The *in vivo* detection and measurement of the unfolded protein response in recombinant cellulase producing *Saccharomyces cerevisiae* strains

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Abstract

The yeast *Saccharomyces cerevisiae* possesses industrially desirable traits for ethanol production and has been engineered for consolidated bioprocessing (CBP) of lignocellulosic biomass through heterologous cellulase expression. However, *S. cerevisiae* produces low titers of cellulases and one suspected reason for this is that heterologous proteins induce the unfolded protein response (UPR). Current methods of measuring the UPR are RNA based and can be inconsistent and cumbersome. We developed vector-based biosensors that will detect and quantify UPR activation. The vector consisted of either the *Trichoderma reesei* xylanase 2 or codon optimized green fluorescent protein (eGFP) reporter genes under the control of the *S. cerevisiae* P_{HAC1} or P_{KAR2} promoters. The eGFP reporter under control of P_{KAR2} was identified as the preferred combination due to its superior dynamic range and its greater sensitivity when measuring UPR induction in cellulase producing strains. To our knowledge, we show for the first time that significant UPR activation differences could consistently be observed for different cellulase candidate genes unlike previous RNA-based tests, which were unable to detect these differences. The ability to quantify UPR induction will assist in identifying candidate cellulase genes that do not greatly induce the UPR, making them favorable for use in CBP yeasts. Volume 67, Number 1, Pages 82–94, 2020

Keywords: *cellulases, consolidated bioprocessing,* Saccharomyces cerevisiae*, unfolded protein response, UPR biosensor*

1. Introduction

Second-generation bioethanol production using lignocellulosic biomass as feedstock has great potential to displace currently utilized petroleum-based liquid fuels [1]. However, the complex structure of biomass necessitates expensive pretreatment prior to hydrolysis and is therefore one of the technical barriers that needs to be overcome [2–4]. Consolidated bioprocessing (CBP) is one of the more cost-effective configurations of producing bioethanol from lignocellulosic biomass in a second-generation process, but no ideal natural microorganism exists that is fit for this purpose [5]. The yeast *Saccharomyces cerevisiae* has many desirable traits for CBP such as high ethanol productivity, high ethanol yield, and comparatively high resistance to inhibitors found in the lignocellulosic hydrolysate [6, 7]. *S. cerevisiae* is also an extensively studied host for heterologous protein production and has thus been the subject of much research to engineer and enhance the expression of necessary cellulases to accomplish the ultimate goal of being able to break down lignocellulose for second-generation production of bioethanol

Abbreviations: *BGL, β-glucosidase; CBH, cellobiohydrolase (exoglucanase); CBP, consolidated bioprocessing; DCW, dry cell weight; EG, endoglucanase; eGFP, codon optimized green fluorescent protein; ER, endoplasmic reticulum; MFI, mean fluorescence intensities; OD, optical density; qPCR, quantitative PCR; RT-qPCR, reverse transcriptase quantitative PCR; U, enzyme unit; UPR, unfolded protein response; UPRE, unfolded protein responsive element; YPD, yeast extract, peptone, glucose.*

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[7–11]. Despite significant success achieved in cellulase expression in yeast, the secretion titers of cellobiohydrolases (CBHs) have been relatively low, and one of the reasons for this phenomenon is the induction of the unfolded protein response (UPR) [4, 12, 13].

Heterologous protein secretion is known to cause the accumulation of unfolded proteins in the endoplasmic reticulum (ER), inducing the UPR with the severity of this response varying with different heterologous genes [12]. The UPR can be described as a highly conserved signaling pathway that is capable of altering gene expression and protein translation to assist with correct folding of proteins during ER stress [13]. The UPR is thus able to signal the nucleus and cytosol information about the protein folding state in the ER lumen, which then buffer the changes caused by the unfolded protein load [15, 16]. When ER stress is irreversible, the UPR will remove the damaged cells through apoptosis. The responsibility of the UPR is thus to re-establish homeostasis in the ER. ER stress caused by accumulation of unfolded proteins is sensed by UPR regulators, Ire1p and Kar2p, which use the transcription factor Hac1p to activate genes with promoters containing unfolded protein response elements (UPREs). As the *HAC1* gene contains an UPRE on its promoter, it is able to upregulate its own transcription. Hac1p is capable of upregulating over 400 genes and cause the secretory pathway to be remodeled in a way that increases the capacity of the ER, resulting in decreased misfolding and increased membrane synthesis to accommodate increased ER function [17]. The UPR generally starts with the dissociation of the Kar2p from the Ire1p complex, activating Ire1p, which in turn results in the splicing of *HAC1* mRNA that encodes Hac1p. Hac1p and Kar2p are thus important regulators of UPR as their activation allows the UPR to achieve its function. The interconnectedness of the UPR with other protein quality control and degradation systems, and its direct impact on the flux of protein through the secretion pathway has led to significant amounts of research focused on manipulating genes involved in the UPR with the aim of enhancing protein secretion [13, 18–21].

It is important to know when the UPR is induced by heterologous protein expression in order to identify gene candidates for strain engineering that will allow for improved secretion of heterologous proteins due to causing lower ER stress. ER stress sensors and UPR detection methods that have been developed and applied in the past were based on the Ire1p-mediated unconventional splicing of *HAC1* mRNA introns and makes use of Northern analysis or reverse transcriptase quantitative PCR (RT-qPCR) [16, 22]. These methods can prove to be cumbersome and expensive, and thus more recently fluorescence reporter constructs and enzyme-based reporter systems have been reported [23–25]. These methods are more sensitive than RT-qPCR and allows for continuous and live detections of the UPR. Due to the simplicity of a biosensor reporter system, it is ideal to use this approach for UPR detection in yeast. However, previous biosensors were often based on chromosomal integration, which may be problematic to use in diploid, or polyploid strains as copy number variance may ensue. Biosensors based on 2μ-plasmids also have copy number issues as strains experiencing secretion stress will downregulate 2μ-plasmid copy numbers [12]. The centromeric-based plasmids that we describe here provide a more stable option for biosensors in a variety of natural and industrial strain isolates and are amenable to high-throughput transformation approaches. To our knowledge, no biosensor to detect UPR in heterologous cellulase producing strains has previously been described.

The aim of this study was to develop a UPR biosensor for the *in vivo* detection and measurement of the UPR in *S. cerevisiae* independent of RNA-based methods. We compared the putative UPR biosensors based on P_{HAC1} and P_{KAR2}, promoter regions of two key regulators and sensors of the UPR. It will be advantageous to know the level to which heterologous cellulases induce the UPR in *S. cerevisiae* as this can help identify heterologous gene candidates more suitable to construct yeast strains for CBP.

2. Materials and Methods

2.1. Media and culturing conditions

The *S. cerevisiae* strain Y294 (ATCC201160) was the background strain for all strain derivatives used in this study. All yeast strains were cultured at 30 °C on yeast extract, peptone, glucose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose), supplemented with 100 μg/mL of the appropriate antibiotic (Geneticin (G418) or Hygromycin B), purchased from Sigma (Darmstadt, Germany) when required for selection of transformants. Y294 transformants were screened for xylanase activity on AZCL-xylan SC^{-URA} medium plates (1.7 g/L yeast nitrogen base w/o amino acids and ammonium sulfate (Difco, Detroit, USA), 5 g/L ammonium sulfate, 20 g/L glucose, 15 g/L agar, 1 g/L AZCL-xylan (Sigma), and supplemented with required amino acids) and incubated for 24 H at 30 °C. For activity assays and eGFP fluorescence measurements, Y294 transformants were cultured in 10 mL YPD medium (10 g/L yeast extract, 20 g/L peptone, and supplemented with 20 g/L glucose) in 100 mL flasks containing 100 μg/mL of the appropriate antibiotic for 120 H at 30 °C while shaking at 180 rpm on a rotary shaker.

