Analysis of 3’ UTR Modifications Using Streamlined Plasmid Production and Cell Growth Systems

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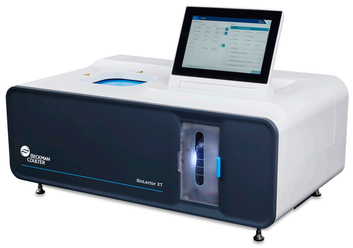
INTRODUCTION

Many different factors regulate mRNA levels in yeast cells, including the 3’ untranslated region (UTR) of the gene. The 3’ UTR affects mRNA stability and translation, leading to differences in protein expression levels. Regulatory motifs within the 3’ UTR have been identified, and different 3’ UTR sequences can have a strong impact on protein expression. Several studies (Savinov et. al. 2021, Shalem et. al. 2015, Shalem et. al. 2013) identified 3’ UTR sequences that increase expression with a constitutive promoter. In this study, a high-expressing 3’ UTR from Shalem 2013 was combined with the ADH2 promoter, which induces expression when the yeast transitions from utilizing glucose as a carbon source to using ethanol. The ADH2 promoter has been used for chemical production as it allows the yeast cells to reach higher biomass levels before starting production and then transition their energy entirely to chemical production. In addition to observing the production of this combination, I plan to mutate the 3’ UTR and observe the impact on protein expression.

To speed up the build and test processes, two new technologies will be utilized: the EMnetik system and the BioLector XT Microbioreactor. PCR and plasmid purification is performed on the Emnetik system, which uses magnetic beads for binding and releasing DNA. The system then mixes the beads using an electromagnet, leading to less variability due to pipette mixing. The process is also faster than column PCR purification kits, and a set of 24 samples can be purified in 16 minutes.

The BioLector XT is plate bioreactor system that can run 48 strains at a time in batch mode or 32 when fed with microfluidics. The instrument can measure biomass, dissolved oxygen, pH, and fluorescence non-invasively in real time while managing humidity, temperature, shaking, and air flow to the samples. The system works with a variety of different cell types, including aerobic and anaerobic bacteria, yeast, and fungi.



METHODS

The ADH2 promoter and Rpl3 3’ UTR were amplified from the genome of *S. cerevisiae* BY4741. eGFP was amplified from a preexisting plasmid. Primers designed for Gibson assembly were used for these amplifications. If yields were low from the initial PCR, an additional PCR was run to increase the DNA fragment yield. The pRS-416 plasmid was cut with Xho1 and Not1-HF to form a linearized plasmid for Gibson assembly. The cut plasmid, as well as ADH2 and GFP fragments which showed multiple bands after PCR, were purified using gel extraction. The 3’ UTR was purified using the EMnetik PCR cleanup kit. The four DNA fragments were combined 1:1:1:1 and concentrated with AMPure XP Reagent to reduce the required volume for Gibson assembly. Gibson assembly was done with NEB Gibson reagent and transformed into *E. coli* cells. Plasmids were isolated from *E. coli* cells using the EMnetick Plasmid Purification kit and cut with ScaI to check for the presence of the ADH2-GFP-UTR inserts. The new pRS-GFP plasmid and an empty pRS-416 were transformed into *S. cerevisiae* BY4741.

To vary the 3’ UTR, mutagenic PCR was performed using the Rpl3 3’ UTR as a template. The mutagenic PCR resulted in a single, clear band and was purified with the EMnetik PCR purification kit. The ADH2-GFP fragment was amplified from the pRS-GFP vector, and gel purified to remove off target DNA. Gibson assembly was performed with linearized pRS 416, ADH2-GFP, and the variable UTR and transformed into *E. coli.* Plasmids were purified with the EMnetik plasmid purification kit and will be sequenced. Plasmids were transformed into *S. cerevisiae* BY4741.

