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Exploring Selective Pressure Trade-Offs for Synthetic Addiction to Extend Metabolite Productive Lifetimes in Yeast

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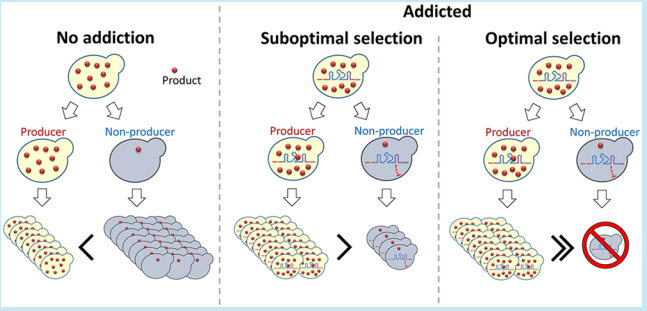
ABSTRACT: Engineered microbes often suffer from reduced fitness resulting from metabolic burden and various stresses. The productive lifetime of a bioreactor with engineered microbes is therefore susceptible to the rise of nonproductive mutants with better fitness. Synthetic addiction is emerging as a concept to artificially couple the growth rate of the microbe to production to tackle this problem. However, only a few successful cases of synthetic addiction systems have been reported to date. To understand the limitations and design constraints in long-term cultivations, we designed and studied conditional synthetic addiction circuits in *Saccharomyces cerevisiae*. This allowed us to probe a range of selective pressure strengths and identify the optimal balance between circuit stability and production-to-growth coupling. In the optimal balance, the productive lifetime was greatly extended compared with suboptimal circuit tuning. With a too-high or -low pressure, we found that production declines mainly through homologous recombination. These principles of trade-off in the design of synthetic addition systems should lead to the better control of bioprocess performance.



KEYWORDS: *industrial biotechnology, synthetic circuit balancing, metabolite production, biosensor, production control, population heterogeneity*

INTRODUCTION

Engineered microorganisms have been extensively used to build a more renewable and sustainable society. However, large-scale operations are required for cost-effective production to compete with chemical processes. The long-term operation of such a bioreactor can suffer from a loss of yield and production due to the natural accumulation of mutations.[1−3] The stability of metabolite production on an industrial scale has been an important issue because the performance of the bioreactor is directly related to the economic competitiveness of this renewable approach. The cause of the reduced yield in the bioreactor can be explained by single-cell variation owing to stochastic gene expression and mass-transfer heterogeneities.[1,4,5] However, another important cause of the long-term reduction of yield would be reduced fitness due to metabolic burden, which, in turn, leads to a complete loss of production over time.

For cost-effective operation, microbes are often heavily engineered to produce a maximum titer of metabolites. Metabolic burden commonly arises from certain metabolite depletions,[6] or toxicities from intermediates and end products[7] can cause the reduced fitness of engineered host organisms. Spontaneous mutations in these engineered microbes will create nonproductive mutants with better fitness. These nonproducers will take the majority of the population, which leads to a complete loss of production in the bioreactor.[3,8] Several attempts have been made to solve the genetic instability of engineered microbes.[9] A common way to achieve this goal is to reduce the mutation rate[10,11] to slow down the emergence of nonproductive mutants; however, this approach will only delay the reduced yield from arising, and thus a more selective way to extend the productive lifetime would be ideal.

Instead of delaying the occurrence of nonproductive mutants, selecting a producer population with a biosensor coupled to essential genes was proven to be effective to extend the productive lifetime in our recent study.[12] In this approach, the engineered *E. coli* was addicted to metabolite production to proliferate, thereby selecting the producer population without any conditioned medium. However, the engineering of stable synthetic addiction systems is difficult due to the required circuit tuning, and it is still limited to a few successful cases and the use of a conditioned medium.[1,12−15] Therefore, to develop better design principles for synthetic product addiction, in this

