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Short Communications

Expanding the Yeast MoClo Toolkit: gene expression control parts for *Saccharomyces cerevisiae* tested in industrially relevant conditions

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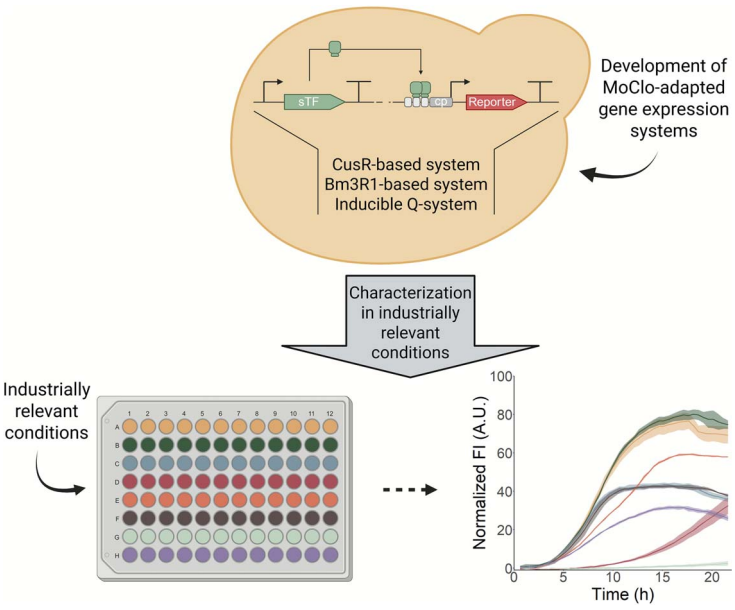
Abstract

Fine-tuning of gene expression is often required to achieve competitive production levels in microbial cell factories. Several orthogonal expression systems based on heterologous regulatory parts have been developed for *Saccharomyces cerevisiae*. In laboratory conditions the systems demonstrate predictable results, but few expression systems have been tested in industrial conditions. Here, a new expression system based on the bacterial gene *cusR* was developed for *S. cerevisiae*, and two previous developed systems, the strong Bm3R1-based system and the quinic acid inducible Q-system, were adapted for compatibility with the Yeast MoClo Toolkit. The bacterial transcription factors CusR and Bm3R1 acted as DNA binding domains, and fused to a viral activation domain, they functioned as transcriptional activators. The Q-system is originally from *Neurospora crassa* and consists of a transcriptional repressor, QS, which in the absence of quinic acid blocks the activity of a transcriptional activator, QF2. Quinic acid binds to QS, inhibiting QS from blocking the activity of QF2 in a dose-dependent manner. The gene expression systems were assessed in industrially relevant conditions, proving a predictable performance at low pH. The performance of the constitutive systems was predictable also at high temperature and in a synthetic lignocellulosic hydrolysate medium. Altogether, the MoClo-compatible expression systems enable fast construction of fine-tuned production pathways for *S. cerevisiae* cell factories used for industrial applications.

Graphical Abstract



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Introduction

Saccharomyces cerevisiae is widely used for production of valuable compounds [1]. To develop economically viable cell factories, fine-tuning of gene expression is often needed [2, 3]. This can lead to a more balanced carbon flow between production formation and cell growth, reduced accumulation of toxic intermediates and may surpass by-products formation and feedback inhibition [2, 3]. For precise metabolic engineering, synthetic gene expression systems have emerged as important tools.

Synthetic gene expression systems offer fine-tuned and adaptable control over gene expression that, due to complex endogenous regulation, is often difficult to achieve with native promoters [3, 4]. Moreover, with two-component designed systems, multiple target genes can be activated simultaneously, enabling coordinated pathway expression [5]. To be suitable for applications in cell factory design, gene expression systems need certain traits: orthogonality to prevent interference with endogenous regulation, predictability for design and optimization of cell factories, and stability across various conditions for performance in industrial settings [2, 5, 6]. Modularity and tunability are also beneficial traits and some applications may require an inducible expression for dynamic gene expression control [5, 6]. Systems compatible with the widely used Yeast Modular Cloning (MoClo) Toolkit [7] are ideal for easy adaptation to various applications. Notably, many previously developed expression systems for *S. cerevisiae* are of orthogonal design [8] and recently, two new sets of inducible promoters compatible with the MoClo Toolkit were reported [9, 10], offering enhanced flexibility for gene expression control. Unlike many native promoters [6, 11], engineered synthetic promoters have demonstrated consistent gene expression across varying carbon sources [2, 5]. Nevertheless, few synthetic expression systems have been tested for robustness in industrially relevant conditions such as increased temperatures, lowered pH, or the presence of inhibitors or metabolic by-products.