2.2. Microbial strains and plasmids

Descriptions of the plasmids and yeast strains that were constructed and used in this study are summarized in Tables 1 and 2, respectively. The *S.c.*yENO1, *S.c.*BGL1, *S.c.T.r.*cbh1, *S.c.T.e.*cbh1, and *S.c.T.e.*cbh1-CCBM strains were obtained from Ilmén et al. [12] and Den Haan et al. [26, 27]. All other plasmids and strains listed in the Tables 1 and 2 were constructed in this study.

2.3. *Escherichia coli* and yeast transformation

E. coli XL Gold was used for plasmid propagation and was cultivated in terrific broth containing 100 μg/mL ampicillin at 37 °C overnight. Plasmid DNA was extracted using the cetyl trimethyl ammonium bromide method (Del Sal et al. [29]) and

TABLE 1 *Plasmids used in this study*

Plasmids	*Description*	*Source*
pRDH182	P_{ENO1}*-*Trichoderma reesei xyn2 (xyn11A) (native)-*T_{ENO1}, *Amp*	[28]
pHK211	P_{PGK1}-*eGFP*-T_{CYC1} *kanMX*	H. Kroukamp
pHK212	P_{PGK1}-*eGFP*-T_{CYC1} *hph*MX	H. Kroukamp
pGC1 (pHK211-P_{HAC1}-*xyn2*)	P_{HAC1}-*xyn2*-T_{CYC1}*kanMX*	This study
pGC2 (pHK211-P_{KAR2}-*xyn2*)	P_{KAR2}-*xyn2*-T_{CYC1}*kanMX*	This study
pGC3 (pHK212-P_{HAC1}-*xyn2*)	P_{HAC1}-*xyn2*-T_{CYC1}*hph*MX	This study
pGC4 (pHK212-P_{KAR2}-*xyn2*)	P_{KAR2}-*xyn2*-T_{CYC1}*hph*MX	This study
pGC5 (pHK211-P_{HAC1}-*eGFP*)	P_{HAC1}-*eGFP*-T_{CYC1}*kanMX*	This study
pGC6 (pHK211-P_{KAR2}-*eGFP*)	P_{KAR2}-*eGFP*-T_{CYC1}*kanMX*	This study

*P denotes promoter and T denotes terminator.

plasmids or ligation mixtures were added to *E. coli* competent cells before incubation on ice for 30 min. The cells were heat shocked at 42 °C for 20 sec and spread plated on Luria Bertani medium plates (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, and 20 g/L agar) containing 100 μg/mL ampicillin.

Yeast genomic DNA was isolated using the quick yeast mini prep method [30]. Yeast transformations were carried out using the LiOAc/DMSO protocol [31]. Following an overnight expression step in YPD at 30 °C, transformants were plated on YPD medium plates containing the appropriate antibiotic. Total genomic DNA of the yeast transformants was extracted [30] and the successful transformation of the yeasts with the plasmids was confirmed with PCR analysis using primers listed in Table 3 with New England Biolabs (Ipswich, USA) Taq polymerase as recommended by the manufacturer.

2.4. UPR biosensor construction

DNA manipulations were performed using standard protocols [32]. Restriction endonuclease-digested DNA was eluted from 1% agarose gels with the freeze and squeeze method [32]. Restriction endonucleases and T4 DNA ligase were purchased from ThermoScientific (Waltham, MA, USA) and used as recommended by manufacturer. The Phusion DNA polymerase that was used for PCR was also purchased from ThermoScientific and used as recommended by manufacturer with an Applied Biosystems 2720 Thermal cycler. The primers used in this study are listed in Table 3. The CloneJET PCR Cloning Kit that was used for initial cloning of putative promoters was purchased from ThermoFisher and used as recommended by the manufacturer.

The UPR biosensors that were developed in this study consisted of the putative promoters of the *HAC1* and *KAR2* UPR genes as predicted by Yeastract software (www.yeastract.com). The respective putative UPR promoters were amplified from extracted genomic DNA of the *S. cerevisiae* 288C strain by PCR using primers P_{HAC1}-L and P_{HAC1}-R, or P_{KAR2}-L and P_{KAR2}-R. The endonuclease restriction sites for *Eco*RI (GAATTC) and *Pac*I (TTAATTAA) were included at the 5′-ends of the left and right primers, respectively. The putative UPR promoter fragments (P_{HAC1} ~600 base pair [bp] and P_{KAR2} ~800 bp) were eluted from a 1% agarose gel and cloned into a pJET1.2 cloning vector using the CloneJET PCR Cloning Kit. The ligation mixture was then transformed into *E. coli* XL Gold heat shock competent cells. The promoters were removed from the pJET1.2 cloning vector, after confirming that the ligation was successful, using the *Eco*RI and *Pac*I restriction endonucleases and cloned into the *Eco*RI and *Pac*I sites of the pHK211, to create plasmids pGC5 and pGC6 (Table 1). The resulting plasmids contain a codon optimized green fluorescent protein (*eGFP*) reporter gene downstream of the putative UPR promoters (Fig. 1A).

To create the UPR biosensors with the *Trichoderma reesei* Xyn2 (*T.r.xyn2*) reporter gene, pRDH182 plasmid DNA was extracted and *T.r.xyn2* was amplified using PCR with primers XYN2-L and XYN2-R (Table 3). The endonuclease restriction sites for *Pac*I (TTAATTAA) and *Asc*I (GGCGCGCC) were included at the 5'-ends of the left and right primers, respectively. The ~700 bp *T.r.xyn2* amplicon was eluted after agarose gel electrophoresis and ligated into the *Pac*I and *Asc*I sites of the pHK211 and pHK212 plasmids downstream of the putative UPR promoters, replacing the *eGFP* reporter genes with *T.r.xyn2*. Subsequently, the promoters in those plasmids were replaced with *Eco*RI/*Pac*I fragments of *Hac1*p or *Kar2*p in order to create plasmids pGC1, pGC2, pGC3, and pGC4 (Fig. 1A). These reporter genes will allow for the direct measurement of the promoter activation in response to UPR stress, through increased fluorescence or xylanase secretion. Successful plasmid

