue: Glucose repression pathway, Green: RAS-cAMP-PKA pathway. Source: Celina Tufvegren.

Three major sugar signaling pathways were analyzed in this project (Fig 1): the Snf3/Rgt2 hexose recognition pathway, the Snf1 glucose repression pathway and the trehalose biosynthesis pathway (stress response pathway), which is closely related to the RAS/cAMP/PKA pathway (Conrad *et al.* 2014; Francois and Parrou, 2001). The Snf3/Rgt2 glucose signaling pathway is in charge of signaling the presence and concentration of glucose and induces the corresponding hexose transporters in order to uptake glucose from the media. Rgt2 senses high concentrations of glucose while Snf3 can sense high and low concentration of glucose; depending on which of these that are activated, an appropriate high or low affinity hexose transporter will be expressed (Özcan *et al.* 1998, Lutfiyya *et al.* 1998). Moreover, the glucose repression pathway is essential for *S. cerevisiae* to adapt to glucose limitation levels and to utilize alternate carbon sources such as

galactose, arabinose or sucrose. For this purpose, this pathway induces the expression of genes involved in the metabolism of other hexoses (Hedbacker and Carlson, 2008). The key regulator of this pathway is the hexokinase 2 (HXK2p), an enzyme involved in the phosphorylation of glucose to glucose 6 phosphate in the initial steps of glycolysis. This enzyme also plays a role as a transcription factor inhibiting the expression of genes involved in glucose repression, when intracellular levels of glucose are optimal (Peláez *et al*, 2010). Lastly, the RAS/cAMP/PKA pathway is a complex signaling pathway, which plays a major role in the regulation of metabolism, stress resistance and cell cycle progression. It has been shown that this pathway can sense extracellular glucose concentrations by the Gpr1 complex and respond to the glucose repression pathway, inducing the biosynthesis of trehalose by the induction of the TPS1 and TPS2 genes (Pescini *et al*, 2012).

1.3. The Green Fluorescent Protein and its Application in Biosensory Systems

The discovery of the green fluorescent protein (GFP), initially isolated from the jellyfish *Aequorea victoria* by Shimomura and colleagues in 1962 (Shimomura *et al*, 1962) has been an unparalleled scientific breakthrough. Since its isolation many variants of this protein have been engineered for different fluorescent excitation peaks, stabilities and purposes, becoming a well-established fluorescent marker for molecular and cell biology studies. Due to its known non-intrusive characteristic (Shibasaki et al, 2009) this protein has also been applied to *in vivo* studies to monitor complex physiological traits in real time without creating any burden to any endogenous physiological attribute.

Whole cell-biosensors, cells that are able to respond to a particular stimulus and produce a signal (Liu *et al*, 2010), have become increasingly popular over the last decade, allowing a variety of *in vivo* studies, from recognition of transient elements in the media for characterization of gene activity (Mehta *et al*, 2016). In order to create a whole cell-biosensor it is necessary to construct a reporter gene made of a regulative region (promoter) and a gene encoding a protein able to give a reliable signal. Both parts of this reporter gene are vital: the promoter will regulate the expression of the reporter protein to specific conditions, and the reporter protein will give a detectable signal, which can be interpreted in a qualitative or quantitative manner (Bereza-Malcolm *et al*, 2015).

1.4. Aim of the Study

The objective of this project was to validate a panel of yeast biosensor strains for three signaling pathways (the Snf3/Rgt2 pathway, the Snf1 glucose repression pathway and the RAS/cAMP/PKA pathway) which are involved in sugar recognition and metabolism. Furthermore, these biosensors will be employed for unveiling the effects of xylose, in the induction of these genes. In this sense, we will understand what are the signaling implications of the presence of xylose in the media, uncovering if *S. cerevisiae* is able to sense this pentose or not and what signaling pathways are affected by it.

2. Material and methods

2.1. Strains

The *S. cerevisiae* strains that were used in this study are listed in Table A1. The W303-1A strain [ATCC® 208352], from which the engineered strains were derived, was purchased from ATCC (Manassas, VA, US). All strains were stored in 25% (v/v) glycerol at -80°C. The yeast strains were maintained on YNB-glucose agar plates (6.7 g/L YNB w.o. amino acids, 20 g/L glucose, 20 g/L agar-agar). Agar cultivation were considered fresh for two weeks before they were restreaked from the -80°C glycerol stock.

2.2. Shake Flask Cultivation Conditions

Pre-pre cultures of the yeast biosensor strains were started by inoculating a single colony into a 50 ml conical tube with 5 ml of YNB 20 g/L glucose, buffered with Potassium Hydrogen Phthalate (KHPh) 50mM pH 5.5; the tubes were grown for 10 hours to achieve sufficient biomass. Pre-pre cultures were used to inoculate 250 ml baffled shake flasks with 25 ml of repressing media; different repressing conditions, OD and culture times were used depending on each biosensor strain (Table 1). Finally, sufficient volume from the pre-culture was harvested to inoculate 1 L baffled shake flasks with an initial OD of 0.5; the harvested volume was centrifuged at 3000 rpm for 5 min and washed twice with buffered YNB 6.7 g/L. After the final wash, cells were pelleted and resuspended in 1 ml of the same YNB media as the wash step, and were used to inoculate 1 L baffled shake flasks with 100 ml of YNB 6.7 g/L, with 1 g/L or 20 g/L glucose and KHPh buffer 50mM pH 5.5. Biological duplicates were performed for each condition tested. All cultivations were performed at 30°C in a shaking incubator.

Table 1. Pre-culture conditions used to repress the biosensor strains

Strain	Pre-Culture for 1L Shake Flask (25 ml in 250 ml shake flask)	Pre-Culture for Microtiter plate (5 ml in 50 ml conical tube)	Time (hours)	Initial OD
TMB3711	40 g/L Glucose	40 g/L Glucose	12	0.05
TMB3712	3% Ethanol	3% Ethanol	32	0.05
TMB3713	40 g/L Glucose	40 g/L Glucose	12	0.05
TMB3714	40 g/L Glucose	40 g/L Glucose	12	0.05
TMB3715	40 g/L Glucose	40 g/L Glucose	12	0.05
TMB3716	40 g/L Glucose	40 g/L Glucose	12	0.05
TMB3717	40 g/L Glucose	40 g/L Glucose	12	0.05
TMB3718	40 g/L Glucose	40 g/L Glucose	12	0.05
TMB3719	20 g/L glucose	20 g/L glucose	24	0.05

2.2.1. Growth and Metabolite Profiles

Growth was monitored by Optical Density (OD) at 620 nm using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden). Extracellular glucose and metabolites (glycerol, acetate and ethanol) were quantified with a Waters HPLC system (Mildford, MA, USA). The separation was performed with an HPX-87H ion exchange column (Bio-Rad, Hercules, CA, USA), using a mobile phase that consisted of 5mM H_2SO_4 at a flow rate of 0.6 mL/min and a column temperature of 60°C. A refractive index detector (RID-6a, Shimadzu, Kyoto, Japan) was used for detection.

