Establishing CRISPR/Cas9 in *Lipomyces starkeyi*

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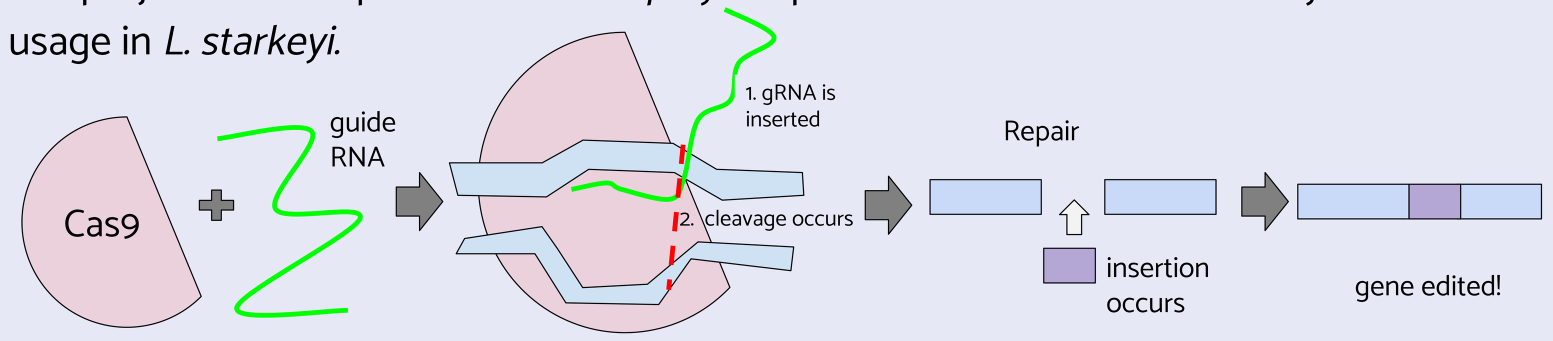
Introduction

Lipomyces starkeyi is an oleaginous yeast, meaning that it synthesizes and stores high amounts of intracellular lipids. This specific yeast can store lipids at concentrations higher than 60% of its dry cell weight.[1] Due to these high concentrations of lipids, *L. starkeyi* is a desired organism for the production of biofuels and other oleochemicals.[2] However, there is a lack of knowledge and of genetic tools when trying to engineer the cells to produce these lipids for our use. The genome editing tool, CRISPR/Cas9 is efficient and simple, therefore desirable for the engineering of *L. starkeyi*.[3] The goal of this project is to adapt the *Yarrowia lipolytica* plasmid based CRISPR/Cas9 system for usage in *L. starkeyi*.



Methods

Replacing the *Y. lipolytica* promoter with *L. starkeyi* P_{PYK1}

- The PYK1p was amplified from *L. starkeyi* genomic DNA template using PCR.
- An Asc1 and Sma1 restriction digest was done on the pCRISPRyl (*Yarrowia lipolytica* optimized) plasmid to cut out the promoter in order to insert the new PYK1 promoter. **(figure 1 b.)**
- An Asc1 and EcoRV restriction digest was done on the PYK1p.
- The PYK1p and pCRISPRyl were ligated together, now referred to as pCRISPR*Ls*.
- The ligated pCRISPR*Ls* was transformed in *E. coli* and plated for colonies.
- Plasmid candidates were purified and verified by restriction digest. **(figure 1 c.)**

Insertion of guide RNA

- pCRISPR*Ls* was digested with AvrII. **(figure 1 d.)**
- Gibson Assembly reaction was performed on the pCRISPR*Ls* and Lig4 hybridized oligos, and then transformed in *E.coli* and plated for colonies. **(figure 1 e.)**
- Plasmid candidates were purified and verified by sequencing.

Homology donor (pUC19 Lig4 Hygro) digestion

- A HindIII restriction digest was performed on the plasmid and then PCR purified.