TABLE 2 **S. cerevisiae *strains used in this study***

Strain name	*Abbreviation used*	*Description*
S. cerevisiae Y294 yENO1	*S.c.*yENO1	*S. cer. fur1::LEU2 with P_{ENO1}-T_{ENO1} (Reference strain)*
S. cerevisiae Y294 BGL1	*S.c.*BGL1	*S. cer. fur1::LEU2 with P_{PGK1}-Saccharomycopsis fibuligera bgl1 (cel3A)-$PGK1_T$*
S. cerevisiae Y294 *T.r.*cbh1	*S.c.T.r.*cbh1	*S. cer. fur1::LEU2 with P_{ENO1}-Trichoderma reesei cbh1 (T.r.cbh1)-$ENO1_T$*
S. cerevisiae Y294 *T.e.*cbh1	*S.c.T.e.*cbh1	*S. cer. fur1::LEU2 with P_{ENO1}-Talaromyces emersonii cbh1 (T.e.cbh1)-$ENO1_T$*
S. cerevisiae Y294 *T.e.*cbh1-CCBM	*S.c.T.e.*cbh1-CCBM	*S. cer. fur1::LEU2 with P_{ENO1}-Talaromyces emersonii cbh1-CCBM (T.e.cbh1-*CCBM*)-$ENO1_T$*
S. cerevisiae Y294 yENO1 with pGC1	*S.c.*yENO1 pGC1	*S. cer. fur1::LEU2 with P_{ENO1}-T_{ENO1} with pGC1*[*]
S. cerevisiae Y294 yENO1 with pGC2	*S.c.*yENO1 pGC2	*S. cer. fur1::LEU2 with P_{ENO1}-T_{ENO1} with pGC2*
S. cerevisiae Y294 yENO1 with pGC3	*S.c.*yENO1 pGC3	*S. cer. fur1::LEU2 with P_{ENO1}-T_{ENO1} with pGC3*
S. cerevisiae Y294 yENO1 with pGC4	*S.c.*yENO1 pGC4	*S. cer. fur1::LEU2 with P_{ENO1}-T_{ENO1} with pGC4*
S. cerevisiae Y294 yENO1 with pGC5	*S.c.*yENO1 pGC5	*S. cer. fur1::LEU2 with P_{ENO1}-T_{ENO1} with pGC5*
S. cerevisiae Y294 yENO1 with pGC6	*S.c.*yENO1 pGC6	*S. cer. fur1::LEU2 with P_{ENO1}-T_{ENO1} with pGC6*
S. cerevisiae Y294 BGL1 with pGC2	*S.c.*BGL1 pGC2	*S. cer. fur1::LEU2 with P_{PGK1}-S.f.bgl1 (cel3A)-P_{PGK1} with pGC2*
S. cerevisiae Y294 BGL1 with pGC6	*S.c.*BGL1 pGC6	*S. cer. fur1::LEU2 with P_{PGK1}-S.f.bgl1 (cel3A)-P_{PGK1} with pGC6*
S. cerevisiae Y294 *T.r.*cbh1 with pGC2	*S.c.T.r.*cbh1 pGC2	*S. cer. fur1::LEU2 with P_{ENO1}-T.r.cbh1 (T.r.cbh1)-T_{ENO1} with pGC2*
S. cerevisiae Y294 *T.r.*cbh1 with pGC6	*S.c.T.r.*cbh1 pGC6	*S. cer. fur1::LEU2 with P_{ENO1}-T.r.cbh1 (T.r.cbh1)-T_{ENO1} with pGC6*
S. cerevisiae Y294 *T.e.*cbh1 with pGC2	*S.c.T.e.*cbh1 pGC2	*S. cer. fur1::LEU2 with P_{ENO1}-T.e.cbh1 (T.e.cbh1)-T_{ENO1} with pGC2*
S. cerevisiae Y294 *T.e.*cbh1 with pGC6	*S.c.T.e.*cbh1 pGC6	*S. cer. fur1::LEU2 with P_{ENO1}-T.e.cbh1 (T.e.cbh1)-T_{ENO1} with pGC6*
S. cerevisiae Y294 *T.e.*cbh1-CCBM with pGC2	*S.c.T.e.*cbh1-CCBM pGC2	*S. cer. fur1::LEU2 with P_{ENO1}-T.e.cbh1-CCBM-T_{ENO1} with pGC2*
S. cerevisiae Y294 *T.e.*cbh1-CCBM with pGC4	*S.c.T.e.*cbh1-CCBM pGC4	*S. cer. fur1::LEU2 with P_{ENO1}-T.e.cbh1-CCBM-T_{ENO1} with pGC4*
S. cerevisiae Y294 *T.e.*cbh1-CCBM with pGC6	*S.c.T.e.*cbh1-CCBM pGC6	*S. cer. fur1::LEU2 with P_{ENO1}-T.e.cbh1-CCBM-T_{ENO1} with pGC6*

[*]See Table 1 for descriptions of the plasmid components.

TABLE 3

Primers used in this study

Primer	*Oligonucleotide sequence (5′-3′)*	*Restriction sites*
P_{HAC1}-L	ATGC**GAATTC**CCTCTTTCATAGCCTGAGATCCG	*Eco*RI
P_{HAC1}-R	ATGC**TTAATTAA**AGTGGCGGTTGTTGTCGTAGG	*Pac*I
P_{KAR2}-L	ATGC**GAATTC**TTACATAAAAGTATCTCAGCAGCATTATTG	*Eco*RI
P_{KAR2}-R	ATGC**TTAATTAA**GGTATGTTTGATACGCTTTTTCCC	*Pac*I
XYN2-L	GATC**TTAATTAA**AATGGTCTCCTTCACCTCC	*Pac*I
XYN2-R	GTAC**GGCGCGCC**CTCCCTTTAGCTGACGGTG	*Asc*I
eGFP-L	ATGGTTTCATTTACATCTTTATTAGCCG	
eGFP-R	TTATTTGTACAATTCATCCATACCATGG	

Restriction sites are shown in bold.

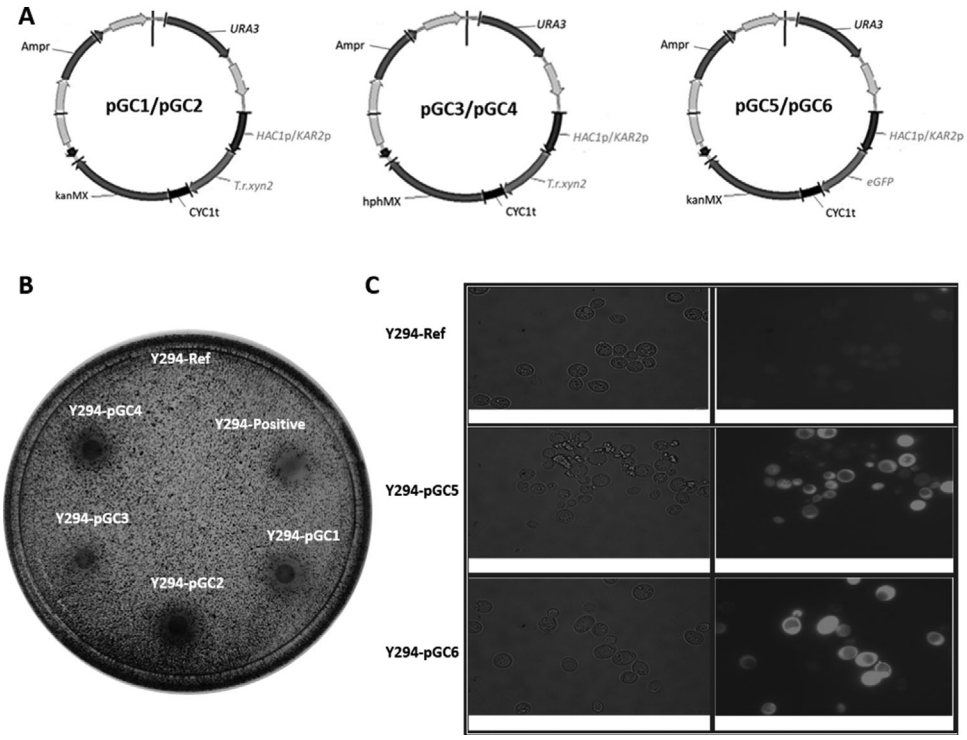

FIG. 1

A: Schematic representation of the plasmids used in this study. B: Yeast strains cultivated on 0.2% AZCL Birchwood - Xylan SC^{-URA} media plates. The plates confirm the presence of T.r.xyn2 *in the yeast transformants by the presence of a halo around the colonies. Plates were incubated at 30 °C for 24 H. C: Zeiss Axioplan 2 imaging fluorescent microscope images to show fluorescing cells. A parental Y294 control strain without a plasmid containing the eGFP gene was compared with a strain containing the pGC5 or pGC6 plasmids. Details of the plasmids and strains shown are given in Tables 1 and 2.*

construction was confirmed using restriction enzyme digestion and sequences were confirmed using Sanger sequencing (Central Analytical Facility, Stellenbosch University, Stellenbosch).