2.2.2. Flow Cytometry

The biosensor strains were evaluated for fluorescence intensity (FI) with a BD Accuri C6 flow cytometer equipped with a BD CSampler autosampler (Becton-Dickinson, NJ, US). A laser with a wavelength of 488 nm was used and fluorescence was measured with a 533/30 bandpass filter. Quality control was performed prior to each experiment using Spherotech 8-peak and 6-peak validation beads (Becton-Dickinson, NJ, US). Only fresh samples were analysed at a flow rate of 14 μL/min and a core size of 10 μm. The threshold was set at 8000 at the forward scatter-height (FSC-H) channel and 100,000 events were collected per sample. After each sample a wash cycle and a 2 minute injection of deionised filtered water was performed to minimize sample carryover.

Flow cytometry raw data was analyzed with the Knijnenburg morphology correction model (Knijnenburg, Roda et al. 2011) in Matlab (Release R2015a, The MathWorks, Inc., Natick, MA, US) with the FCS data reader function (version 28 May 2014; L. Balkay, University of Debrecen, Hungary; downloaded from http://www.mathworks.com/matlabcentral). A custom, in-house Matlab script was developed to allow high-throughput pipeline for processing data.

2.2.3. Transcription Profiling

Samples for mRNA analysis were quenched in cold methanol (-80°C; 1.4 ml methanol per 1 ml cell culture (Ismail *et al*, 2014)) and centrifuged at 3000 RPM and 0°C for 5 min. The supernatants were decanted and the cell pellets were stored at -80°C. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The quenched cell samples were resuspended and lysed mechanically by bead beating in a Precellys 24 (6500 rpm, 3 cycles á 60s with 30s pauses in-between cycles; Bertin Technologies, France) with a Cryolys temperature controller (Bertin Technologies, France) cooled with liquid nitrogen with the air flow setting at 2. DNA was removed with rDNase I (Ambion, Life Technologies, Carlsbad, CA, US). Conventional PCR was used to verify that no residual DNA was left in the RNA extract. The RNA content was quantified using a BioDrop (BioDrop Ltd, Cambridge, UK). Superscript IV Reverse Transcriptase kit and Oligo$(DT)_{20}$ primers (Invitrogen, Life Technologies, Carlsbad, CA, US) were used to synthesize cDNA from the extracted mRNA (using 0.5

mg/mL of mRNA extract per sample). RT-qPCR was performed using Ex Taq DNA polymerase kit (Takara Bio, Kusatsu, Shiga, Japan), EvaGreen dye (Biotium, Hayward, CA, US), bovine serium albumin (20 mg/mL;) and a LightCycler 2.0 (Roche Life Science, Basel, Switzerland) and quantification cycle (C_q)-values and melting curve analysis were determined using the LightCycler software 4.1. ACT1 was chosen as the reference gene and yEGFP3 and SUC2 were used as the target genes (Table A2 for primers sequence). The following RT-qPCR program was used to analyze all three genes: denaturation 95°C 2min; 45 cycles of 95°C 10s, 55°C 10s, 72°C 30s; melting curve analysis: 50°C 1 min hold time, ramp to 95°C with 0.05 °C/s; cooling: 40°, 30s. Standard curves for calculation PCR efficiencies, and assessment of relative expression were performed as previously described (Pfaffl, 2000). Each sample was analyzed in biological and technical triplicates.

2.2.4. Invertase Enzyme Assay

Enzymatic activity of invertase (encoded by SUC2) was assessed in yeast cell extracts according to previous protocols (Weiß, Huppert et al. 2008, Harkness and Arnason 2014) with the exception that o-dianisidine was replaced with 4-aminophenazone/phenol (Bauminger 1974). Samples were taken from cell cultures of TMB3711 and TMB3715 during inducing conditions (1 g/L glucose). The assay was performed in biological triplicates.

2.2.5. Sampling procedures

The 100 ml batch cultures from 1 L shake flasks were sampled for OD (500µl) and FI (200µl) measurements every hour, for metabolite concentrations every second hour (2 ml) for the first 8 hours and a final sample at 24 hours. Samples for RNA extraction were taken at 0, 15, 30, 45 and 60 minutes (4 ml). Enzyme assay samples were collected every 30 minutes for the first two hours.

2.3. Microtiter Plate Assay

Yeast strains were pre-pre cultivated for 10 hours by inoculating a single colony from YNB agar plates (20 g/L glucose) into a 50 ml conical tube with 5ml of 20 g/L glucose buffered YNB 6.7 g/L media. 50 ml conical tubes were used for pre-cultures of the biosensor strains, with different repressing conditions and culture times were used depending on the strain (Table 1). The tubes were incubated at 30°C in a shaking incubator with the lids loosely screwed (and secured with tape) in order to improve oxygenation for 12 hours. 1.5 ml of the culture was harvested at 5000 RPM for 3 min in a bench top centrifuge and the cells were washed twice with buffered 6.7 g/L YNB media. After the washing steps, a micro culture of 250 µl was prepared per well with an initial OD 0.5. In the microtiter plate, two technical replicates per strain and one blank well (media only) were done for each condition. A graphical scheme of the microtiter plate can be seen in figure A4. The microtiter plate was analyzed in the flow cytometer at 0, 3 and 6 hours; between samplings the plate was

incubated at room temperature in a micro-plate shaker at 800 RPM. Each strain was analysed in biological duplicates.

Cell specific fluorescence analysis was performed using an Accuri C6 flow cytometer (BD, NJ, US) equipped with an automated sampler. Each sample was run at 14 µl/min with plate shaking and washing features after each sample. 10 000 events per sample were recorded for analysis. Cell size normalization was carried out according to Knijnenburg (Knijnenburg *et al.* 2011) and a custom script implementing the normalization model was developed for high-throughput processing of the data in Matlab (Brink *et al*, manuscript under preparation).

3. Results & Disucussion

3.1. Validation of the Biosensor Strains

In order to validate the fluorescent behavior of each biosensor strain, 100 ml batch cultivations were performed under low (1 g/L) and high (20 g/L) glucose concentrations, as previous studies have shown that these are known inducing and repressing conditions for each promoter (Table 2). The cellular auto-florescence at the GFP emission wavelength was determined using fluorescence values from the negative control TMB3711 (Figure A1). Repression of the GFP biosensor cassette at the start of the experiment (0h) was highly desired in order to achieve a clear induction pattern and be able to characterize the strains. Furthermore, to rule out any effect of pH in the analyzed signaling pathways, buffered media was employed. Overall, the fluorescence intensity from the biosensors over time when cultivated on glucose showed good resemblance to previous findings (Table 2 and Figs. 2 & 3). The fitness of yeast did not seem to be affected by the presence of these exogenous cassettes, as OD and HPLC results for glucose uptake and metabolite production (acetate, ethanol and glycerol) for all strains showed no significant difference (Figs. A2 and A3).