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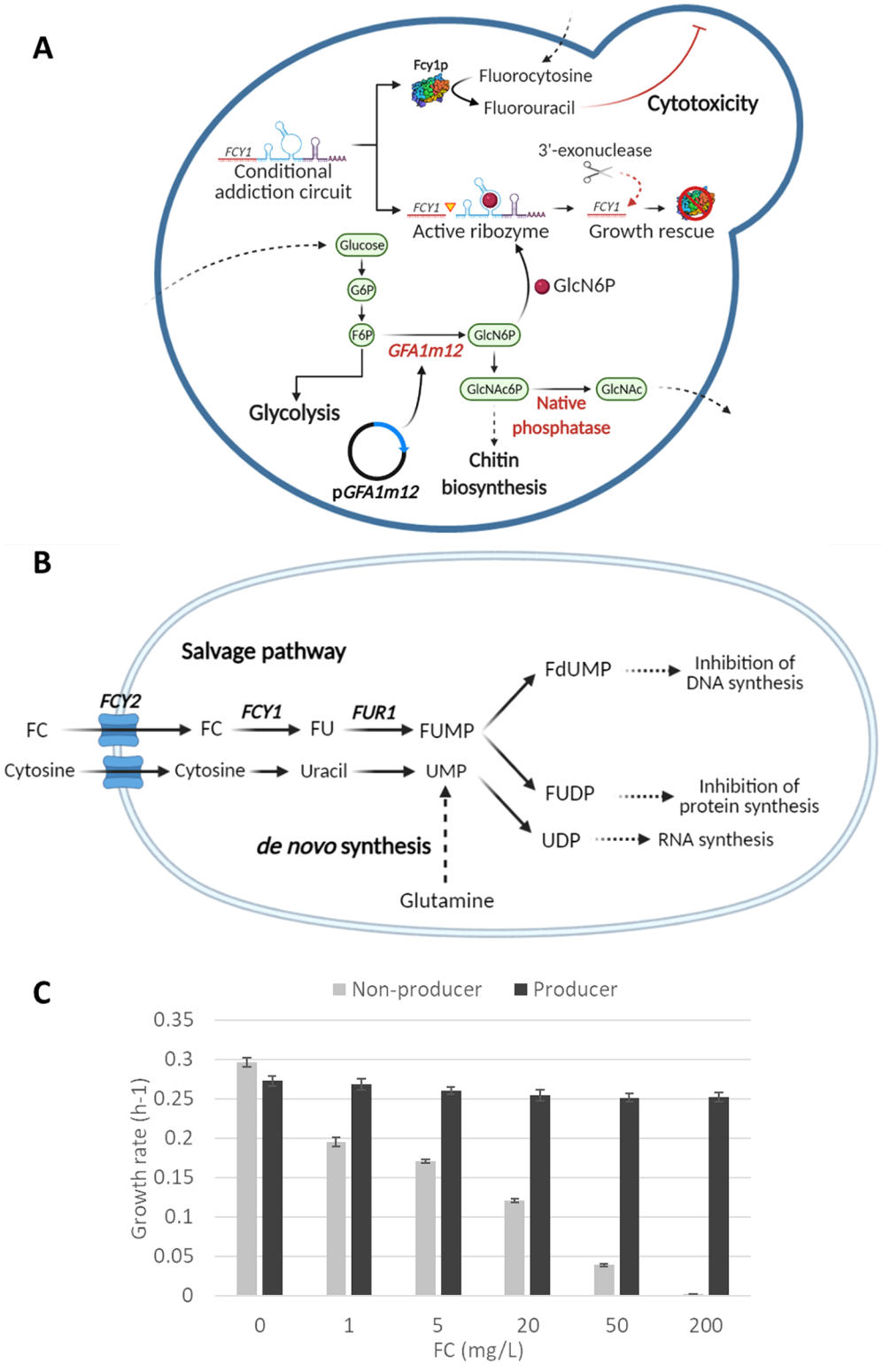

Figure 1. Conditional addiction circuit for yeast *S. cerevisiae.* (A) Suicide gene *FCY1* converts fluorocytosine (FC) to fluorouracil (FU). *glmS* ribozyme controls the expression of the suicide gene *FCY1*. A sufficient level of GlcN6P activates *glmS* ribozyme, which leads to degradation of the *FCY1* transcript, thereby restoring the cell growth (synthetic addiction). (B) Mechanism of FC toxicity. FC was imported and converted by three steps (*FCY2*, *FCY1*, and *FUR1*) in the salvage pathway. Disrupting the salvage pathway or activating the *de novo* synthesis of UMP can show FC resistance. FC: fluorocytosine, FU: fluorouracil, UMP: uridine monophosphate, UDP: uridine diphosphate, FUMP: fluoro-UMP, FdUMP: fluoro-deoxy-UMP, FUDP: fluoro-UDP, UPRTase: uracil phosphoribosyltransferase. This figure was inspired by Hope et al., 2004.[21] (C) Comparison of the specific growth rates of the producer and the nonproducer. Different levels of fluorocytosine (FC) were used to adjust the selective pressure. The producer showed only a minor change in response to the increased selective pressure. The nonproducer, however, showed a significantly reduced growth rate, confirming the usefulness of this biosensor in yeast. Error bars represent standard deviation (n = 3).