In this study, a new MoClo compatible gene expression system, based on the bacterial transcriptional activator CusR was developed, and two previously developed expression systems, the strong Bm3R1-based system [4] and the quinic acid inducible Q-system originally derived from *Neurospora crassa* [12], were adapted for MoClo compatibility. Bm3R1 has previously been applied in *S. cerevisiae* in both a synthetic activator and a repressor system [4], as well as in an acetic acid-sensing biosensor [13]. This system has previously demonstrated among the strongest transcriptional activation [4], emphasizing its potential for application in cell factory engineering. The Q-system has previously been applied in *S. cerevisiae* as a quinic acid inducible synthetic activator/inhibitor system and was further evolved into an optogenetic system inducible by darkness or blue light [12]. In comparison to widely used inducers such as isopropyl β-D-1-thiogalactopyranoside (IPTG) or anhydrotetracycline (aTc), quinic acid offers greater suitability for industrial use and scale. Quinic acid is inexpensive [14], non-toxic at low concentrations [15], and, unlike aTc, neither classified as an antibiotic nor subject to specialized waste disposal requirements. To assess the utility of the systems in industrial applications, the expression systems were characterized in various industrially relevant conditions. All systems proved robust in acidic conditions and the CusR- and Bm3R1-based systems also proved robust at high temperatures and in lignocellulosic hydrolysate, demonstrating their potential for use in industrial production strains. Together, these synthetic gene expression systems span a wide range of expression levels and enable adaptable or inducible expression across various industrially relevant conditions.

Materials and methods

Plasmid and strain construction

All plasmids were designed and assembled according to the MoClo method [7]. *Escherichia coli* TOP10 cells were grown in Luria-Bertani medium (5 g/L yeast extract, 10 g/L bacto tryptone, and 10 g/L NaCl) and used for construction and propagation of all plasmids. All plasmids that were constructed and used in this study are listed in Tables S1–S3 (Supporting Information) [12, 13, 16–19] and all primers used for construction and verification are listed in Table S4 (Supporting Information). The sequences of the synthetic promoters used in the study are listed in Table S5 (Supporting Information). Yeast transformations were performed according to the LiAc/salmon sperm carrier DNA/polyethylene glycol method [20]. Transformants were selected on yeast extract–peptone–dextrose medium (10 g/L yeast extract, 20 g/L bacto peptone, and 20 g/L glucose) and supplemented with 200 mg/L G418 sulphate or synthetic defined (SD) medium lacking uracil (0.77 g/L complete supplement mixture [CSM] without uracil, 6.7 g/L yeast nitrogen base without amino acids [YNB w/o AA] and 20 g/L glucose) and supplemented with 200 mg/L G418 sulphate. The *S. cerevisiae* strain that was used as background strain in this study was the CEN.PK113-5D-derivative IMX581 [21]. Genomic constructs were integrated in the *X2* and *HO* loci. All constructed strains are listed in Table S6 (Supporting Information). Detailed descriptions of the plasmid and strain construction are available in the Supporting Information.

Cultivation conditions and fluorescence measurements

Yeast cells were inoculated from solid medium into 3 ml of SD medium (0.79 g/L CSM, 6.7 g/L YNB w/o AA, and 20 g/L glucose) at pH 5.5 adjusted with NaOH titration and grown overnight at 30°C, 200 rpm. From precultures, cells were inoculated into 100 μl of the desired media at an initial OD_{600} of 0.1. A detailed description of the different media and conditions that were used is available in the Supporting Information. Cells were cultivated in CELLSTAR black clear-bottom 96-well plates (Greiner Bio-One) with 85% humidity, 900 rpm, at a temperature set to 30°C or 35°C using a microbioreactor device able to detect fluorescence (Biolector, Beckman Coulter Life Sciences). The cultures were analysed every 30 min using filters for biomass detection (excitation 620 nm, emission 620 nm, and gain 10), mCherry detection (excitation 580 nm, emission 610 nm, and gain 100), and mTurquoise2 detection (excitation 436 nm, emission 488 nm, and gain 100).