2.5. Enzyme activity assays

The *S. cerevisiae* Y294 transformants with the pGC1, pGC2, pGC3, and pGC4 UPR biosensors (Table 2) were initially screened on AZCL-xylan plates to confirm xylanase activity. These transformants were then cultured in 100 mL flasks containing 10 mL YPD growth media and 100 μg/mL of appropriate antibiotic (G418 or Hygromycin B). To evaluate tunicamycin (Tm) response, required volumes of a 1 mg/mL Tm stock was used to obtain 0.2, 0.5, and 2 μg/mL Tm final concentrations. Xylanase activity in the culture supernatant was measured using 1% (w/v) Beechwood xylan (Sigma) resuspended in 0.05 M sodium acetate buffer (pH 5) as previously described [33]. Samples were diluted as required to determine the optical density (OD_{600nm}) of the cultures using an LKB ULTROSPEC II Spectrophotometer. Dry cell weight (DCW) was estimated from OD_{600nm} readings and used to normalize the volumetric values of the yeast cultures [9]. Xylanase activities were determined as units/g DCW in which one enzyme unit (U) was defined as the amount of enzyme needed to release 1 μmol of reducing sugar equivalent per minute. All assays were performed in triplicate and measured at 48, 72, 96, and 120 H, except for the Tm test where activity was measured at 0, 6, 24, and 48 H.

2.6. Fluorescence microscopy

The *S. cerevisiae* Y294 transformants with the pGC5 and pGC6 UPR biosensors (Table 2) and the appropriate control strains were initially screened though fluorescence microscopy using a Zeiss Axioplan 2 imaging fluorescence microscope (Oberkochen, Germany) to confirm *eGFP* expression using excitation and emission of 495 and 530 nm, respectively. Fluorescence images were captured using a Zeiss AxioCam HRm camera and the AxioVision software (Zeiss) was used to create false color (Green) images.

2.7. Flow cytometry

S. cerevisiae Y294 transformants were cultured in 100 mL flasks containing 10 mL YPD growth media and 100 μg/mL G418. For Tm tests, concentrations were added in the same way as described in the enzyme activity assays above. Relative eGFP fluorescence was measured with a BD FACS Aria III flow cytometer using the 100 μM nozzle and BD sheath fluid. The machine was calibrated using CS&T beads as recommended by manufacturer (BD Biosciences, Franklin Lakes, USA). The FITC channel (488 nm) was used for measuring GFP fluorescence signals. The number of events recorded for each sample was 500,000. The software used for collecting data was FACS Diva version 8.0.2 (BD Biosciences) and files were exported as FCS 3.1. The fluorescence of all strains was measured in triplicates. Florescence intensities of the eGFP were measured at 24, 48, and 72 H, except for the Tm test in which it was recorded at 0, 24, and 48 H. The mean fluorescence intensities (MFI) were determined using Flowjo software version 10 (www.flowjo.com) and all values were normalized by dividing arbitrary fluorescence units by lowest MFI obtained for each result to determine relative fluorescence levels.

3. Results

3.1. Construction of UPR biosensors and yeast strains

The two plasmids used to create our UPR biosensors, pHK211 and pHK212, are low copy number centromeric plasmids that contain the *kanMX* (G418 resistance) or *hphMX* (hygromycin B resistance) selection markers, respectively. The plasmids contain the *eGFP* reporter under the transcriptional control of the constitutive *S. cerevisiae PGK1* promoter. The UPR biosensors were developed by removing the *PGK1* promoter and replacing it with the putative UPR promoters P_{HAC1} or P_{KAR2}, upstream of the *eGFP* reporter genes to create plasmids pGC5 and pGC6 (Fig. 1A). The pGC1, pGC2, pGC3, and pGC4 UPR biosensors consisted of the *T.r.xyn2* reporter gene cloned downstream of the putative UPR promoters, on either the *kanMX* or *hphMX* plasmid backbone (Fig. 1A and Table 1). Plasmid constructions were confirmed through restriction enzyme digests and Sanger sequencing (results not shown). The putative UPR biosensors were subsequently transformed into *S. cerevisiae* Y294 strains (Table 2). Transformants were confirmed through PCR analysis (data not shown) using putative UPR promoter and reporter gene primers (Table 3). The *S. cerevisiae* yENO1 reference strain was used in this study as it was a strain in which the UPR was uninduced and was thus used to compare UPR induction levels of recombinant cellulase secreting *S. cerevisiae* Y294 strains. The *S. cerevisiae* Y294 CBH and β-glucosidase (BGL) producing strains used here were previously described [9, 10, 12].

The reporter genes for the UPR biosensor, *T.r.xyn2* and *eGFP*, were selected due to the minimal effect that they had on the yeast cells and their ability to be easily be assayed. *T.r.*Xyn2 was selected as it is a relatively small, easily secreted protein that is easy to assay, and had no apparent impact of yeast growth [33]. The codon optimized GFP variant that was used in this study is known for its fluorophore that can spontaneously form intracellularly without requiring cofactors and thus provides emitted fluorescence intensities that are a direct representation of the GFP expression [34]. GFP fluorescence or xylanase activity in the culture supernatant was thus measured using flow cytometry or DNS assays, respectively, and the results were observed as a measure of the level of expression of the putative UPR promoters.

3.2. Screening of *S. cerevisiae* strains carrying the UPR biosensors

One of the objectives for the biosensor that was developed in this study was to be able to indicate whether the UPR was active through a simple screening method. When the UPR is active in the cell, the *HAC1* and *KAR2* gene promoters are able to sense that and express the reporter genes producing either xylanase (*T.r.xyn2*) or GFP fluorescence (*eGFP*) depending on

the relative transformant. Xylanase activity can be detected on AZCL-xylan containing SC^{-URA} medium plates [35]. AZCL xylan plates provide a good initial plate screen to determine if the UPR is active in the cell by means of indicating the presence of *T.r.*Xyn2, which can be observed by blue halos around the colonies (Fig. 1B). Positive transformants containing the pGC1, pGC2, pGC3, and pGC4 biosensor plasmids were spot plated on AZCL-xylan containing SC medium plates and grown overnight at 30 °C before analysis. The AZCL-xylan plates confirmed xylanase activity for the strains indicated by the formation of dark zones around the colonies. It is clear by the zones that were obtained that xylanase was produced by each of the *S.c.*yENO1 reference strains containing the biosensors (Fig. 1B). This also indicated that the cloned P_{HAC1} and P_{KAR2} allowed basal levels of expression during these uninduced conditions. This can be used as a way of readily revealing the presence of xylanase as shown in a previous study by Guo et al. [35] confirming successful transformation.