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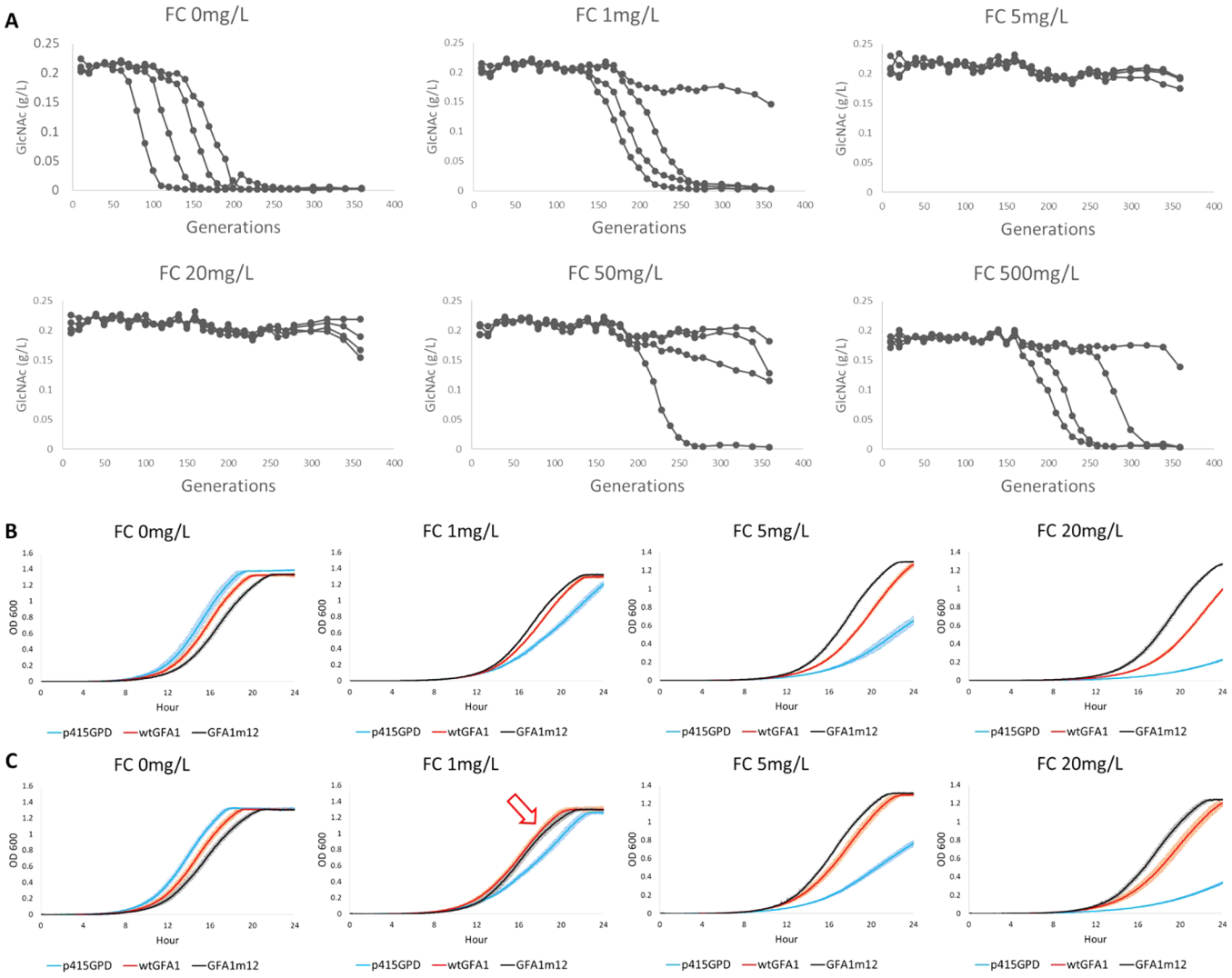

Figure 2. Optimal selection pressure improved production stability. (A) Long-term production was monitored with various selection pressure strengths. Without selective pressure (FC), populations lost production drastically after 100 generations (FC 0 mg/L). At low selective pressure (FC 1 mg/L) and high selective pressure (FC 50 and 500 mg/L), they also lost production after 150 generation. Moderate selective pressure (FC 5 and 20 mg/L) maintained production up to 350 generations, proving that optimal selective pressure can significantly extend the productive lifetime. Each line represents a different replicate. (B) Fresh strain with synthetic addiction can select *GFA1m12* from *wtGFA1* with selective pressure (FC). (C) Strain pregrown in FC 1 mg/L failed to select *GFA1m12* from *wtGFA1* only at FC 1 mg/L (red arrow). Fast adaptation to mild FC stress in yeast seemed to reduce the lag phase, thereby failing selection at the lowest selective pressure (FC 1 mg/L). Error bars represent the standard deviation (n = 4).

study, we are exploring its design in Baker's yeast, *Saccharomyces cerevisiae*, focusing on the tuning of the biosensor-selection interface through a conditional selection gene, *FCY1*. *S. cerevisiae* is a commonly used industrial microbe, yet there is limited knowledge of its genetic instability during long-term bioproduction. In *S. cerevisiae*, a biosensor-based production extension was recently introduced for vanillin β-glucoside production and monitored on relatively short cultivation scales of 60 generations.[14] Therefore, it remains unknown if *S. cerevisiae* can sustain production significantly longer, whether such synthetic stabilization circuits themselves collapse, and what are the design principles to sustain addiction over very long-term or even in continuous manufacturing. By understanding the nature of genetic instability, we can also suggest countermeasures to circumvent the emergence of nonproductive mutants, which may greatly improve the economic competence of bioprocess.