Confirming protein expression in *L. starkeyi* strains transformed by *Agrobacterium tumefaciens*

- Strains were grown up to midlog phase.
- Proteins were extracted via TCA. (trichloroacetic acid)
- Proteins were separated by SDS-PAGE. (sodium dodecyl sulfate - polyacrylamide gel electrophoresis)
- A semi dry transfer to a PVDF membrane was completed..
- The Western blot was performed with FLAG-HRP (horseradish peroxidase) antibodies and enhanced with chemiluminescence to expose on xray film. **(figure 2.)**

Results

figure 1 a. Strategy for constructing pCRISPR*Ls* and insertion of guide RNA

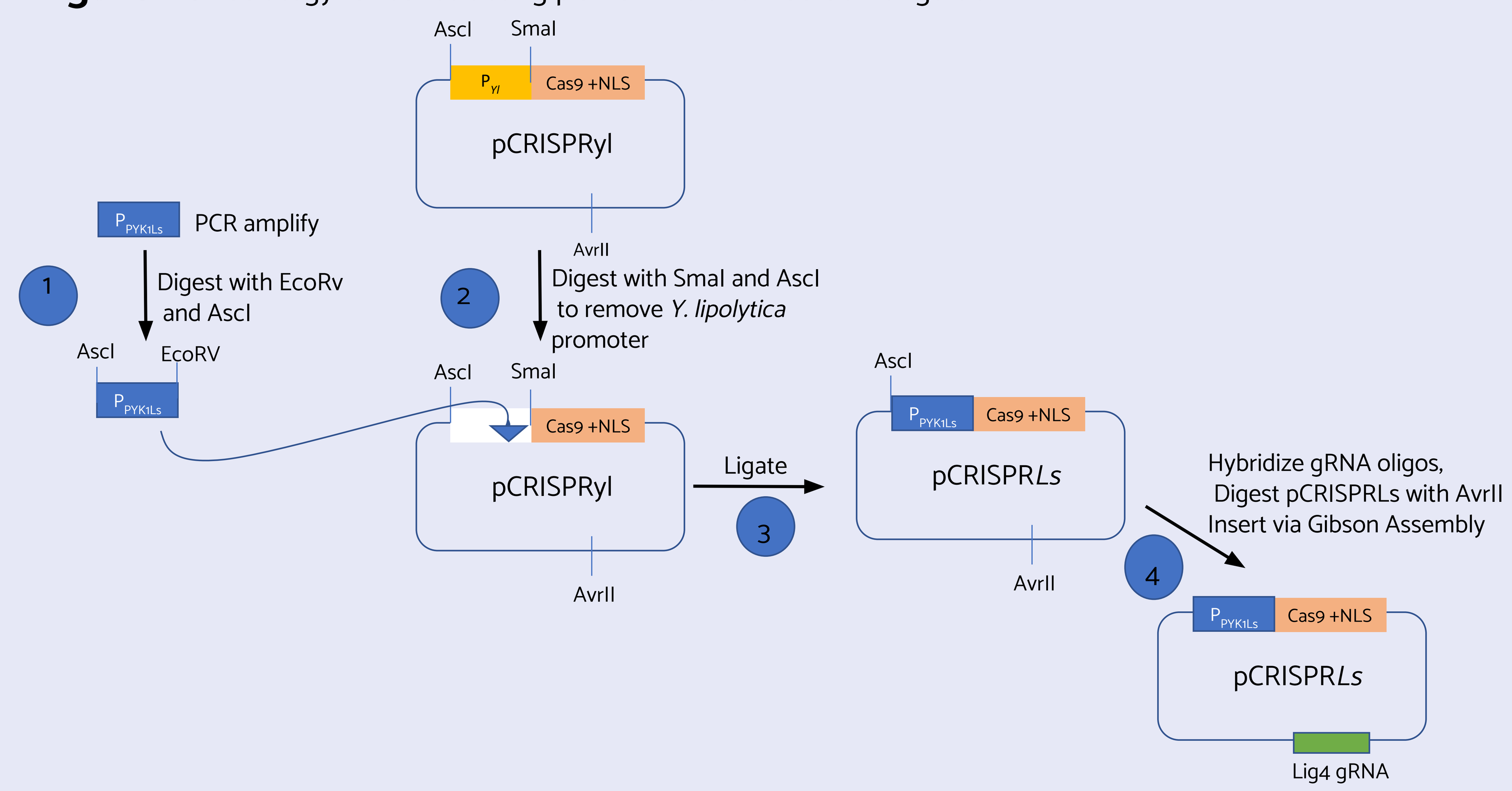


figure 1 b. pCRISPRyl Asc1 & Sma1 restriction digest gel

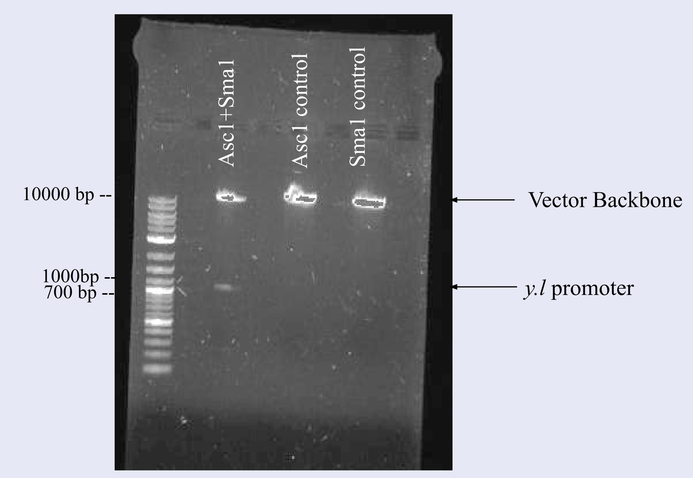


figure 1 c. pCRISPR*Ls* ligated plasmid candidates

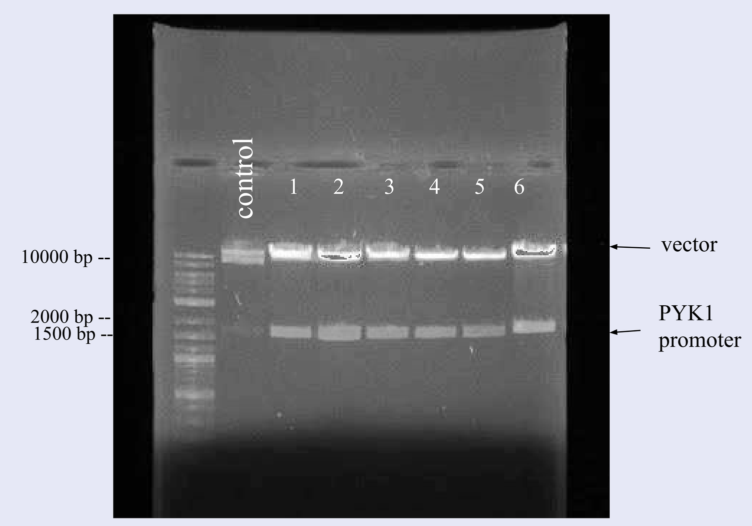


figure 1 d. pCRISPR*Ls* AvrII restriction digest gel

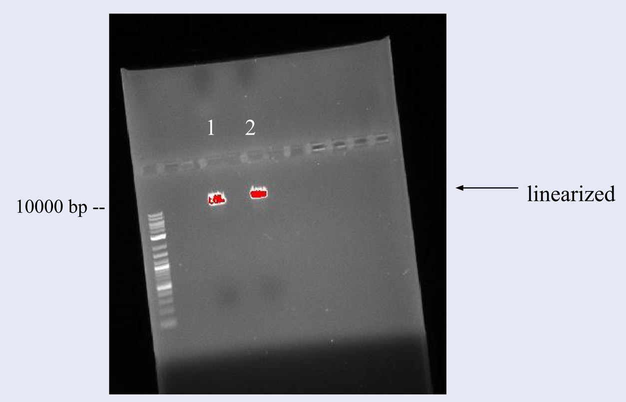


figure 1 e. Gibson Assembly reaction pCRISPR*Ls* candidates: mini preps of plasmid

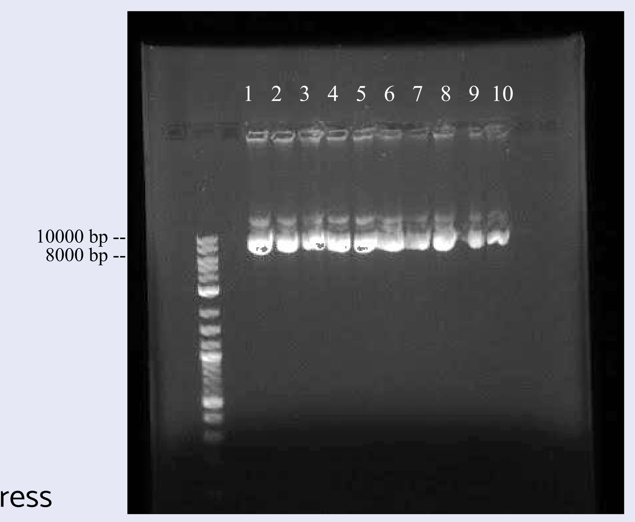
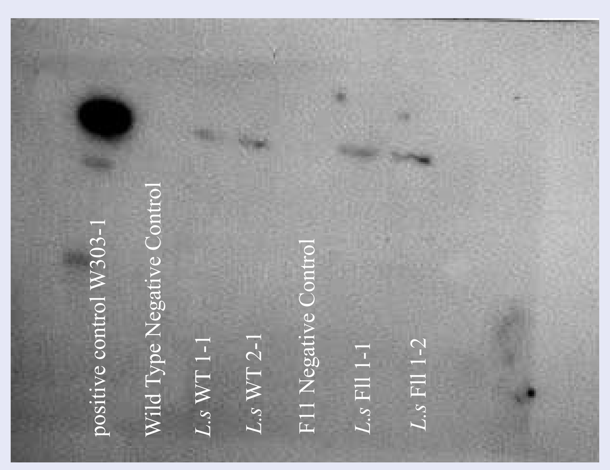