Statistical analysis

Statistical analyses were performed in R using Student's *t*-test with Holm–Bonferroni correction. Statistical significances were designated as follows: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$. Comparison of expression levels of the CusR-based systems in control condition was performed after 12.5 h of cultivations when the cells were around mid-exponential phase. Comparison of expression levels of the systems in various industrial conditions were performed after 17 h of cultivation to account for the increased lag phase in the more challenging conditions.

Results and discussion

MoClo-compatible DNA regulatory parts expand the Yeast MoClo Toolkit

To expand the MoClo Toolkit for gene expression control in *S. cerevisiae*, a CusR-based system was developed along with MoClo

adaptation of the Bm3R1-based system [4] and the Q-system [12]. The systems consist of a donor unit containing a synthetic transcription factor (sTF), and a receiver unit containing a reporter gene controlled by an sTF-dependent synthetic promoter (Fig. 1A). For the CusR- and Bm3R1-based systems, the sTFs are composed of a bacterial DNA binding domain coupled to a viral activating domain and a nuclear localization signal. For the Q-system, the sTF is composed of two parts, a transcriptional repressor, QS, and a transcriptional activator, QF2. The sTF-dependent promoters were designed with binding sites for the sTF directly upstream of a yeast core promoter (Fig. S1). The CusR- and Bm3R1-systems are constitutive whereas the Q-system is inducible by quinic acid. In the Q-system, the repressor prevents reporter gene expression by blocking the activator's activity in the absence of quinic acid. When quinic acid is present, it binds to the repressor, relieving it from blocking the activator in a dose-dependent manner, allowing an activator-mediated expression of the reporter gene. To assess the basal activity, controls containing the receiver units alone were designed for the respective system. For the Q-system, an additional control containing the receiver unit together with the activator was constructed. For comparison of expression level to native promoters, two strains, containing the red fluorescence protein mCherry and the cyan fluorescence protein mTurquoise2 expressed under the native *TDH3* promoter, respectively, were constructed.