RESULTS

Tunable Circuit for Artificial GlcNAc Addiction in Yeast. Synthetic addiction can couple the growth rate to the production through product-sensitive biosensors and essential genes to stabilize production in long-term cultivation.[12] We decided to investigate this strategy further in *S. cerevisiae* to understand the constraints and design considerations of bioproduction and synthetic stabilization. We hypothesized that an optimized window for selective pressure would provide better production stability and therefore opted for a tunable selection circuit design. In a previous study, we developed a suicide genetic circuit that can isolate efficient enzyme mutants from directed evolution with high-throughput screening in *S. cerevisiae*.[16] Because this conditional addiction circuit allows a tunable selective pressure through medium supplementation and without additional genetic modification, it was further utilized in this study (Figure 1A). The conditional addiction

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Table 1. Summary of Production Monitoring with Various Selective Pressure Strengths[a]

		GlcNAc production	*GFA1m12* seq	FC sensitivity	*FCY1*(circuit) seq	*FCY2* seq	*FUR1* seq
FC 0 mg/L	**rep1**	**complete loss**	***wtGFA1***	intact			
	rep2	**complete loss**	***wtGFA1***	intact			
	rep3	**complete loss**	***wtGFA1***	intact			
	rep4	**complete loss**	***wtGFA1***	intact	**R105L**		
FC 1 mg/L	**rep1**	**decreasing**	intact	intact			
	rep2	**complete loss**	***wtGFA1***	intact			
	rep3	**complete loss**	***wtGFA1***	**resistant**			
	rep4	**complete loss**	***wtGFA1***	intact			
FC 5 mg/L	**rep1**	intact	intact	**diauxic**			
	rep2	intact	intact	**diauxic**			
	rep3	intact	intact	**partially resistant**			
	rep4	intact	intact	**diauxic**			
FC 20 mg/L	**rep1**	intact	intact	**resistant**		**T380P**	
	rep2	intact	intact	**resistant**		**T380I**	
	rep3	intact	intact	**resistant**			
	rep4	intact	intact	**diauxic**			
FC 50 mg/L	**rep1**	**complete loss**	***wtGFA1***	**resistant**	**D92Y**		
	rep2	**decreasing**	intact	**resistant**	**G96D**		
	rep3	**decreasing**	intact	**resistant/diauxic**	**frame shift**		
	rep4	**decreasing**	intact	**resistant**		**repeat**	
FC 500 mg/L	**rep1**	**complete loss**	***wtGFA1***	**resistant**	**G14S**		
	rep2	**decreasing**	intact	**resistant**			
	rep3	**complete loss**	***wtGFA1***	**resistant**	**D11G**		
	rep4	**complete loss**	**MultipleSNPs**	**resistant**	**G14C**		

[a]Samples at the end of production monitoring were analyzed. The GlcNAc production and the sequencing results for production plasmid (*GFA1m12*), *FCY1*, *FCY2*, and *FUR1* are listed.

circuit expresses the suicide gene of *S. cerevisiae FCY1* in response to the intracellular metabolite level of the target product intermediate glucosamine-6-phosphate (GlcN6P). This feedback is attained through an allosteric self-cleaving ribozyme inserted into a 3′-untranslated region of *FCY1* that reacts with the GlcN6P level and controls the expression of *FCY1*. The translated Fcy1p converts fluorocytosine (FC) to fluorouracil, inducing cytotoxicity. Therefore, different levels of selective pressure can be applied by the FC level in the medium. This conditional addiction circuit is based on a different design than that of our previous report on production control[12] or another study exploiting auxotrophy/antibiotic resistance in *E. coli*.[1] We hypothesized that an FC-dependent genetic circuit could be used to probe which selective pressures will maintain the producer population in long-term cultivation, experimentally simulating the industrial growth duration and the failure modes of the circuit.

To biosynthesize elevated concentrations of GlcNAc (ca. 0.2 g/L extracellular), we overexpressed a mutant version of *GFA1* (*GFA1m12*) that was isolated from high-throughput screening using the GlcN6P-responsive biosensor in our previous study.[16] *GFA1* is responsible for a rate-limiting step of cell-wall biosynthesis in *S. cerevisiae*. Because we aimed to monitor the long-term production stability, we overexpressed only *GFA1m12* to make a simplified pathway with a minimal burden rather than having a heavily engineered pathway for *N*-acetylglucosamine (GlcNAc) production in this study (Figure 1A). We cloned *GFA1m12* into the low-copy plasmid with a strong constitutive GPD promoter to make the GlcNAc production plasmid pGFA1m12. Next, we engineered *S. cerevisiae* CEN.PK2-1C to carry both the conditional addiction circuit and the pGFA1m12 and tested for the stability of GlcNAc production in this study.