Transcription profiles from both the biosensor cassette (Suc2p::GFP) and the endogenous SUC2 gene were compared in strain TMB3715 to further validate the GFP signal. Relative expression experiments between yEGFP3 and SUC2 showed that both ORFs have the same trend in transcription, showing an abrupt increase in transcription activity after the first 15 minutes, followed by a decrease over the lapse of 1 hour. However, the transcription levels of the biosensor cassette appeared to be weaker than the endogenous gene (Figs. 4 and 5). To assess whether the biosensor cassette was hindering the transcription of the endogenous gene, we compared the transcription profile of the SUC2 gene of strains TMB3715 and the negative control TMB3711, which did not have any GFP construct. The results showed no interference of the biosensor cassette in the normal transcription dynamics of the endogenous gene (Fig. 6).

Table 2. Known inducing and repressing conditions for the promoters used

Promoter	Name/Function	Induced/ derepressed by	Repressed by	References
HXT1	Low-affinity hexose transporter	High glucose (4% w/v)	Low glucose (0.1% w/v)	Özcan and Johnston (1995)
HXT2	High-affinity hexose transporter	Low glucose (0.1% w/v)	High glucose (4% w/v)	Özcan and Johnston (1995)
HXT4	High-affinity hexose transporter	Low glucose (0.1% w/v)	High glucose (4% w/v) More glucose-repressed than HXT2	Özcan and Johnston (1995), Özcan and Johnston (1996)
SUC2	Invertase	Low glucose (0.1% w/v)	High glucose (2% w/v) *and* depleted glucose (0% w/v)	Özcan, Vallier et al. (1997)
CAT8	*Alternative carbon source response*-activator	Low glucose (0.2 % w/v)	High glucose (4% w/v)	Hedges, Proft et al. (1995)
TPS1	Trehalose-6-Phosphate Synthase (56 kD subunit)	Glucose limitation, stress conditions (e.g. heat, nutrient starvation, oxidative stress)	High glucose (RAS-cAMP-activity); however, a basal expression level has been observed when growing on rapidly fermentable sugars	Parrou, Enjalbert et al. (1999), Winderickx, deWinde et al. (1996)
TPS2	Trehalose-6-Phosphate Synthase (102.8 kD subunit)			
TEF4	Translation Elongation Factor	-	Stress conditions	Olarewaju, Ortiz et al. (2004), Grousl, Ivanov et al. (2013)

To further test for any intrusive effect of the biosensor cassette at the protein level, enzymatic activity for invertase (encoded by the SUC2 gene) was tested in strains TMB3711 and TMB3715 (induced by 1g/L glucose). Results from protein extracts confirmed that enzyme activity increased over time and that there were no significant difference in activity throughout the tested time points (Fig. 7). With these findings we concluded that there was no invasive effect of the biosensor cassette or GFP, additionally two promoter of the same ORF did not alter the normal physiological traits of the strains developed in this study.

3.2. Microtiter Plate Assay

After validation of the biosensor strains, a high-throughput protocol was developed to assess the effect of different carbon sources on the biosensor strains. The signal of the biosensors when exposed to glucose 1g/L and 20 g/L confirmed that the downscaling of the experiment volume did not disturb the behavior of the biosensor (Fig 2 & 3; Table 3). The time required to complete the sampling from the microtiter plate was approximately 90 minutes, due to this technical limitation the raw values of fluorescent intensity from the different conditions are not comparable with each other. In order to have a more time-independent assessment of the microtiter plate results, fold change values were calculated by dividing the values for each time point by its 0 hour value (within each condition). A complete overview of the fold changes in fluorescent intensity is shown in Table 3.

All biosensor strains lacked induction when exposed to YNB without carbon source, xylose 50 g/L, glycerol 3% or xylose 50 g/L and glycerol 3% combined media condition. Xylose 50 g/L and glucose 5 g/L media resulted in induction of the

biosensor strains, which are induced under low concentrations of glucose and resembled the effect of 5 g/L glucose condition (strains TMB3713-3714). However, TMB3715 exhibited an induction pattern higher in the combined condition of xylose with glucose than the one observed in glucose 5 g/L alone. In addition, induction values from TMB3713 and TMB3714 were higher in the combined condition of glucose and xylose than in xylose alone, but nevertheless these values were below 2 in fold change.

Table 3. Fold change induction of the biosensor strains under different conditions. A fold change of 2 or higher was determined as an induction pattern, and is indicated by the grey colored cells.

Strain	**YNB only (no carbon source)**		**Glucose 1 g/L**		**Glucose 20 g/L**		**Glucose 5 g/L**	
	3h	**6h**	**3h**	**6h**	**3h**	**6h**	**3h**	**6h**
TMB3711 (No GFP)	0.92 ± 0.023	0.91 ±0.024	0.98 ±0.062	0.89 ±0.040	0.96 ±0.026	0.87 ±0.010	1.00 ±0.010	0.92 ±0.056
TMB3712 (*HXT1p*)	0.84 ±0.010	0.83 ±0.031	1.15 ±0.010	1.21 ±0.012	1.82 ±0.01	4.14 ±0.17	1.21 ±0.016	1.23 ±0.013
TMB3713 (*HXT2p*)	1.13 ±0.034	1.28 ±0.091	3.62 ±0.130	3.01 ±0.068	0.75 ±.013	0.67 ±0.021	1.10 ±0.264	1.34 ±0.11
TMB3714 (*HXT4p*)	1.04 ±0.088	1.15 ±0.18	4.27 ±0.50	3.67 ±0.21	1.25 ±0.21	1.22 ±0.21	1.77 ±0.058	2.47 ±0.22
TMB3715 (*SUC2p*)	1.10 ±0.22	1.44 ±0.56	6.45 ±0.37	7.66 ±0.089	1.07 ±0.071	1.03 ±0.059	0.98 ±0.15	3.51 ±0.75
TMB3716 (*CAT8p*)	1.01 ±0.010	1.27 ±0.041	1.48 ±0.18	1.69 ±0.030	0.97 ±0.11	0.92 ±0.094	0.98 ±0.002	1.14 ±0.039
TMB3717 (*TPS1p*)	1.04 ±0.001	1.08 ±0.018	1.25 ±0.056	1.37 ± 0.095	0.66 ±0.033	0.60 ±0.051	0.74 ±0.051	1.23 ±0.075
TMB3718 (*TPS2p*)	1.00 ±0.017	1.11 ±0.022	1.72 ±0.023	2.06 ±0.18	0.81 ±0.018	0.70 ±0.024	0.85 ±0.026	1.61 ±0.084
TMB3719 (*TEF4p*)	0.95 ±0.025	1.01 ±0.043	1.20 ±0.025	1.25 ±0.010	1.24 ±0.018	1.44 ±0.010	1.35 ±0.013	1.46 ±0.010
Strain	**Glycerol 3% (v/v)**		**Xylose 50 g/L**		**Xylose 50 g/L + Gluc. 5 g/L**		**Xyl. 50 g/L + Glyc. 3% (v/v)**	
	3h	**6h**	**3h**	**6h**	**3h**	**6h**	**3h**	**6h**
TMB3711 (No GFP)	0.82 ±0.024	0.79 ±0.017	0.79 ±0.017	0.78 ±0.023	1.04 ±0.010	1.02 ±0.041	0.76 ±0.014	0.72 ±0.013
TMB3712 (*HXT1p*)	0.75 ±0.013	0.79 ±0.051	0.83 ±0.015	0.84 ±0.010	1.48 ±0.011	1.70 ±0.011	1.22 ±0.097	1.14 ±0.064