Development of a CusR-based expression system

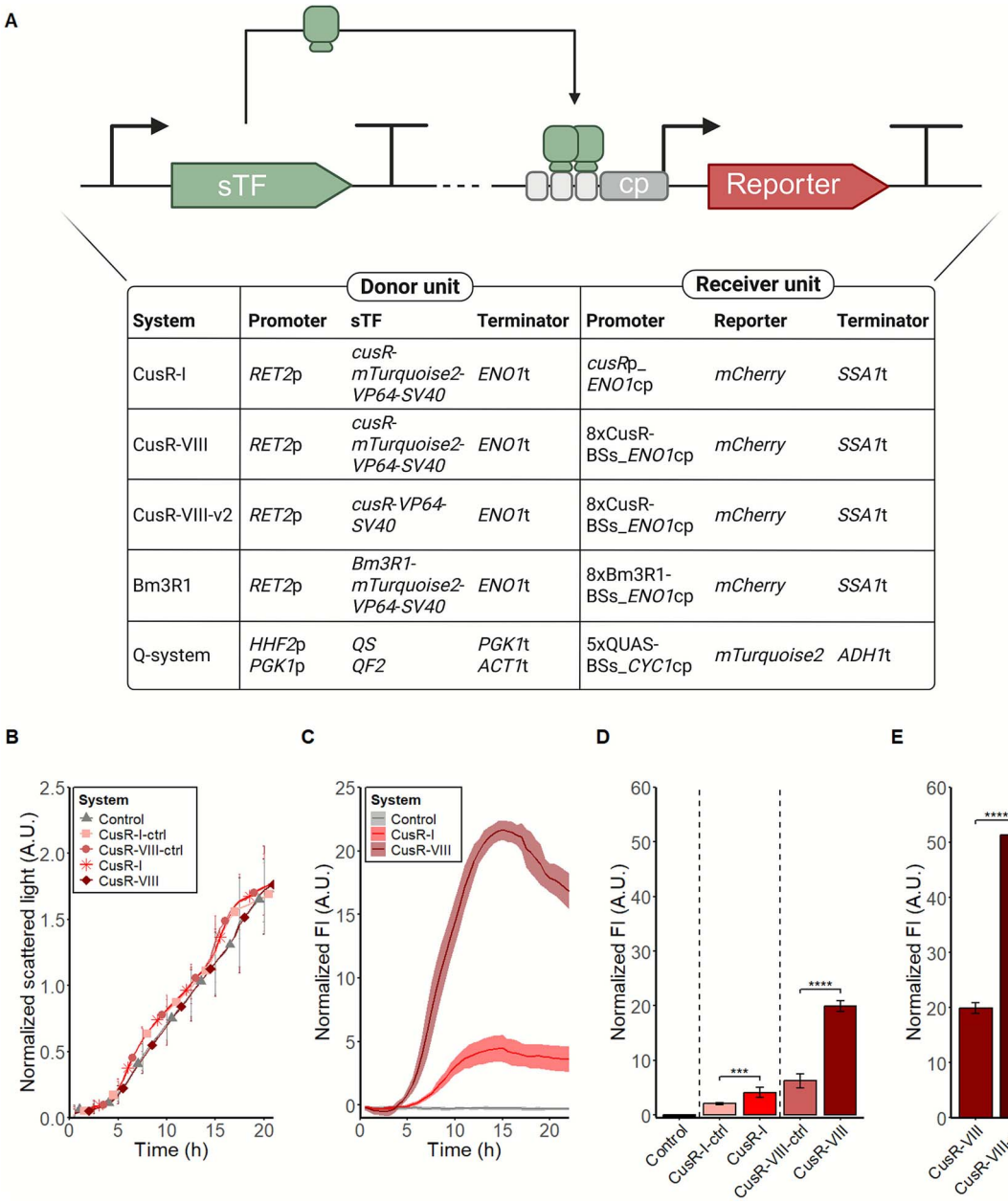
To develop a novel orthogonal expression system, the bacterial TF CusR was explored for its potential to function as a DNA binding domain in yeast. *CusR* is part of the *cusRS* two-component system involved in copper response in bacteria and its role as an activator has been well characterized in bacteria [22]. Together with the CusR-based sTF, two designs of synthetic promoters were evaluated. One consisted of the native *cusR* promoter, holding one binding site for CusR, coupled to a core promoter (CusR-I). The other contained eight repeats of the CusR binding site upstream of a core promoter (CusR-VIII), according to similar systems described by Rantasalo *et al.* [4] and Mormino *et al.* [13]. To assess if the CusR-based systems affected the physiology of the strains, the biomass formation was monitored over time (Fig. 1B). All strains carrying expression systems had similar maximum specific growth rate as the parental strain (control; Fig. S2). High expression of viral activation domains has earlier been reported to have negative impact on growth rate [5, 16] but this was not seen with the CusR-systems (Fig. 1B and Fig. S2).

Next, the reporter expression of the CusR-systems was monitored over time (Fig. 1C). The sTF activated the reporter unit in both versions of the system (Fig. 1C and D). The basal expression of the CusR-VIII-system harbouring eight binding sites for CusR (CusR-VIII-ctrl) was higher compared to the basal expression of the CusR-I-system harbouring only one binding site for CusR (CusR-I-ctrl; Fig. 1D). This may suggest that there are intrinsic TFs in *S. cerevisiae* that are able to interact with CusR's binding sequence, thus inducing a slight activation of the reporter gene. Indeed, two native activators, Rtg3 and Yap1, were found to have binding sites within the binding sequence of CusR [23] which could explain the elevated basal expression. Although the CusR-VIII-system demonstrated higher basal expression, the system had a distinct increase in sTF-induced expression with a 3.2-fold activation at 12.5 h of cultivation compared with a 2.0-fold activation of the CusR-I-system at the same timepoint (Fig. 1C and D). The sTF-induced expression of the respective systems demonstrates that CusR successfully functioned as an sTF together with a viral activating domain in *S. cerevisiae*. A stronger activation was achieved with multiple binding sites as demonstrated earlier for similar systems [4]. Compared with previously developed gene expression systems [4, 5], the level of activation remained low, even in the CusR-system with eight binding sites. Removing the fluorescent protein in the sTF of the CusR-VIII-system (CusR-VIII-v2) increased reporter activation by 2.6 times (Fig. 1E), suggesting the fluorescent protein hinders sTF function, possibly by impeding DNA binding or destabilizing the overall fusion protein. Several aspects such as promoter strength, number of binding sites, and sTF binding affinity may influence activation [4, 5]. The modularity of the system allows rapid further development and optimizing expression within a pathway may demand expression also at lower levels.