To evaluate the activity of our conditional addiction circuit, we engineered GlcNAc producer and nonproducer strains and compared them. These engineered strains were grown in different FC concentrations to compare their specific growth rates (Figure 1C). Different concentrations of FC will apply different selective pressures, causing producers and non-producers to react differently. Nonproducers (strain with empty plasmid) showed an 82% growth rate reduction when high selective pressure (FC 50 mg/L) was applied, whereas producers (strain with GlcNAc production plasmid) showed only a 7.5% reduction (Figure 1C at FC 50 mg/L), as previously observed in a different strain background.[16] The growth rate of producers showed an 8% reduction compared with that of nonproducers when no selective pressure was applied (Figure 1C at FC 0 mg/L). This fitness cost of production was caused by expressing *GFA1m12*, and we therefore hypothesized that spontaneous mutations on the production plasmid will restore the growth rate. Therefore, the overall production will decrease over time in long-term cultivation without any selective pressure.

To validate our conditional addiction circuit, we monitored engineered strains with the GlcNAc production plasmid pGFA1m12 in long-term cultivation to compare with and without selective pressure as a preliminary test. 0.1% of cultivated samples with the engineered strain were transferred to fresh medium every 48 h, and the GlcNAc level was monitored in each transfer. As we expected, populations without selective pressure (without FC) lost their production drastically after 100 generations (around 10 transfers) (Supplementary Figure 1). On the contrary, populations with selective pressure (with FC 25 mg/L) kept their production until 250 generations, proving the usefulness of the production control of our conditional addiction circuit. We hypothesized

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that proper selective pressure can further extend the productive lifetime; therefore, we tried to exploit the tunability of our conditional addiction circuit in the next step.

Production Monitoring Showed Moderate Selective Pressure Is Favorable. We hypothesized that optimal selective pressure should be important to extend the productive lifetime when using synthetic addiction systems. If selective pressure is too weak, then we anticipated that nonproducers will not be eliminated properly, and over time, they will eventually dominate the population. At the same time, we speculated that strong pressure will cause an additional burden that could compromise the integrity of the selection system. To address this question, we decided to monitor our engineered strain with various ranges of selective pressure, expecting that an unknown, optimal point could extend the productive lifetime significantly better.

Serial transfer and GlcNAc level detection were carried out as in a preliminary test to experimentally simulate long-term cultivation using different selection pressures. As we expected, control samples without any selective pressure started to lose their production around 100 generations, similar to the previous test (Figure 2A, FC 0 mg/L). Interestingly, samples with the lowest and highest selective pressures started to lose their production around 150 generations following the control sample (FC 1 and 500 mg/L, respectively). Samples with relatively high selective pressure (FC 50 mg/L) also showed poor stability, whereas moderate selective pressure (FC 5 and 20 mg/L) performed the best. Indeed, moderate selective pressure could extend a productive lifetime of our strain from 100 generations to at least 350 generations, proving that a more cost-effective operation for the industrial scale is possible with synthetic selection.

Declining Production and Circuit Function Are Genetically Explained by Homologous Recombination and FC Resistance Development. We observed that moderate selective pressure greatly the extended productive lifetime, whereas populations experiencing low or high selective pressure lost their GlcNAc production much earlier in our production monitoring (Figure 2A). To verify the underlying mechanism of their behavior, we further collected and analyzed samples after the last transfer. Their GlcNAc production and sequencing results for the production plasmid (*GFA1m12*), *FCY1* (addiction circuit), *FCY2*, and *FUR1* are summarized in Table 1. In all examples of lost GlcNAc production, we found mutations on the *GFA1m12*-encoding gene of the production plasmid. Developed FC resistance could be explained by mutations on the *FCY1*, *FCY2*, and *FUR1* loci from the genome.

Interestingly, the common error mode on the production plasmid was the gene conversion of *GFA1m12* to wild-type *GFA1* (*wtGFA1*) by homologous recombination (Table 1). Because *GFA1m12* requires a 4 base pair (bp) substitution to revert to *wtGFA1*, it is unlikely that this evolution is caused by a spontaneous mutation. All samples that showed a complete loss of GlcNAc production had wild-type *GFA1* instead of *GFA1m12* on their production plasmid, except for FC 500 mg/L rep4, which had multiple mutations. *GFA1m12* was isolated from directed evolution with the error-prone polymerase chain reaction (PCR) and has four amino acid substitutions (V12L, Q96H, Q157R, and E343V) compared with the wild-type *GFA1* sequence.[16] These mutations seemingly eliminated the allosteric inhibition of Gfa1p by uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), an end product of the chitin biosynthetic pathway.[16] Therefore, we can expect that reversion to wild-type *GFA1* will significantly reduce the GlcNAc production.