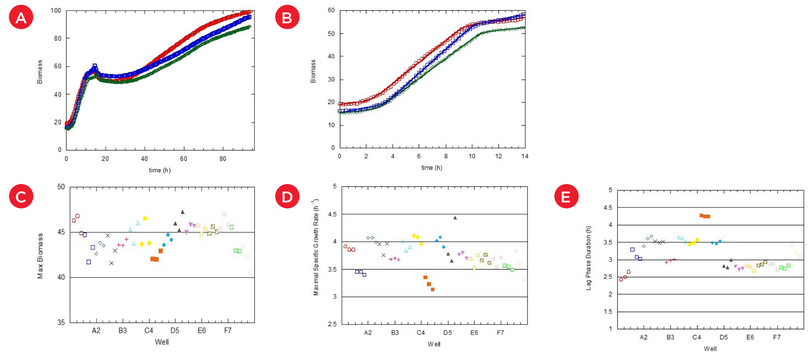
Yeast strains with an empty pRS-416 vector, pRS-GFP, and plasmids with 15 3’ UTR variants were grown in triplicate in a BioLector XT device in FlowerPlate microtiter plates for improved gassing transfer rates. Strains were grown in batch culture with 1 mL of SD – Ura media with 800 rpm shaking and 30 mL/min air. Biomass was measured at three different gains (2, 4, and 6), as well as pH, dissolved oxygen, and GFP fluorescence.

RESULTS

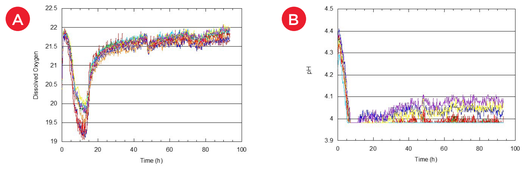
Biomass measurements showed two phases of yeast growth, with an early phase correlating to glucose utilization and a second phase that occurs after the yeast starts utilizing ethanol **(A)**. The first growth phase was fit to a Baranyi equation that included a linear phase to account for the impact of the second growth phase **(B)**.

$$y(t) = y_{max} + \ln\frac{-1 + e^{\mu_{max}\lambda} + e^{\mu_{max}t}}{-1 + e^{\mu_{max}t} + e^{\mu_{max}\lambda + y_{max} - y_0}} + At$$

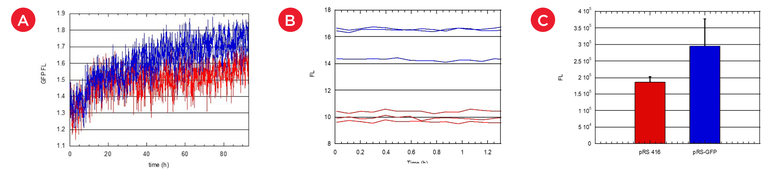
Y_{max} is the maximal OD, μ_{max} is the maximal specific growth rate, λ is the lag phase duration, y_0 is the initial OD, and A is the linear component of the second growth phase. All strain variants grew consistently, with similar maximum biomass, maximum specific growth rates, and lag times **(C-E)**. Different plasmid variants are shown in different colors.



The dissolved oxygen **(A)** and pH **(B)** were also measured; results were similar across all strains. The measurements from row A are shown below.



GFP fluorescence was very low with this promoter/3’ UTR combination. The three replicates on the BioLector XT microreactor were averaged to get a clearer picture of the fluorescence changes **(A)**. Fluorescence starts increasing at about 24 hrs, which is consistent with timing for ethanol utilization. The GFP plasmid **(blue)** shows significantly more fluorescence than an empty plasmid **(red)**. After the BioLector run, different gain setting were tried, and increasing the gain to 6 from 1 significantly increased the separation between an empty plasmid and the plasmid with GFP **(B)**. These same samples were then measured on a plate reader and the low fluorescence seen there was consistent with that seen on the BioLector **(C)**, incdicating that the low fluorescence is a biological issue and not an instrument issue.



CONCLUSIONS

- The combination of the ADH2 promoter and the Rpl3 3’ UTR resulted in very low levels of gene expression, indicating that the effect of the 3’ UTR is promoter dependent.

FUTURE WORK

- The 3’ UTR mutations will be tested again with different GFP gain settings to increase the sensitivity of those readings and determine if the mutations affect GFP mRNA stability.