Characterization of the expression systems in industrially relevant conditions

To assess the utility of the expression systems in industrial applications, strains expressing the systems were examined in control conditions (30°C, pH 5.5), acidic conditions (30°C, pH 3.5), at high ethanol concentration (10% v/v, 30°C, pH 5.5), and in synthetic lignocellulosic hydrolysate (SLH; 30°C, pH 5.5) [24]. Strains expressing the CusR-VIII-, CusR-VIII-v2-, and Bm3R1-systems as well as the native system for mCherry expression (TDH3p-mCherry) were also assessed in high temperature (35°C, pH 5.5) and in combinations of high temperature and the listed conditions. Only the strain harbouring the native system for mTurquoise2 expression (TDH3p-mTurquoise2) had a significantly ($P < .05$) lower maximum specific growth rate compared with the control, and that only in SLH medium (Fig. 2A and Fig. S3). On the contrary, all systems except Bm3R1-ctrl and TDH3p-mTurquoise2 unexpectedly increased the maximum specific growth rate of the strains compared with the parental strain when grown in SLH. Unlike the other strains, both the parental strain and the TDH3p-mTurquoise2 carrying strain are *URA3* negative. Consequently, their reduced growth rate may result from limited uracil availability under these specific growth conditions. Previous studies also report slower growth in uracil auxotrophic strains compared to prototrophic strains, even when cultured in rich media [25, 26]. A large variation in the maximum specific growth rate was observed both between the strains and among replicates of the same strain in high concentration of ethanol (Fig. 2A). Indeed, in high ethanol concentration the maximum specific growth rate was significantly reduced compared to the control condition (Fig. 2A). Moreover, a significant increase in the lag phase was observed in the presence of ethanol, compared with the control condition (Fig. S4). High ethanol concentrations are well known to challenge the growth of cells [27, 28]. The lag phase across all strains was also significantly increased in SLH medium (Fig. S4). An increased lag phase in SLH has also been reported before [29], likely due to the stressors present in hydrolysate that cells must adjust to [30]. This suggests that the expression systems *per se* do not have any noticeable negative impact on the strains' physiology, but that harsh industrially relevant conditions do.

The performance of the CusR-VIII-, CusR-VIII-v2-, and Bm3R1-systems as well as of TDH3p-mCherry in the industrially relevant conditions were assessed by monitoring the reporter expression over time (Fig. 2B and Fig. S3). At 17 h of cultivation, when cells in all conditions had entered the exponential phase, the basal expression was comparable between the systems and remained low in all tested conditions (Fig. 2C). In acidic conditions, all systems performed similarly as in the control condition (Fig. 2B and C). However, in high temperature and in SLH the sTF-induced



	Donor unit			Receiver unit		
System	**Promoter**	**sTF**	**Terminator**	**Promoter**	**Reporter**	**Terminator**
CusR-I	*RET2*p	*cusR-mTurquoise2-VP64-SV40*	*ENO1*t	*cusR*p_*ENO1*cp	*mCherry*	*SSA1*t
CusR-VIII	*RET2*p	*cusR-mTurquoise2-VP64-SV40*	*ENO1*t	8xCusR-BSs_*ENO1*cp	*mCherry*	*SSA1*t
CusR-VIII-v2	*RET2*p	*cusR-VP64-SV40*	*ENO1*t	8xCusR-BSs_*ENO1*cp	*mCherry*	*SSA1*t
Bm3R1	*RET2*p	*Bm3R1-mTurquoise2-VP64-SV40*	*ENO1*t	8xBm3R1-BSs_*ENO1*cp	*mCherry*	*SSA1*t
Q-system	*HHF2*p *PGK1*p	*QS QF2*	*PGK1*t *ACT1*t	5xQUAS-BSs_*CYC1*cp	*mTurquoise2*	*ADH1*t