Wild-type *GFA1* on the genome of CEN.PK 2-1C was not deleted in this study because it is an essential gene for chitin and cell-wall biosynthesis. However, strong homologous recombination activity in yeast reverted *GFA1m12* to wild-type *GFA1* in long-term cultivation, which leads to reduced fitness costs caused by GlcNAc production. Indeed, we observed that expressing wild-type *GFA1* on the same plasmid backbone had a better growth rate than *GFA1m12*, although it was lower than that of the empty plasmid (Figure 2B, FC 0 mg/L). The strain with a plasmid expressing wild-type *GFA1* did not secrete a detectable amount of GlcNAc; however, this strain was able to grow slightly faster than the *GFA1m12* strain when the strain was pregrown at FC 1 mg/L once and tested (Figure 2C, FC 1 mg/L). This explains why the producer population was not properly selected at the lowest selective pressure (Figure 2A and Table 1, FC 1 mg/L). Fresh strains with a conditional addiction circuit can select *GFA1m12* over wild-type *GFA1*, even at FC 1 mg/L, when they encounter selective pressure for the first time (Figure 2B, FC 1 mg/L); however, yeast strains seemed to adapt quite quickly to mild FC stress,[17] and this leads to a slightly reduced lag time, resulting in the failure to select the producer population at the lowest selective pressure (Figure 2B,C at FC 1 mg/L).

Too-High Selective Pressure Selects for Resistance Development and Lost Addiction Function. Another interesting observation from our analysis is that relatively high selective pressure developed strong FC resistance and eventually disabled the genetic circuit. FC is a commonly used antifungal drug, and the molecular mechanism to confer resistance is well studied in the pathogenic yeast background.[18−21] This antifungal drug can inhibit DNA replication, transcription, and protein synthesis.[22,23] There are two major routes to confer resistance. One is by disrupting the pyrimidine salvage pathway (*FCY1*, *FCY2*, and *FUR1*), and the other is by increasing the *de novo* pyrimidine synthesis (Figure 1B).[20]

At higher selective pressure (FC 50 and 500 mg/L) in our monitoring, populations tend to disrupt the pyrimidine salvage pathway to survive severe stress from FC toxicity (Table 1). Evolved populations under this condition seemed to disrupt *FCY1*, *FCY2*, and *FUR1* directly to confer strong FC resistance. Indeed, populations under higher selective pressure (FC 50 and 500 mg/L) almost completely lost FC sensitivity whether or not they had intact *GFA1m12* (Table 1 and Supplementary Figure 2). A disruption in the salvage pathway was known to show high FC resistance in *S. cerevisiae*;[24−27] therefore, cells under higher FC concentration lose FC sensitivity and disable the genetic circuit to survive in high selective pressure, resulting in a loss of production in long-term cultivation. We confirmed this theory by observing populations in decreasing production at the highest selective pressure (FC 500 mg/L rep3 at #22 transfer). Nonproducers were the dominating producers because the conditional addiction circuit was disabled under this condition (Supplementary Figure 3). On the contrary, populations at lower selective pressure (FC 5 mg/L and lower) mostly maintained an intact FC sensitivity and therefore showed different responses when they had intact *GFA1m12* or *wtGFA1* (Table 1 and Supplementary Figure 2). Interestingly, populations showed a diauxic growth pattern mostly at moderate selective pressure (FC 5 mg/L), suggesting that populations adapted to mild FC stress without disrupting

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the pyrimidine salvage pathway (Table 1 and Supplementary Figure 2).

Homologous Recombination Was a Major Mechanism of Production Decline. Because 10 out of 11 samples that completely lost production had converted *GFA1m12* to *wtGFA1* on their production plasmid (Table 1), the major mechanism of production decline in our system is likely to be homologous recombination. To further characterize the mechanisms of decline, we investigated single colonies from streaks of several samples featuring decreasing production but not a complete loss (Supplementary Figure 4). Replicate 1 from FC 1 mg/L and replicate 2 from FC 50 mg/L consisted of a mixed population with producer cells (intact *GFA1m12*) and escaper cells (*wtGFA1*). Replicate 3 from FC 50 mg/L and replicate 2 from FC 500 mg/L showed only a minor decrease at the end of the production monitoring. This explains why a production decline was not captured from eight selected colonies. It also suggests that there will be a minor population showing a complete loss of production by homologous recombination.