Initial detection of the *eGFP* reporter in transformants was done through fluorescence microscopy [36]. Colonies of the positive transformants containing the pGC5 or pGC6 biosensors were submerged in buffer on a microscope slide and viewed using a Zeiss Axioplan 2 imaging fluorescent microscope and the images were captured using a Zeiss AxioCam HRm camera (Fig. 1C). The strains containing the two biosensors both showed fluorescence when viewed under the fluorescence microscope. The strains were compared with an untransformed *S.c.*yENO1 reference strain, which showed that Y294 had low background fluorescence. Fluorescence microscopy was thus able to show that the *eGFP* reporter gene was expressed but does not provide information on the number of fluorescing cells or a way of quantifying the intensity of fluorescence and does thus not provide a means of knowing the level of UPR present in the cells. Although screening of the reporter genes used in this study could be done using basic methods such as the AZCL-xylan plate screen and fluorescence microscopy, determining the relative level of UPR induction will require quantitative assays. However, these screening methods provide an adequate initial screen to determine if the biosensor is present and active in the yeast transformants.

3.3. Determination of preferred promoter and selection marker combination

In order to select the best combination of promoters, selectable markers, and reporter proteins for the UPR biosensor, the different components had to be directly compared. The differences between the two selection markers yielding resistance to G418 or hygromycin was tested, as well as the amount of background activity observed for either reporter in the control strains for the P_{HAC1} and P_{KAR2} promoters. It is important for the promoter in the UPR biosensor to have as little as possible background activity in uninduced conditions and that all other potential inducers of the systems (e.g., the influence of the heat-shock element present in the P_{KAR2}) give a constant noise level as that will provide a better depiction of the level of UPR induction and allow for more accurate measurement of the stress response pathway. The *S.c.*yENO1 reference strain was used for comparison of the two promoters due to the strain not expressing any heterologous genes, despite carrying a reference plasmid and therefore providing an uninduced environment. Transformants of the *S.c.*yENO1 reference strain carrying biosensor plasmids with different promoters and selection markers were cultivated under the same conditions for a period of 48 H after which DNS assays were performed in 24 H intervals up to 120 H.

Higher xylanase activities per gram DCW were observed in the control strain with the P_{HAC1} containing biosensor (Fig. 2A). As this was observed in uninduced conditions it shows that P_{HAC1} is active in yeast cells at a higher basal level compared to P_{KAR2}. The strain expressing *T.r.xyn2* under the P_{KAR2} yielded much lower xylanase activity (Fig. 2A), suggesting that P_{KAR2} is a preferred candidate for the biosensor. As P_{KAR2} is a stress sensor in the UPR pathway, it is expected to be highly active when there is an accumulation of unfolded proteins such as those caused by heterologous protein expression in yeast [12]. In addition, we observed that there was no significant increase in xylanase production between 48 and 120 H.

The selection marker is an important part of the biosensor as this will be used to select positive transformants during transformation [37]. It is thus important that the selection marker does not have a negative effect on the strain, UPR promoter, or reporter gene, and therefore does not affect the reporter activity levels. Some markers may also yield varying amounts of plasmid copy numbers that may cause variable levels of reporter activity through a copy number effect. However, it would be useful to create biosensor plasmids with a variety of selectable markers to compensate for different selectable markers already used to create the recombinant strains whose UPR induction one might want to determine. The comparison between our selection markers was done in a *S. cerevisiae* Y294 strain with CBH induction of the UPR (*T.e.*cbh1-CCBM producing strain) containing the biosensor under control of the same promoter (P_{KAR2}) (Supplementary Fig. S1). The strains containing the biosensors were cultivated in YPD media that contained the corresponding selection marker and grown for a period of 120 H. There were no significant differences between the xylanase activities per gram DCW of the strains with the different selection markers that were used (Supplementary Fig. S1) as determined by a two-tailed *T*-test assuming unequal variance. The xylanase activity followed a similar trend under both selections. The selection markers thus did not have an effect on the performance of the biosensors or the growth of the strains. Due to these results, the biosensor plasmids with P_{KAR2} and G418 selection were selected for further studies.

3.4. Testing the dynamic range of the UPR biosensors with Tm

It was subsequently important to test the sensitivity and range of P_{KAR2} in the biosensor as this will be an indication of how the biosensor will react when subjected to various levels of stress. The UPR can be activated through various chemicals