TMB3713 (*HXT2p*)	1.09 ±0.037	1.28 ±0.064	0.89 ±0.039	0.89 ±0.046	1.37 ±0.26	2.01 ±0.55	0.89 ±0.012	0.91 ±0.009
TMB3714 (*HXT4p*)	0.98 ±0.087	1.06 ±0.16	1.08 ±0.25	1.31 ±0.51	2.15 ±0.31	3.45 ±0.62	0.96 ±0.14	1.09 ±0.33
TMB3715 (*SUC2p*)	0.98 ±0.20	1.20 ±0.40	0.95 ±0.12	1.06 ±0.21	1.13 ±0.10	5.15 ±0.62	0.91 ±0.17	1.02 ±0.27
TMB3716 (*CAT8p*)	0.89 ±0.027	1.09 ±0.049	0.82 ±0.003	0.84 ±0.018	1.08 ±0.037	1.13 ±0.037	0.80 ±0.004	0.78 ±0.009
TMB3717 (*TPS1p*)	0.92 ±0.006	0.91 ±0.029	0.92 ± 0.006	0.91 ±0.029	0.70 ±0.048	0.77 ±0.047	0.91 ±0.007	0.91 ±0.026
TMB3718 (*TPS2p*)	0.93 ±0.020	1.03 ±0.029	0.85 ±0.012	0.85 ±0.026	0.95 ±0.076	1.05 ±0.032	0.84 ±0.012	0.82 ±0.025
TMB3719 (*TEF4p*)	0.97 ±0.015	1.07 ±0.040	0.87 ±0.012	0.96 ±0.010	1.28 ±0.018	1.42± 0.0032	0.87 ±0.010	0.95 ±0.0027

3.3. Biosensor Characterization

Throughout this study we have characterized 8 different biosensor strains and confirmed their employability for the Snf3/Rgt2, Snf1/AMPK and RAS-cAMP-PKA signaling pathways. In addition, we have showed that these synthetic constructs do not alter any physiological trait of *S. cerevisiae.* In fact, no significant difference was observed when comparing growth pattern, metabolite profile, glucose uptake, transcription profile and enzyme activity between the biosensor strains and the control strain (TMB3711). As a result of the successful validation, these biosensors were applied in high-throughput screenings and valuable data was acquired.

For this study, a single-copy integration approach by homologous recombination was chosen to guarantee the integration of only one copy of the biosensor cassette and its stability over time. Other studies (Shibasaki *et al*, 2009) have shown that it is possible to have multiple copies of the biosensor cassettes if episomal plasmids are used or by anomalies in a single cross over integrations that could affect the accuracy of the fluorescent signal through the experiments and therefore affecting their reproducibility and reliability. In fact, previous findings have proven the variability in signal strength by having episomal biosensor constructs. However, when these constructs were integrated the signal variability decreased dramatically (Knudsen *et al*, 2014).

Even though most of the biosensors show a very distinct pattern in induction and repression, strains TMB3714 and TMB3716 exhibit a very mild induction (Figs. 2 and 3). A recent study suggests that by having a triple GFP variant fusion instead of the yEGFP could result in higher resolution for weak promoters (Rugbjerg *et al*,

2015). In this sense, a better resolution in induction pattern could be achieved for these “weak” biosensor cassettes and thus give new insights about their behavior.

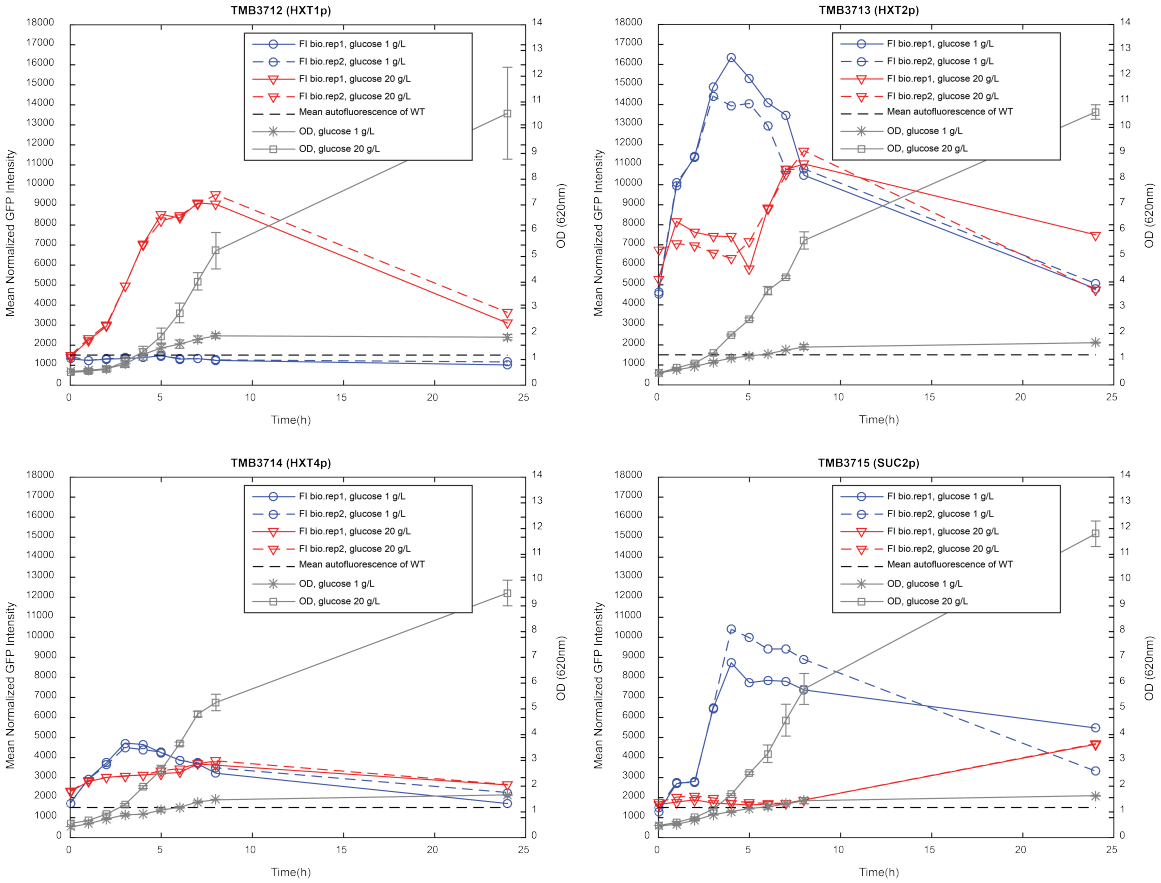

Figure 2. Shake flask cultivations of TMB3712-3715. Gray lines show averaged OD values for each condition. Blue lines show biological replicates for 1g/l glucose. Red lines show biological replicates for 20g/l glucose. Black dashed line shows autofluorescence threshold.