Interestingly, replicate 4 from FC 50 mg/L showed a linear decrease in production monitoring, and the major population showed a ∼20% reduced production; however, the GPD promoter and *GFA1m12* ORF sequence were intact (Supplementary Figure 4), suggesting that mutations downstream of the GlcNAc pathway, outside of the production plasmid, caused the slightly reduced production. Further studies should include whole-genome sequencing, but we speculate possible mutation targets to include the gene encoding Gna1p converting GlcN6P to GlcNAc6P or native phosphatase (Figure 1A).

DISCUSSION

Microorganisms used in bioreactors are heavily engineered to focus on the higher yield and production of the metabolite;[28] however, once they lose their production by spontaneous mutations, nonproductive mutants will dominate the entire population in the reactor due to the better fitness. Therefore, the original population producing a high level of metabolite will be outgrown by these nonproducers. This problem leads to reduced production and impaired overall performance, thereby hampering the economic operation of sustainable alternatives against chemical processes.

In this study, our genetic circuit selected high-performing producers against nonproducers (Figure 1C). One advantage of this circuit is that it can apply different selective pressure by modulating the FC concentration in the medium without introducing genetic modifications. With tunable selective pressure control, we showed that optimal selective pressure can significantly extend the productive lifetime (Figure 2A).

In our observation, synthetic addiction could not enrich producers (strain with intact *GFA1m12*) from escapers (strain with reverted *wtGFA1*) at the lowest selective pressure (Figure 2C, FC 1 mg/L). Although this condition can select producers (strain with *GFA1m12*) from nonproducers (strain with empty plasmid), evolved populations mostly have reverted *wtGFA1* due to the strong homologous recombination activity in yeast. Our previous study in engineered *Escherichia coli* showed that the common error mode was disruption by mobile elements.[8,12] These results imply that one should consider a common error mode of engineered microbes to find an optimal biosensor design. Strong recombination activity in yeast can be applied for various purposes[29−32] but can also cause instability in the engineered strain.[14] We expect that deleting the potential homology region (*GFA1* locus on the genome in our case) will further improve the production stability; therefore, understanding the common error mode will be critical to operating long-term cultivation with engineered hosts.

At extreme selective pressure (FC 500 mg/L), populations maintained production slightly longer than at low selective pressure (Figure 2A); however, we observed that evolved populations under this condition tend to develop/evolve FC resistance before the production plasmid is damaged (Supplementary Figure 3). A loss of FC sensitivity caused by the disrupted pyrimidine salvage pathway leads to the disabling of our conditional synthetic addiction. Therefore, it could not select the producer population and was susceptible to the rise of nonproducers, which explains its poor performance at maintaining production stability. In contrast, a moderate selective pressure (FC 5 mg/L) maintained both the FC sensitivity and the GlcNAc production. The GlcNAc productive lifetime was greatly extended to 350 generations (Figure 2A). Samples from FC 20 mg/L developed FC resistance (Table 1 and Supplementary Figure 2), although GlcNAc production was maintained, suggesting that the addiction circuit cannot select the producer population any longer, as we can observe a slight decline at the end of monitoring (Figure 2A, FC 20 mg/L). Therefore, we can conclude that the optimal selective pressure for this addiction circuit is around FC 5 mg/L. These results confirmed that a proper balance in selective pressure is important for the long-term cultivation of engineered microbes.

Although our genetic circuit is useful for stable long-term production, it requires additional fluorocytosine in the medium, which could cause additional cost and downstream processing. Therefore, it is preferable to replace fluorocytosine, an environmentally problematic antifungal drug, for large-scale industrial operations. A modified genetic circuit that does not require additional cost can be used instead. Several other candidates to induce cytotoxicity can be tested to replace the suicide gene *FCY1*, which requires fluorocytosine. Genes inducing cytotoxicities such as *GIN11M86*[33] or *PKA3*[34] can be tested in the future to improve our strategy. In this case, it will require fine-tuning of the expression level to optimize the selective pressure properly. For continuous fermentation processes, these approaches for studying genetic heterogeneity could be even more important and should be conducted in settings that more closely mimic the continuous fermentation process.

Synthetic addiction can enrich the producer population against the nonproducers, but their performance depends on their context in long-term cultivation.[12,14] Transcription-factor-based biosensors controlled the essential gene expression in these examples. On the basis of our observation from conditional synthetic addiction in this study, fine-tuning essential gene expression levels can potentially reduce the occurrence of escaper mutants resistant to selective pressure and further improve the productive lifetime. Applying various translational strengths (including ribosome binding site (RBS) optimization) for essential gene expression, for example, can help us to find an optimal point for synthetic addiction in the future.