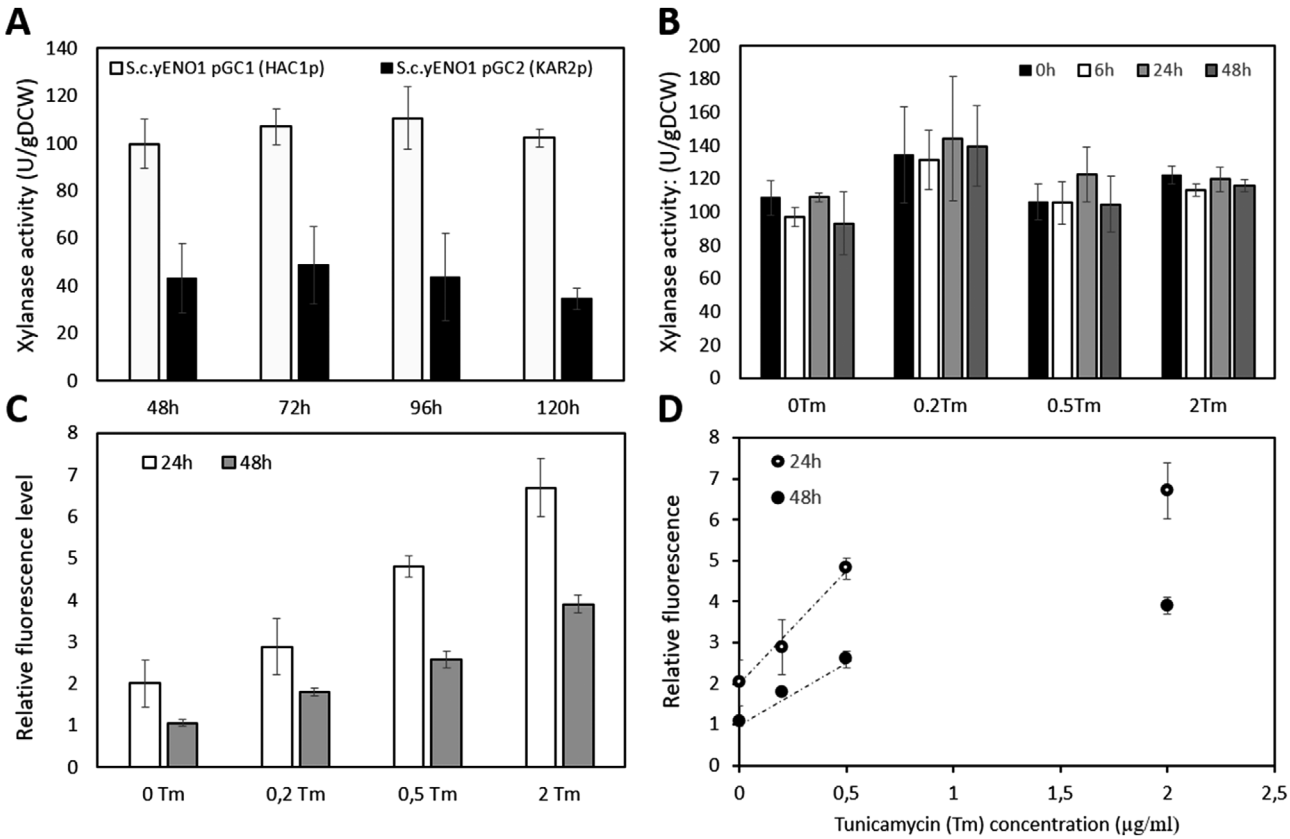

FIG. 2

A: Comparison of the influence of promoters used for the biosensor in this study. Xylanase activity of the control strains containing P_{HAC1} *and* P_{KAR2} *were compared. B: Xylanase activity of biosensors induced by tunicamycin in the* S.c.*yENO1 pGC2 strain. Xylanase activity was measured at 0, 6, 24, and 48 H and induced with 0.2, 0.5, or 2 µg/mL Tm. The activities represent the means of three biological repeats. C: Relative fluorescence levels of the* S.c.*yENO1 pGC6 strain were measured at 0, 24, and 48 H and induced with 0.2, 0.5, and 2 µg/mL Tm. Relative fluorescence levels of the* S.c.*yENO1 pGC6 strain against the increased tunicamycin concentrations show the linearity of the eGFP response over 0, 0.2, and 0.5 µg/mL Tm. Arbitrary fluorescence values were normalized against the lowest obtained fluorescence value (0 Tm, 48 H). The activities represent the means of biological triplicates and error bars represent the standard deviation. Error bars for both graphs represent the standard deviation.*

that are able to induce ER stress, such as Tm and dithiothreitol [38, 39]. Tm functions by inhibiting the initial glycoprotein biosynthesis in the ER, causing an accumulation of unfolded glycoproteins which will result in ER stress, providing an ideal way to test the sensitivity range of the biosensor. The pGC2 (P_{KAR2} with *T.r.xyn2*) and pGC6 (P_{KAR2} with *eGFP*) biosensor containing strains were thus subjected to Tm in the uninduced conditions of the control strain, to determine the sensitivity of the reporter genes to various levels of stress. There is no set concentration and duration for Tm treatment that will induce ER stress as each system is different and should thus be determined independently [38]. Therefore, we created a Tm gradient of 0, 0.2, 0.5, and 2 µg/mL for both xylanase activity assays and *eGFP* fluorescence assays (Figs. 2B and 2C). The relevant strains were grown in YPD media supplemented with Tm for a period of 48 H.

No significant differences were observed across the Tm concentrations used for the pGC2 (P_{KAR2}-*xyn2*) biosensor (Fig. 2B). All xylanase activity levels obtained were comparable to the uninduced (0 µg/mL Tm) *S.c.*yENO1 strain, which would indicate that the concentrations of Tm used did not have a notable effect on the activity. These quantitative xylanase results were similar to the AZCL-xylan plate screen results as it depicted a notable background activity. However, the pGC6 biosensor displayed an increase in the relative eGFP fluorescent levels with increased Tm concentrations after 24 H (Fig. 2C). A notable linearity was observed for the induction up to 0.5 µg/mL Tm concentrations at the low end of the scale (Figs. 2C and 2D). The results also indicated that the best eGFP expression measurements were made after 24 H, which is most likely due to the short half-life of the eGFP, which was previously shown to be approximately 8 H [40]. This means that due to the relatively rapid decay of the eGFP protein, relative fluorescence will decrease appreciably in the timeframes used in our study if new eGFP is not constantly synthesized. The rapid decrease we observed after 24 H therefore likely indicates that there was no more accumulation of GFP after this time. The eGFP

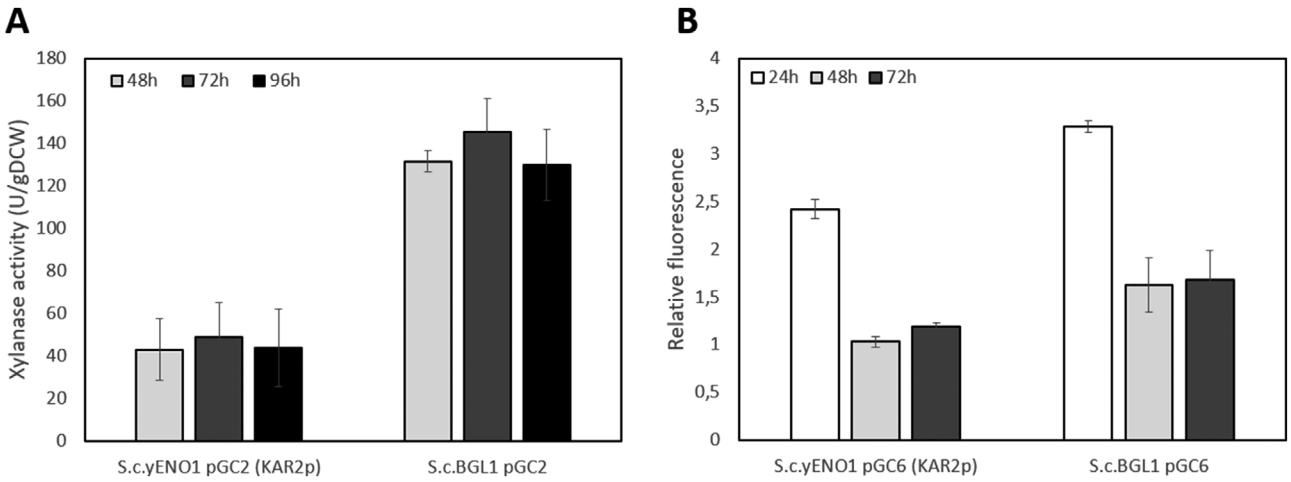

FIG. 3

Induction of UPR by BGL expression in S. cerevisiae *Y294 transformants. A: Comparison of the xylanase activity of a* S. cerevisiae *Y294 yENO1 strain and the BGL1 producing strain containing the pGC2 plasmid. The activities represent the means of biological triplicates. B: Relative fluorescence levels of the* S. cerevisiae *Y294 yENO1 reference strain and the BGL1 producing strain containing the pGC6 plasmid. Arbitrary fluorescence values were normalized against the lowest obtained fluorescence value (*S.c.*yENO1 pGC6, 48 H). Error bars for both graphs represent the standard deviation of three biological repeats.*

levels that were observed after 48 H still displayed linearity in terms of the Tm level but the relative fluorescence that was observed had decreased by ~50%. This result also indicated the sensitivity of detecting fluorescence using flow cytometry for determining fluorescence levels compared with the DNS assay for measuring the xylanase activity. As the xylanase does not seem to be a good indicator of the range of levels of UPR induction that the cells may be experiencing, it makes the *eGFP* the preferred choice of reporter gene for measuring the UPR.

3.5. UPR induction by BGL production

The main objective for developing this UPR biosensor was to be able to determine when and to what extent the UPR is active within yeasts that produce a heterologous protein, as this will assist in the development of better recombinant *S. cerevisiae* strains for CBP. The pGC2 and pGC6 biosensor plasmids were transformed into recombinant cellulase secreting *S. cerevisiae* Y294 strains with the purpose of measuring the level of UPR (Table 2). These strains were compared with the *S.c.*yENO1 reference strain. The UPR induction data of the strains used in this study are known through previous RNA measurements [12, 41]. Our results would thus be a comparison of the biosensors to the RNA data of these strains, which will allow for comparison of the biosensors created in this study to the RNA-based methods for determining UPR induction. For the purpose of this study, we tested heterologous BGL and CBH induction of the UPR. DNS-based xylanase assays were performed for a period of 120 H and fluorescence levels were measured using flow cytometry for a period of 72 H.