Figure 1. General design of the expression systems and characterization of the CusR-based systems. (A) Schematic illustration of the expression systems and a detailed description of their parts. A synthetic transcription factor (sTF) binds to its binding sites upstream of a core promoter (cp), inducing expression of a reporter gene. (B) Development of biomass over time in the CusR-based system-harbouring strains and the parental strain (control). Biomass formation was measured by scattered light. (C) Reporter expression of the CusR-I- and CusR-VIII-system compared with the parental strain (control) monitored over time. (D) Reporter expression of the CusR-I- and CusR-VIII-system and their respective controls compared with the parental strain (control) after 12.5 h of cultivation when the cells were around mid-exponential phase. (E) Reporter expression of the CusR-VIII- and CusR-VIII-v2-system after 12.5 h of cultivation. Statistical analyses were performed between the expression systems and their respective control using the Student's *t*-test with Holm–Bonferroni correction; significance is displayed above the bars, ***$P < .001$, and ****$P < .0001$. Reporter expression was measured by mCherry fluorescence intensity (FI) and normalized against the biomass. The values represent the mean of one (CusR-VIII-v2) or three experiments with three replicates each ± standard deviation (SD). A.U.: arbitrary unit.

expression was reduced for all synthetic systems (Fig. 2C). The lowest sTF-induced expression was observed in high ethanol concentration (Fig. 2C). The expression of TDH3p-mCherry was strongly reduced in high concentrations of ethanol while the expression was not much affected by high temperature or SLH (Fig. 2B). The poor performance of the systems in the presence of ethanol can likely be explained by the significantly hampered growth. At high temperature, the expression of the synthetic systems was initially comparable to the control conditions but plateaued for all systems after around 10 h of cultivation (Fig. 2B). While the growth rate was slightly reduced around the same timepoint, exponential growth continued, thus not explaining the plateaued expression. Notably, the expression of TDH3p-mCherry did not plateau in high temperature as clearly as the expression of the synthetic systems. Though the synthetic systems are primarily constructed by orthogonal parts, one limitation is the use of native promoters to express the sTFs. While the *TDH3* promoter was the least affected by the