It is a well-established fact that the maturation of GFP is crucial for fluorescent intensity; this process can be quick in bacterial models (Hebisch *et al.*, 2013), however in yeast, maturation lasts approximately 1 hour (Gordon *et al*, 2007). This feature of GFP can easily be noticed in our batch cultivations experiments, which show a slight increase in fluorescence in the first hour, but a drastically higher increase in the second to third hour, reaching the peak in cumulative fluorescence intensity around the 4 and 5 hour (Figs. 2 and 3). On the other hand, after the peak in fluorescence is reached a slow trend of reduction in fluorescence is distinguished, due to the long half-life of yEGFP around 7.5 hours (Mateus & Avery, 2000) and possibly the dilution effect of successive generations of yeast. Therefore, our sensors allow a higher resolution for induction patterns than for repression. Even though a possible solution could be to employ CLN-PEST tagged yEGFP3, which reduces the half-life of GFP to approximately 34 minutes (Mateus & Avery, 2000), the

dependence on ATP makes it inapplicable to our study due to the fact that dependence on ATP could affect physiological traits in yeast, making it an intrusive construct for the study of signaling pathways.

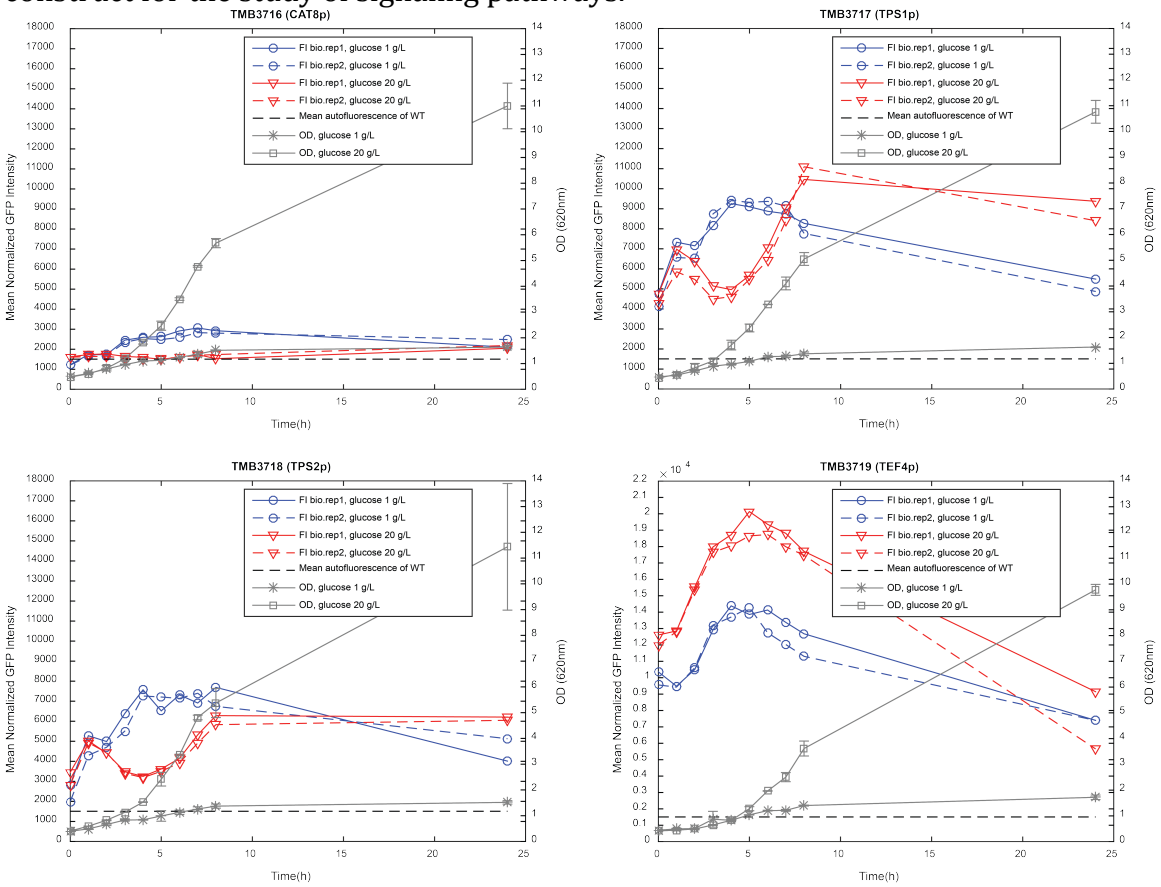

Figure 3. Shake flask cultivations of TMB3716-3719. Gray lines show averaged OD values for each condition. Blue lines show biological replicates for 1g/l glucose. Red lines show biological replicates for 20g/l glucose. Black dashed line shows autofluorescence threshold.

3.4. Validation Experiments

Validation experiments were performed to certify that the biosensor cassettes did not affect normal cellular fitness. A difference in transcription was observed for the biosensor cassette in comparison with endogenous gene under control of the same promoter (Figs. 4 and 5). It is important to understand that the biosensor cassette was integrated in a different locus than the endogenous SUC2. There are three possible explanations to explain this difference, the first is due to the locus in which the cassette was integrated and the difference in strength of expression between the different loci, adding to this hypothesis, a study showed the difference in transcription strength between different chromosomal regions using *LacZ* as a reporter gene (Flagfeldt *et al*, 2009). The second reason involves regulatory motifs in the promoter region that might have been lost when cloning the promoter, as

only 1 kb upstream the open reading frame (ORF) was cloned, excluding possible enhancers. The last scenario could be a combination of the last two explanations.

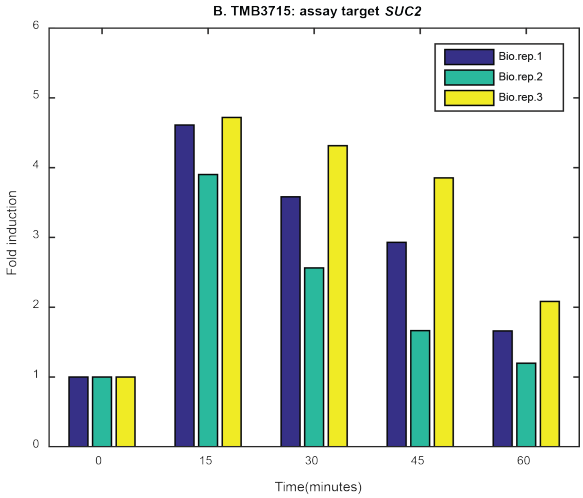

Figure 4. SUC2 transcription profile in TMB3715. Colors represent different biological replicates.