An appropriately threefold increase was observed for the xylanase activity in the *S.c.*BGL1 strain compared with the control at all corresponding time points (Fig. 3A). The highest activity was observed after 72 H. The difference in the xylanase activity was determined to be highly significant ($P < 0.0001$) using the *T*-test. The level of increase is much less in the eGFP fluorescent strains (Fig. 3B). Based on the relative eGFP fluorescence of the strains, eGFP expression was highest after 24 H after which fluorescence halved before levelling off. This is likely due to the previously mentioned short half-life of the eGFP. The *T*-test showed that eGFP levels differed significantly only at the 24 H mark, though the increase in fluorescence was only ~36%. Decreases in xylanase activity could be attributed to the changes brought about by the UPR that allowed for a return to homeostasis but can also be as a result of cell death caused by failure of the UPR to adapt cells to the conditions [42, 43]. These results do show that the heterologous expression of BGL1 caused a significant impact in the level of UPR compared with uninduced conditions in the *S.c.*yENO1 pGC2 strains and the results are in agreement with the results shown previously using RT-qPCR [41]. The difference between *xyn2* and *eGFP* gene expression was very significant, however, it should be noted that due to the xylanase being a heterologously secreted protein, it could have itself contributed to the higher level of UPR induction observed.

3.6. UPR induction by CBH production

The results obtained from the CBH induction of the UPR depicted clear differences between the CBH producing strains that were compared. Previous RNA-based work that was done on these strains, using densitometry analysis of Northern blots, showed *T.e.cbh1* to have low UPR induction, *T.e.cbh1*-CCBM to have medium UPR induction, and *T.r.cbh1* to have high UPR induction though these results were variable and inconsistent [12]. In contrast, RT-qPCR could not show significant differences in UPR induction between these strains [41]. Across the 120 H of cultivation for the CBH producing strains with the pGC2

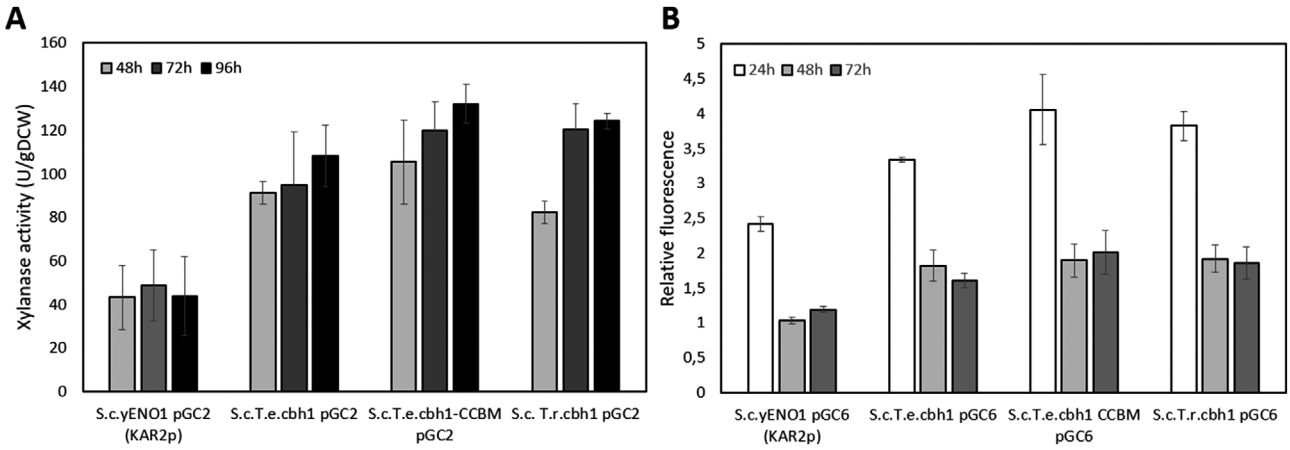

FIG. 4

Induction of UPR by CBH expression in S. cerevisiae *Y294 transformants. A: Comparison of the xylanase activity of a* S. cerevisiae *Y294 yENO1 strain and the three CBH producing strains containing the pGC2 plasmid. The activities represent the means of biological triplicates. B: Relative fluorescence levels of the* S. cerevisiae *Y294 yENO1 reference strain and the three CBH producing strains containing the pGC6 plasmid. Arbitrary fluorescence values were normalized against the lowest obtained fluorescence value (*S.c.*yENO1 pGC6, 48 H). Error bars for both graphs represent the standard deviation of three biological repeats.*

biosensor, the highest xylanase activity levels for each strain was observed after 96 H, though the differences were not statistically significant in all cases (Fig. 4A). It was clear that the *S.c.T.e.cbh1* strain had the lowest xylanase activity per gram DCW at 96 H, and can thus be said to have the lowest level of UPR induction. This would indicate that expression of the *T.e.cbh1* cellulase caused the least amount of stress on the secretory pathway, corresponding with the RNA measurements of the UPR for this strain [12]. Despite this, activity levels were still more than twofold higher than that of the *S.c.*yENO1 pGC2 control strain, and therefore the stress levels were still significantly higher than what we would describe as normal in this study. The *T.e.cbh1*-CCBM (132.8 U/gDCW) and the *S.c.T.r.*cbh1 (124.21 U/gDCW) strains were comparable in xylanase activities obtained at 96 H, which were higher than that of the *S.c.T.e.*cbh1 (108.37 U/gDCW).

The eGFP fluorescence results obtained for the pGC6 biosensor showed that the *S.c.T.e.*cbh1-CCBM strain had the highest UPR induction after 24 H (67.98%) followed by the *S.c.T.r.*cbh1 strain, whereas the *S.c.T.e.*cbh1 displayed the lowest induction (38.26%) (Fig. 4B) compared with the control strain. There is thus a marked similarity in the ranking of the UPR induction of these strains with the two reporter genes. The primary difference was that the relative fluorescence levels of the CBH strains producing their peak fluorescence after 24 H, and then decreased in relative fluorescence by 50% after 24 H before leveling off. This, as mentioned, is as a result of the eGFP that has a relatively short half-life. In comparison, the recombinant *T.r.xyn2* xylanase was shown to be very stable, surviving in the growth media for over 144 H [33]. This result thus shows that when measuring UPR levels with the pGC6 biosensor, it should be done within 24 H for the most accurate result.

4. Discussion

Low heterologous protein secretion in general and low CBH secretion titers in particular are among the main obstacles to developing *S. cerevisiae* for CBP of lignocellulosic biomass to commodity products. RNA-based methods were previously used to show that low heterologous protein titers were due in part to an upregulation of the UPR, though these results were variable and inconsistent [12, 41]. Furthermore, these methods are often cumbersome and lack sensitivity, prohibiting discrimination of the levels of UPR induction by various cellulases. We therefore sought to develop a biosensor that could rapidly quantify UPR induction while indicating which heterologous genes may be more amenable to heterologous expression through avoiding metabolic burden. As different versions of closely related genes were shown to yield vastly varying amounts of secreted activity [12], such a tool could help select the best gene candidates for construction of CBP yeast strains. The biosensors constructed in this study differed in promoter (P_{HAC1} and P_{KAR2}), reporter gene (*T.r.xyn2* and *eGFP*) and selection marker (Geneticin G418 and Hygromycin B) (Fig. 1A). When cells display activation of the components of the UPR, they are generally described as undergoing ER stress and two inherent factors of this response are the splicing of *HAC1* mRNA and upregulation of Kar2p protein production [44]. *HAC1* and *KAR2* are thus important regulators of the UPR and the promoters of these genes were therefore strong candidates for developing a biosensor. The UPR upregulates P_{KAR2} for the production of the ER chaperone BiP in order to enhance secretory protein folding [43]. Similarly, it is the production of the Hac1p transcription factor by expression of the spliced *HAC1* mRNA that is responsible for the upregulation of many genes involved in the UPR, including *KAR2* and *HAC1* itself, that increase the cell's folding capacity and allow the UPR to achieve its function of assisting cells with correct folding of

proteins during ER stress [15, 45]. P_{HAC1} and P_{KAR2} levels will thus always be overexpressed when ER stress occurs.