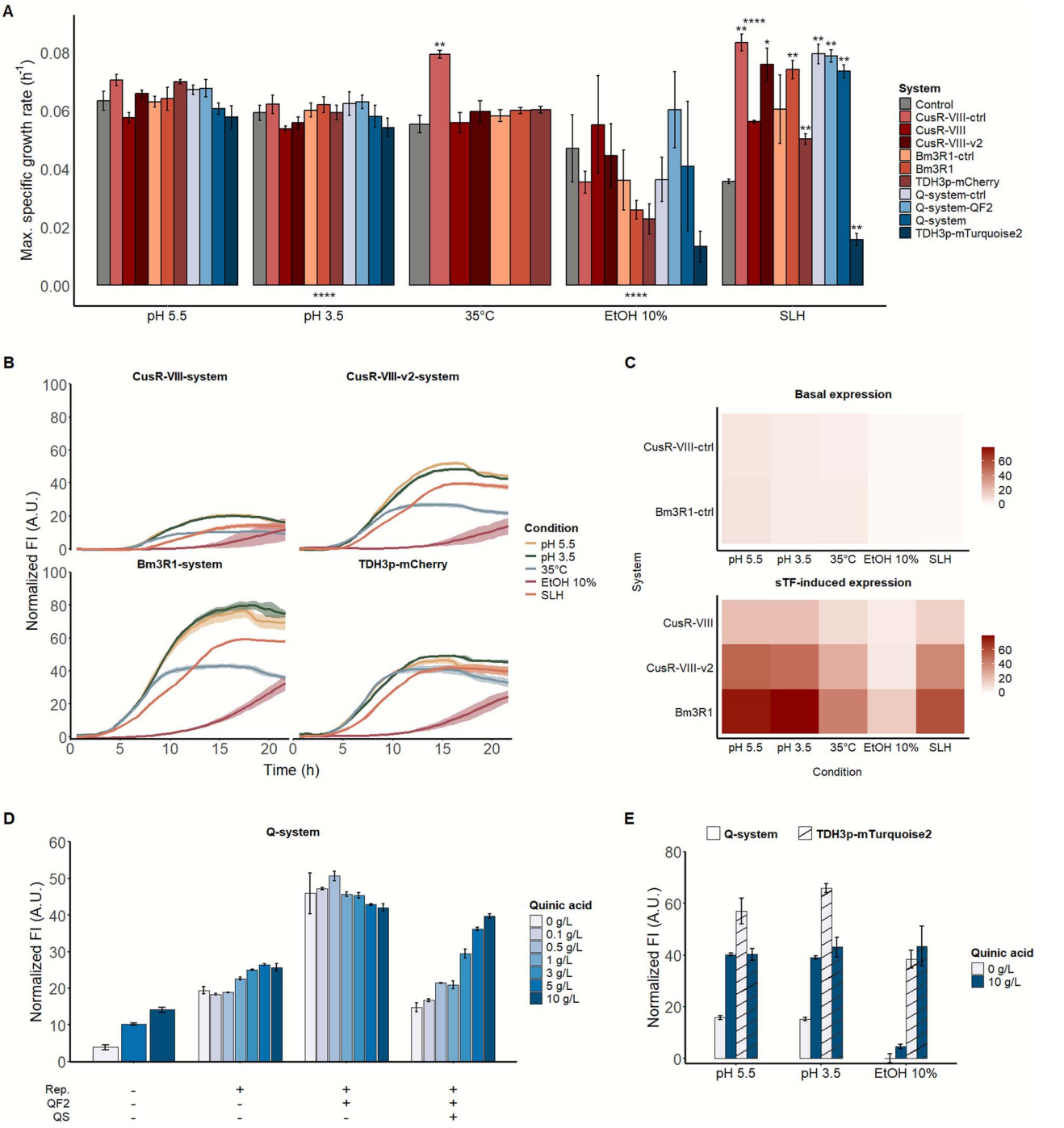


Figure 2. Characterization of MoClo-adapted expression systems in various industrially relevant conditions. (A) The maximum specific growth rate of the parental strain (control), the expression system-harbouring strains, and their respective control strains. Statistical analyses were performed between the parental and all other strains for each medium using the Student's *t*-test with Holm–Bonferroni correction; significance is displayed above each bar. Statistical analyses were also performed between the control condition (pH 5.5) and all other industrially relevant conditions using the Student's *t*-test with Holm–Bonferroni correction; significance is displayed below the bars, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$. (B) Reporter expression of the CusR-VIII-, CusR-VIII-v2-, and Bm3R1-systems as well as of TDH3p-mCherry monitored over time. (C) Heatmap of reporter basal expression (top) and sTF-induced reporter expression (bottom) in the CusR-VIII-, CusR-VIII-v2-, and Bm3R1-systems after 17 h of cultivation when cells in all conditions had entered the exponential growth phase. (D) Assessment of the Q-system and its controls after 17 h of cultivation in control conditions with 0–10 g/L of quinic acid. The background expression of the parental strain and expression of the reporter without QF2-activation, with QF2-activation and with QS-mediated QF2-activation. (E) Reporter expression of the Q-system and TDH3p-mTurquoise2 after 17 h of cultivation with and without 10 g/L of quinic acid. Reporter expression was measured by fluorescence intensity (FI) and normalized against biomass. The values represent the mean of three replicates ± standard deviation (SD). pH 5.5: control condition, pH 3.5: acidic condition, 35°C: high temperature (not tested for the Q-system), EtOH 10%: high ethanol concentration, SLH: synthetic lignocellulosic hydrolysate, A.U.: arbitrary unit.

increased temperature, the activity of native promoters is often influenced by environmental factors [6]. Assessing the expression levels in the control condition, the CusR-VIII-system showed half the expression of TDH3p-mCherry, while the CusR-VIII-v2- and the Bm3R1-system showed 1.2- and 1.8-fold higher expression, respectively, after 17 h of cultivation.