figure 2. Western blot indicating that the strains express the cas9 gene

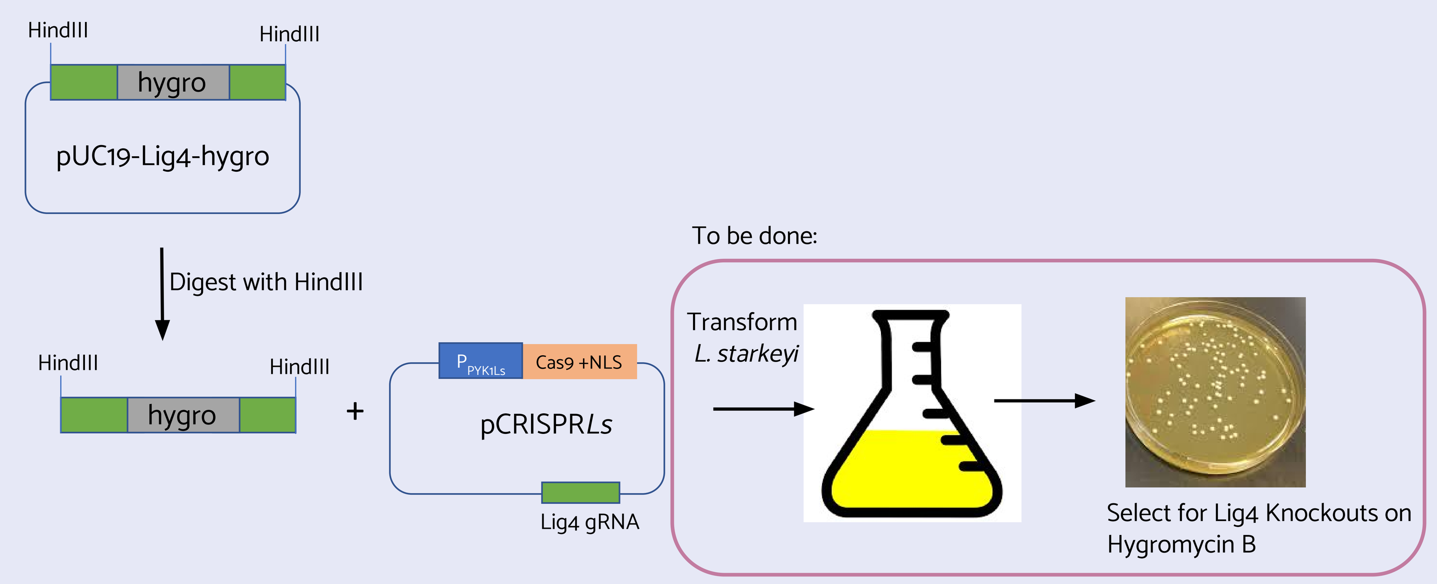


Conclusions

- pCRISPR*Ls* vector was successfully constructed.
  - PYK1p promoter was successfully inserted
  - gRNA insertion requires validation
- The homology donor was successfully digested.
- Western Blot indicated expression of the cas9 protein.

Future Directions

Use CRISPR/Cas9 to knockout Lig4 as proof of concept



Literature Cited

(1) Lin, J., Shen, H., Tan, H., Zhao, X., Wu, S., Hu, C., Zhao, ZK. (2011) Lipid production by Lipomyces starkeyi cells in glucose solution without auxiliary nutrients. http://dx.doi.org/10.1016/j.jbiotec.2011.02.010

(2) Shuobo, S., & Huimin, Z. (2017) Metabolic Engineering of Oleaginous Yeasts for Production of Fuels and Chemicals. http://dx.doi.org/10.3389/fmicb.2017.02185

(3) Schwartz C., Hussain M., Blenner M., Wheeldon I. (2015) Synthetic RNA Polymerase III Promoters Facilitate High-Efficiency CRISPR-Cas9-Mediated Genome Editing in *Yarrowia lipolytica*. http:/dx.doi.org/10.1021/acssynbio.5b00162

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