A good biosensor should also contain an easily assayable reporter protein. The *T. reesei* xylanase encoding gene *xyn2* was selected as reporter gene candidate for the biosensor as this gene was previously shown to express well in *S. cerevisiae* [33]. It is a small (21–27 kDa depending on glycosylation) secreted protein that is easy to screen and quantify. A UPR sensor developed by Lajoie et al. [43] consisting of GFP-tagged KAR2/BiP was found to express fluorescence levels that directly correlated with UPR activity and the GFP was thus found to be a robust reporter when doing high throughput analysis. Ducrest et al. [34] compared a GFP and luciferase reporter system and showed that the GFP system was as sensitive as an enzyme-based system but more advantageous as its expression could be quantified within single cells by flow cytometry. Both xylanase and eGFP reporters were easy to screen on AZCL-xylan plates and fluorescence microscopy, respectively (Figs. 1B and 1C). An improvement of the biosensors used in this study over the previously mentioned biosensors is that it is based on low copy centromeric plasmids. The 2μ-based plasmids previously used have been shown to vary in copy number in yeast strains with different levels of stress induction [12], which may cause variation in the readout of the biosensor.

We observed higher background activity when using the P_{HAC1} with either reporter protein and less range of induction when using the xylanase reporter (Fig. 2). P_{HAC1} is known to be constitutively expressed at low levels in *S. cerevisiae* and studies have shown the effect that this has on heterologous secreted proteins [45]. It would thus likely be preferable to measure the *HAC1* mRNA splicing event when using *HAC1* to measure the level of UPR induction as was reported previously [46]. P_{KAR2} was previously found to be expressed in high levels when yeasts were exposed to chemical agents that interfere with folding as well as in cells overexpressing heterologous proteins that induce the UPR [45], and thus provides a better candidate for a UPR biosensor. We observed significantly different induction levels of the *xyn2* and *eGFP* gene expression (Figs. 2 and 3). However, as the xylanase itself is a heterologously secreted protein, it may have contributed to the higher level of UPR induction observed. Furthermore, the xylanase was previously shown to be a stable protein that can accumulate significantly over time [33].

Tm concentrations of 2.5–5 μg/mL are usually sufficient for induction of the UPR in most cell types [38]. Based on this, the observation that an increase in the eGFP fluorescence levels could be detected from a concentration of 0.2 μg/mL indicates the sensitivity of the pGC6 biosensor (Figs. 2C and 2D). The *eGFP* coupled with P_{KAR2} (pGC6) was also shown to have better dynamic range in response to Tm, compared with xylanase. It is clear that the xylanase reporter in the pGC2 UPR biosensor plasmid should only be used for qualitative measurements of the UPR and that the eGFP reporter in the pGC6 UPR biosensor plasmid should be used for quantitative analysis of UPR induction. However, it should be noted that the eGFP tended to lose resolution after 24 H due to its shorter half-life, thus measurements of UPR induction are best done within the first 48 hours of cultivation. The xylanase reporter may be useful if applied to high-throughput plate screening approaches in the absence of sophisticated flow cytometry equipment.

In the results obtained for cellulase induction of the UPR for both the *T.r.*xyn2 and eGFP, it was clear that the UPR induction was present from the early stages of cultivation and was observed to level off over time (Figs. 3 and 4). The high levels of UPR induction in these recombinant cellulase producing *S. cerevisiae* Y294 strains were likely as a result of the general challenges that are faced in the recombinant yeast such as the incorrect folding, and the secretory mechanisms not being able to cope with heterologous protein production [47, 48]. Therefore, these results should take into consideration that any type of heterologous protein production can induce the UPR in yeast. It was clear in the results obtained that there were small differences between the two biosensors but they detected similar levels of UPR stress in recombinant cellulase producing yeast strains. In the Tm test, the pGC2 biosensor did not react to the concentration gradient that was used. However, it reacted similarly to the pGC6 biosensor when testing the level of UPR in cellulase producing strains. All xylanase activity that was observable existed at 48 H of cultivation after which no further accumulation was observed. This is in contrast to relative eGFP fluorescence, in which the 24 H reading was observed to be the best reading to measure due to the half-life of eGFP. Future tests could include readings at 8 and 16 H of cultivation to test whether these give a different indication of UPR induction compared with the 24 H observations, but it should be kept in mind that fluorescence levels measured for cultivations prior to significant levels of growth may be more variable.

Using an RNA blot and densitometry-based method, CBH expression was shown to induce the UPR at levels that were equal to or higher than UPR induction by BGL expression [12]. Studies by Van Zyl [41] showed that, when measuring *HAC1* splicing using RT-qPCR measurements of UPR induction, there was little induction differences between control *S. cerevisiae* Y294 and various CBH producing Y294 strains. The UPR biosensor developed in this study was able to detect significant differences in UPR induction between different CBH producing Y294 strains, which demonstrated the increased sensitivity of measuring P_{KAR2} induction compared to measuring *HAC1* splicing. Comparing the *T.e.*cbh1 strains, we noticed that the CBH, which had the carbohydrate-binding module (CBM), had higher UPR induction. The CBM was previously shown to cause an increase in the complexity of CBH production yeast, and it was also suggested that this fusion protein results in higher ER stress [4, 12]. This addition of the CBM is thus likely the cause of the increased activity that was observed here. This was the first time that a plasmid-based UPR biosensor was shown to successfully measure UPR induction in cellulase producing *S. cerevisiae* strains and corroborated the results of Ilmén et al. [12] who used an RNA-based approach.

It is important to note that CBHs have generally been reported to have low secretion titers with reports stating that it can vary from 0.002 to >1% of the total cell protein [4, 12, 49]. Despite this, CBH expression induces UPR levels that were similar to or higher than those induced by BGL-expression, depending on the assay method used (Figs. 3 and 4). Based on the results obtained in this study, it seems reasonable to assume that their low secretion titers are due in part to the burden that CBH production causes on the secretory pathway.

In conclusion, we developed a UPR biosensor that was shown to have greater sensitivity for changes in UPR induction than the currently applied RT-qPCR method and will thus allow for more accurate detection and measurement of UPR induction in cellulase secreting *S. cerevisiae* strains. This tool will help to identify the best cellulase gene candidates for CBP strain engineering that yield the required activity while not inducing high levels of secretion-based stress. Furthermore, we can also use this biosensor to investigate whether or not enhanced secretion phenotypes observed in previously engineered strains were partly due to decreased levels of UPR induction [9, 10, 50–52]. These CEN-based plasmids are amenable to high-throughput transformation approaches, in addition to providing more stable biosensors in the variety of natural and industrial strain isolates often used to develop CBP yeast strains [50, 53].

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