The Q-system was first assessed in control conditions with 0–10 g/L quinic acid (Fig. 2D). Quinic acid has previously been shown to inhibit QS repression in a dose-dependent manner [12], and the behaviour was confirmed for the MoClo-adapted system (Fig. 2D). At 10 g/L quinic acid, 95% of the reporter expression was recovered compared with the system lacking the QS repressor (Q-system-QF2). The previous report of the Q-system in *S. cerevisiae* described just above 40% recovery of the reporter expression at 10 g/L quinic acid [12]. The reporter gene used in our MoClo-adapted system is different, but the systems and strains used are similar [10]. In the study by Lalwani *et al.* [12] the recovery was increased by expressing a quinate permease, leading to a 96% recovery at 100 mg/L quinic acid. Indeed, an improvement in induction recovery at lower concentrations of quinic acid was observed when a quinate permease [17] was co-expressed with the Q-system (Fig. S5).

Like the other systems, the Q-system and TDH3p-mTurquoise2 performed similarly in acidic conditions as in the control condition at 17 h of cultivation (Fig. 2E). At high ethanol concentration, the quinic acid induced expression for the Q-system was only about 10% of the expression in the control condition and there was no expression without quinic acid at this timepoint (Fig. 2E). However, the expression of TDH3p-mTurquoise2 in high ethanol concentration remained around 70% of the expression in the control condition at 0 g/L quinic acid and was even slightly increased compared with the control condition at 10 g/L quinic acid (Fig. 2E). The poorer expression in high ethanol concentration for the Q-system may nonetheless reflect the reduced growth in ethanol rather than a change in de-repression capacity by quinic acid. In SLH supplemented with 10 g/L quinic acid the cells failed to grow (Fig. S6). This suggests that the synergistic effects of quinic acid and the inhibitory components in SLH were too harsh for the cells. In control conditions no growth inhibition was observed up to 10 g/L quinic acid (Fig. S6). Still, previous studies have reported reduced growth in the presence of ≥ 10 g/L quinic acid [15], indicating its inhibitory effects. The difference in expression level for TDH3p-mTurquoise2 with or without quinic acid suggests that quinic acid may interfere with the mTurquoise2 expression measurement (Fig. 2E). While quinic acid is a weak acid capable of influencing the medium's pH, pH-dependent quenching is unlikely given that the media was buffered and mTurquoise2 remains stable over a broad pH range with a pK_a of 3.1 [31]. Another alternative is optical interference, where quinic acid may absorb or scatter light [32], thereby diminishing the measured fluorescence signal of mTurquoise2. Relative to the native *TDH3* promoter at 10 g/L quinic acid, the Q-system's expression in the control condition ranged from approximately 40% uninduced to 100% of the expression of the *TDH3* promoter upon induction (Fig. 2E). This highlights the advantage of the Q-system as a gene expression system with a broad dynamic range, capable of tuning expression from low to high levels.

Conclusion

A variety of gene expression systems have previously been developed for *S. cerevisiae* [8–10, 33]. However, such systems have generally only been tested in laboratory conditions. Here, three expression systems were evaluated in industrially relevant conditions, the two versions of the newly developed CusR-based system and the two previously developed Bm3R1- and Q-systems, which were adapted for MoClo compatibility. This study shows that although the expression systems do not affect growth in standard growth conditions and have an orthogonal design which presumably results in minor interaction with the native regulation, the performance of the systems is also affected by environmental factors. While it may be difficult to engineer a system that is completely unaffected by the environment, awareness of the limitations of an applied system is necessary. This study shows that the CusR-VIII-, CusR-VIII-v2-, and Bm3R1-systems are stable and predictable in acidic conditions whereas minor variations can be expected in high temperatures and in lignocellulosic hydrolysate. The Q-system was stable and predictable in acidic conditions but dysfunctional in SLH. In high concentrations of ethanol, quite large variations can be expected for all systems. With the newly developed CusR-based systems and a deeper understanding of the systems' performance in industrially relevant conditions, the systems can be better utilized in the design of new cell factories for industrial applications.

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Author contributions

E.B. performed the experiments and wrote the manuscript. Y.N. supervised and corrected the manuscript. E.B. and Y.N. designed the study together.

Supplementary data

Supplementary data is available at *SYNBIO* online.

Conflict of interest: No potential conflict of interest was reported by the authors.

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Data availability

Supplementary data available at SYNBIO online.

Material availability

The main plasmids that were constructed in this study are available through the Addgene repository (https://www.addgene.org).

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Synthetic Biology, 2025, **10**, 1–7
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