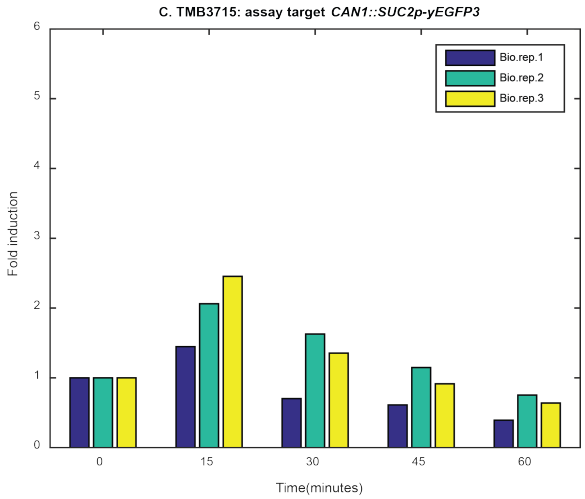

Figure 5. yEGFP transcription profile in TMB3715. Colors represent different biological replicates.

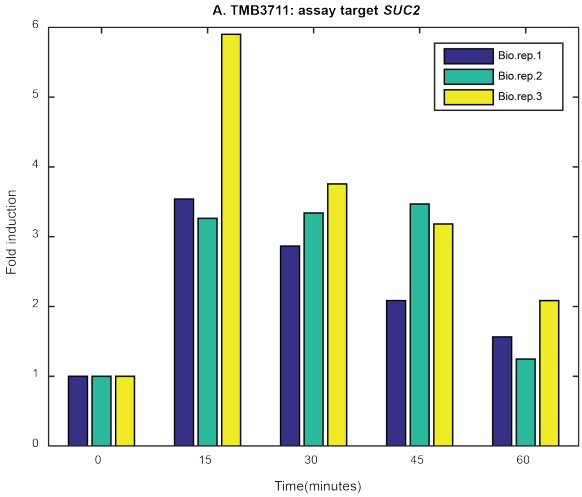

Figure 6. SUC2 transcription profile in TMB3711. Colors represent different biological replicates.

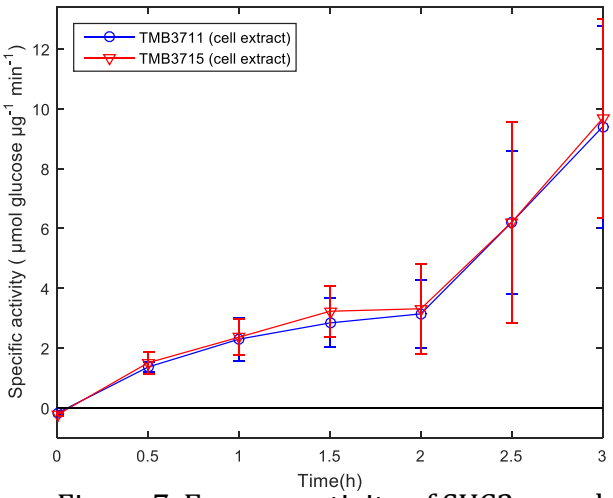

Figure 7. Enzyme activity of SUC2 encoded invertase in TMB3711 and TMB3715.

3.5. The Investigated Signaling Pathways

Overall, the biosensors have responded as expected according to previous studies of these gene targets (Table 2). In the case of the Snf3/ Rgt2 pathway, HXT1 biosensor strain TMB3712 showed a clear induction pattern on 20 g/L glucose (Fig. 2). Even though this strain had been grown on Ethanol 3% as repressing conditions it did not affect the biosensor capacity despite the respiratory pre-culture. However, a slight difference was observed in the growth and metabolites profiles (measured by OD and HPLC), showing a minor increase in the lag phase and minor decrease in the glucose uptake (Figs. 3, 9 and 10), possibly due to the effect of its metabolic machinery adapted to non fermentable carbon sources. The TMB3713 and TMB3714 biosensor strains for HXT2 and HXT4 respectively showed similar trends of induction (on glucose 1 g/L), as both of them encode high affinity hexose transporters.

TMB3715 showed to be the best biosensor for monitoring of the glucose repression pathway (Sng1/Mig1), as clear induction and repression patterns were achieved for low and high glucose concentrations (Fig.2). An interesting event was observed during the first and second hour, as a plateau in the cumulative FI signal induction was detected (Fig 2), which was shortly followed by resumed induction with the fluorescence intensity drastically increasing. This behavior has been previously reported for the SUC2 gene (transcriptomics data) in yeast growing on sucrose and was catalogued as a biphasic induction (Geng and Laurent 2004, Weiß, Huppert et al. 2008). Having caught this event with the biosensor shows how precise and reliable the results are. On the other hand, TMB3716, the biosensor for CAT8 (another gene induced by the glucose repression pathway) did not show such dramatic patterns, and therefore TMB3715 is a better choice for evaluating the activity of this pathway.

Both biosensor strains for the RAS-cAMP-PKA pathway, TMB3717 and TMB3718 showed similar unique patterns of induction and repression. Both conditions tested for both strains showed an increase of fluorescent intensity in the first couple of hours, but from the third hour and on the different conditions showed completely opposite behaviors (Fig. 3). As both of these genes encode enzymes for the biosynthesis of trehalose, and knowing the role of TPS1 in controlling the influx of glucose for initiating the glycolysis (van Heerden *et al.*, 2014), induction in the first hours is understandable. The induction will remain under low levels of glucose, as the cell does not sense more fermentable carbon source, leading totrehalose being produced as an energy reservoir (Petitjean *et al.*, 2015). However, when exposed to high concentrations of glucose (20 g/l) a repression pattern is observed until glucose concentrations in the media start to decrease dramatically around 8 hours.

3.6. Xylose Response

The main objective of evaluating the effect of xylose in the Snf3/Rgt2, Glucose repression and RAS-cAMP-PKA pathways was to unveil any influence that this pentose had in normal fermentable carbon sensing, signaling and metabolism. Previous findings suggest that *S. cerevisiae* responds to a lack of fermentable carbon sources rather than presence of xylose (Matsushika *et al*, 2014), presumably due to the fact that it lacks the adequate sensing machinery. Therefore, xylose induces glucose repression in the cell affecting the glycolytic pathway or central carbon metabolism essential for further processing of fructose-5-phosphate, the same one which is formed after xylose has been up taken by engineered transporters and converted by the pentose phosphate pathway. The microtiter plate assay showed no response to xylose by the genes involved in these signaling pathways from most of the biosensor strains. TMB3714 showed a slight increase in induction, however this induction was less than a 2-fold value, and was therefore not being considered as a clear induction pattern (Table 3).