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METHODS

Strain and Plasmid Construction. Similar to our previous study,[16] a conditional addiction circuit and production plasmid were cloned and introduced to the *Saccharomyces cerevisiae* CEN.PK2-1C strain to make the *N*-acetylglucosamine (GlcNAc) producer strain in this study. In brief, the integration fragment consisting of the *glmS* ribozyme and the *URA3* cassette synthesized as a gBlock (IDT, Coralville, IA) was PCR-amplified using FGU insertion F/R primers and inserted into 3′-UTR of the *FCY1* locus in the CEN.PK2-1C strain by the LiAc transformation method to make a conditional addiction circuit strain SAY. *GFA1m12* (synthesized by IDT as a gBlock) from the previous study[16] and wild-type *GFA1* were cloned into the p415GPD plasmid[35] by USER cloning[36] using GFA1m12 USER F/R and p415GPD BB F/R primers to make plasmids pGFA1m12 and pGFA1, respectively. p415GPD, pGFA1, and pGFA1m12 plasmids were transformed into the SAY strain to construct a nonproducer (with p415GPD), escaper (with pGFA1), and producer (with pGFA1m12) strain. Strains, plasmids, and primers used in this study are listed in Supplementary Table 1.

Cultivation Conditions for Production Monitoring. The strains were cultivated on a synthetic defined minimal medium containing 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids, and 1.6 g/L yeast synthetic drop-out medium supplements without leucine (SD-Leu). For long-term production monitoring, strains were cultivated in a 24-deep-well plate with 1 mL of the medium. 1 μL of the previous culture was transferred to a new plate containing fresh medium every 2 days (0.1% transfer). We applied various selective pressures with different FC concentrations (0, 1, 5, 20, 50, and 500 mg/L), and four replicates were monitored for each concentration. After each transfer, cultures were centrifuged, and cell pellets were freeze-stored. The supernatant was also stored to determine the extracellular GlcNAc level.

Determination of GlcNAc Titer by High-Performance Liquid Chromatography. The extracellular GlcNAc level was determined by high-performance liquid chromatography (HPLC), similar to a previous study.[37] In brief, the culture medium was centrifugated, and the supernatant was analyzed by HPLC. The GlcNAc peak was detected at 205 nm wavelength by a UV detector (Ultimate 3000 HPLC system) at 12 min in the Aminex HPX-87H column (300 × 7.8 mm) using 5 mM H_2SO_4 as an eluent at 60 °C.

Sequencing Analysis of Plasmid and Salvage Pathway Genes from Production Monitoring. We extracted the genomic DNA and plasmid from samples after #36 transfer to verify the sequence of the production plasmid and salvage pathway genes. The freeze-stored cell from the #36 transfer was cultivated again in SD-Leu medium, and cells were extracted for genomic DNA[38] and plasmid DNA (Zymoprep Yeast Plasmid Miniprep II). To check *FCY1* and the addiction circuit sequence, we used primer FGU seq F/R for the PCR amplification from the extracted genomic DNA and the subsequent Sanger sequencing. Similarly, FCY2 seq F/R and FUR1 seq F/R were used for the *FCY1* and *FUR1* sequences, respectively. The GPD promoter and *GFA1m12* sequence on the production plasmid were PCR-amplified and verified by Sanger sequencing with the GPD promoter F and GFA1m12 USER F/R primers from the extracted plasmid DNA. Sequencing data were aligned to check the mutation sites compared with the wild-type sequence.

Growth Monitoring of Engineered Strains with a Microtiter Plate Reader. We monitored the growth profile of the engineered strains with a microtiter plate reader (Synergy H1) similar to our previous study.[12] In brief, freeze-stored strains were streaked on the SD-Leu plate. A single colony from each strain was cultured and monitored with double-orbital shaking (550 rpm) at 30 °C in 48-well plates (Corning 3548) containing 500 μL of SD-Leu medium. The OD_{600} was measured every 5 min with different FC concentrations in the medium to compare their growth profiles.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00240.

Supplementary Table 1. Strain, plasmid, and primers. Supplementary Figure 1. Preliminary production monitoring with an addiction system. Supplementary Figure 2. FC sensitivity of different samples after production monitoring. Supplementary Figure 3. Confirmation that FC resistance was developed before production decline in the highest selective pressure. Supplementary Figure 4. Samples with reduced production were also likely to be caused by homologous recombination (PDF)

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Author Contributions

S.-W.L., P.R., and M.O.A.S. designed the research. S.-W.L. performed the experiment. S.-W.L., P.R., and M.O.A.S. analyzed the data. S.-W.L., P.R., and M.O.A.S. wrote the manuscript.

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The authors declare the following competing financial interest(s): P.R. has a financial interest in Enduro Genetics ApS, which holds proprietary synthetic addiction technology.

ABBREVIATIONS

GlcNAc, *N*-acetylglucosamine; GlcN6P, glucosamine-6-phosphate; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; FC, fluorocytosine; *wtGFA1*, wild-type *GFA1*; *GFA1m12*, efficient mutant of *GFA1*

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