Intracellular xylose has been shown to inactivate Hexokinase 2 by an irreversible autophosporylation, affecting its enzymatic activity and its role to repress genes involved in alternate carbon metabolism (Belinchón & Gancedo, 2003; Roca *et al*, 2004; Bergdahl *et al*, 2013). Supporting these findings, we see that the co-condition of 5 g/L glucose and 50 g/L xylose induces the glucose repression pathway in strain TMB3715, although fold change does not differ much from the 5 g/L glucose condition (Table 3). Even though no engineered transporter has been used in this project, we speculate that transient levels of xylose could be localizing inside the cell due to reported promiscuity of the hexose transporters (Goncalves *et al*, 2014). In spite of all these facts, there seems to be a changing effect of intracellular xylose depending on its concentration. It is important to state that the cultivations performed and strains used by the authors cited in this paragraph deviate from the ones used in this study, as we did not investigate the effects of the conditions used in any recombinant xylose fermenting strain.

High xylose concentration (50 g/L) is commonly used by researchers to facilitate the uptake by pentose metabolizing yeast strains (Karhumaa *et al*, 2006). As a result of the lack of response from the biosensors to the xylose condition we performed another screening to assess whether the concentrations of xylose was affecting the signal. This time, the biosensor strains were exposed to different concentrations of xylose (25 g/L, 50 g/L, 75 g/L and 100 g/L) and an extended sampling time (9 hours) to rule out any lag in the fluorescent intensity response. However, no induction was recorded for any of the concentrations. Therefore, it can clearly be stated that there is a lack of response of these signaling pathways to extracellular xylose in these strains.

4. Conclusions and Outlook

Through the results of this study we have demonstrated that these biosensor strains are capable of producing reliable fluorescent signals that correspond to the activity of their endogenous genes. Furthermore, it has been proven by HPLC, relative expression analysis and enzyme activity that these biosensor cassettes do not hinder the fitness or any physiological trait of yeast, being in this sense non invasive nor intrusive. Moreover, the implementation of this technology in a high throughput methodology represents a milestone for real time assessment of signaling pathways against a wide variety of conditions in an accurate manner.

In addition, this study has proven for the first time that presence of extracellular xylose does not induce any of the signaling pathways studied in this project. However, further experiments are needed in order to uncover possible intracellular effects of xylose or xylose metabolism. Therefore it is of great interest to further manipulate these biosensor strains to add xylose uptake mechanisms and the appropriate metabolic pathways to further characterize the effect of this pentose on the *S. cerevisiae* signaling. This will potentially increase the identification rate of new targets for metabolic engineering and the future biotechnological application of yeast in lignocellulosic biorefineries.

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Appendix

Table A1. Genotype and Phenotype of the strains used in this study

Strain	Relevant Genotype	Phenotype
TMB3711	Can1::Ylplac211, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-
TMB3712	Can1::Ylpgfp-HXT1p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+
TMB3713	Can1::Ylpgfp-HXT2p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+
TMB3714	Can1::Ylpgfp-HXT4p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+
TMB3715	Can1::Ylpgfp-SUC2p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+
TMB3716	Can1::Ylpgfp-Cat8p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+
TMB3717	Can1::Ylpgfp-Tps1p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+
TMB3718	Can1::Ylpgfp-Tps2p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+
TMB3719	Can1::Ylpgfp-Tef4p, SPB1/PBN1::YLPLAC128	Trp+,his+,ade+,ura+,leu+,can-,GFP+

Table A2. Primers used for RT-qPCR

Name	**Description**	**Sequence**	**Reference**
ACT1_F	RT-qPCR reference gene	TGGATTCCGGTGATGGTGTT	Ismail, Sakamoto et al. (2013)
ACT1_R	‘’	TCAAAATGGCGTGAGGTAGAGA	Ismail, Sakamoto et al. (2013)
yEGFP3_F1790_RT	RT-qPCR assay of the yEGFP3 gene	TGGTGATGGTCCAGTCTTGTT	This study
yEGFP3_R1918_RT	‘’	TGGGTAATACCAGCAGCAGT	This study
SUC2_F263_RT	RT-qPCR assay of the SUC2 gene	AACCCATTGCTATCGCTCCC	This study
SUC2_R397_RT	‘’	AAGTCCAAATCGCAACGCAT	This study

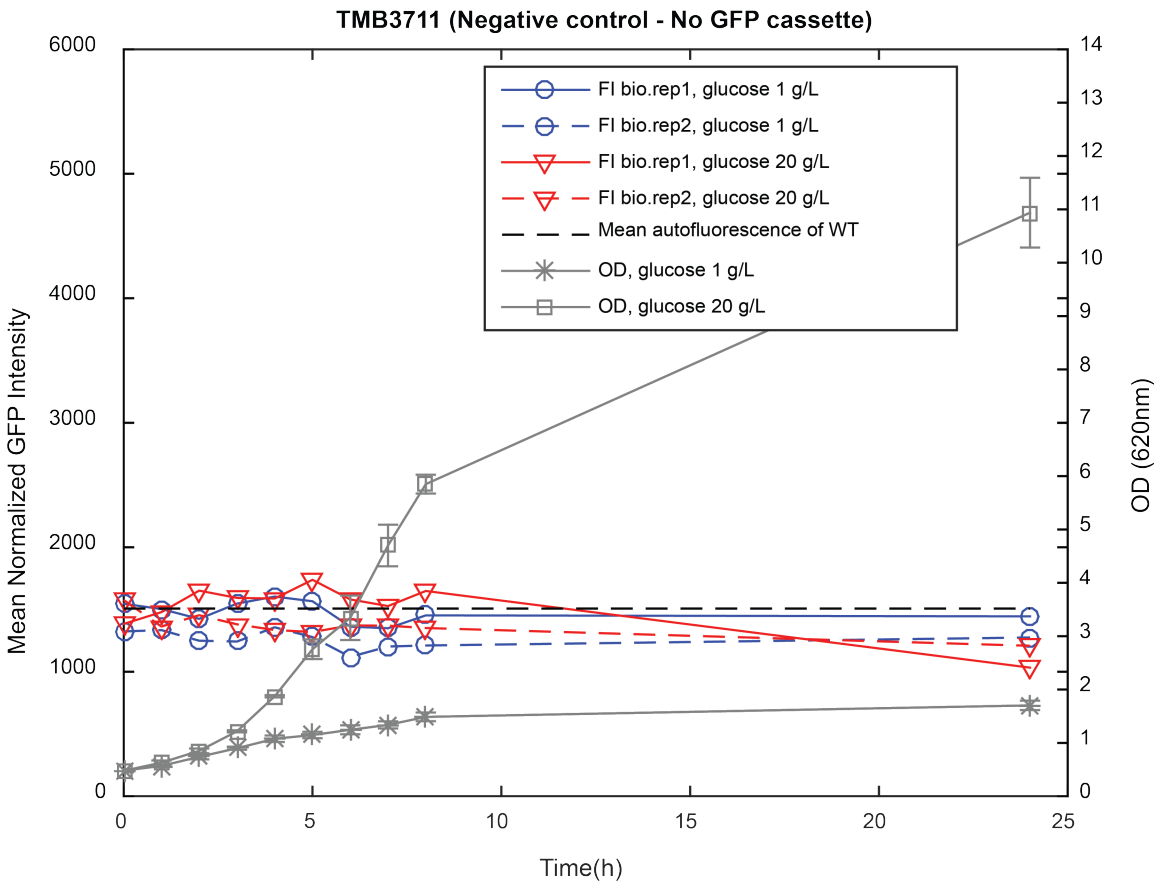

Figure A1. Shake flask cultivation of TMB3711 (no GFP cassette). Gray lines show averaged OD values for each condition. Blue lines show biological replicates for 1g/l glucose. Red lines show biological replicates for 20g/l glucose. Black dashed line shows the calculated aurofluoprescence of this strains on the GFP channel, derived from the time-independent mean of the FI-signal for this strain (TMB3711).

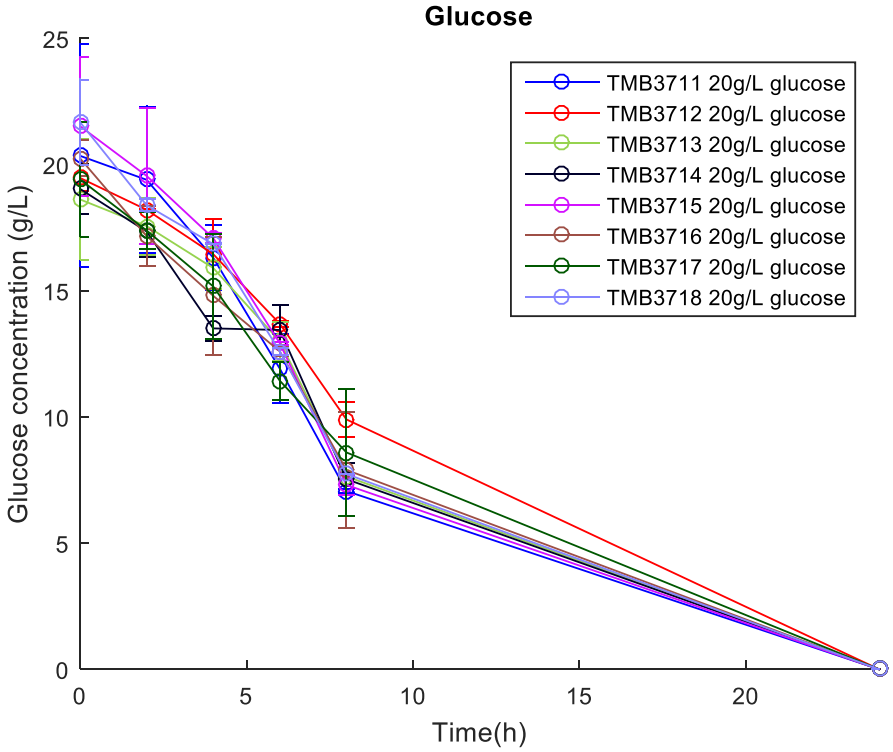

Figure A2. HPLC results for glucose uptake in the shake flask cultivations

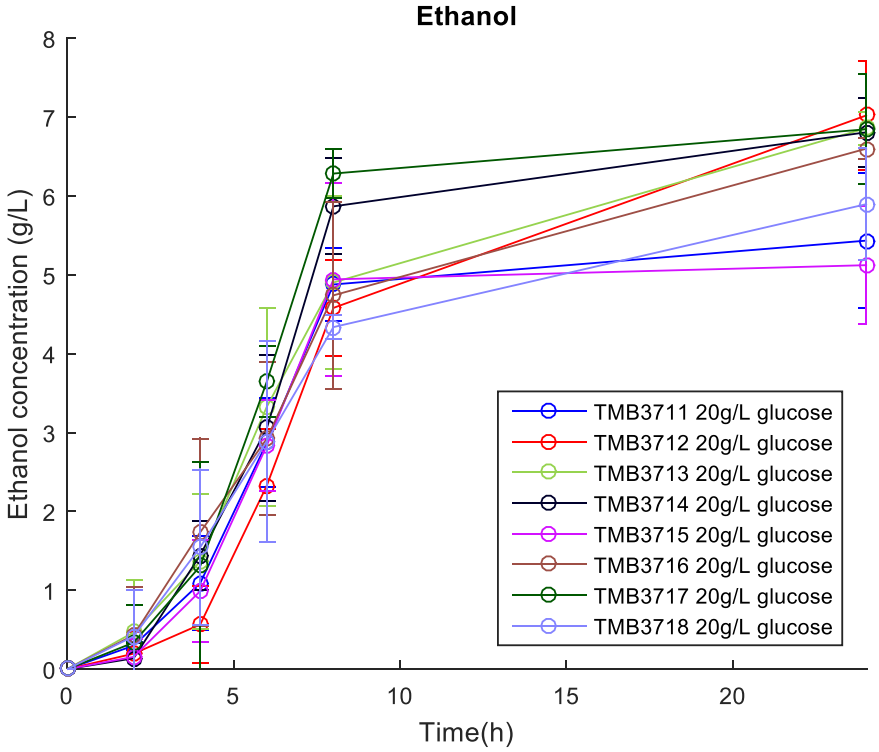

Figure A3. HPLC results for ethanol production in the shake flask cultivations

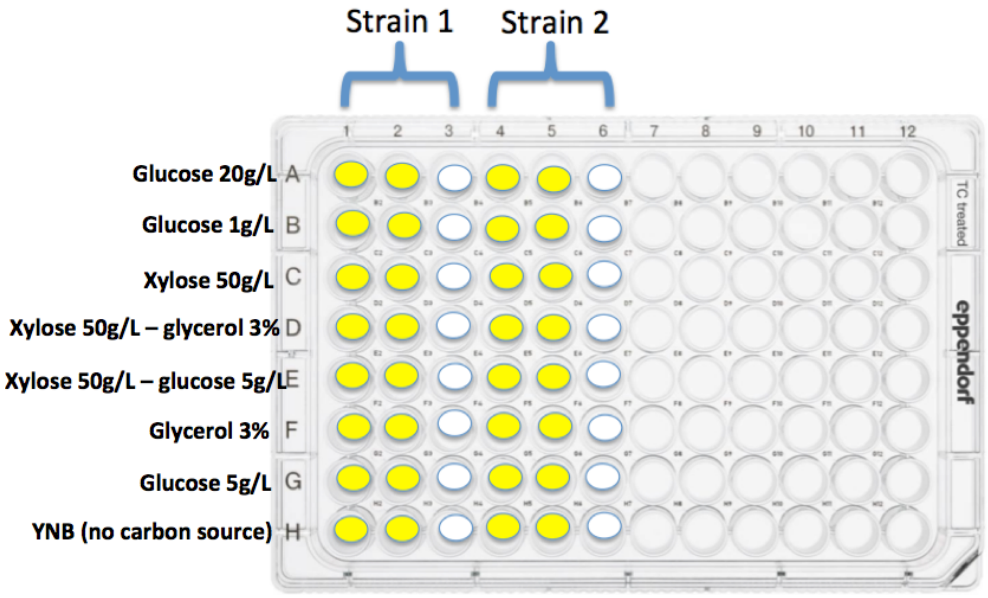

Figure A4. Schematic representation of the microtiter plate layout