Novel methods for accelerating the development of more inhibitor tolerant yeast strains for cellulosic ethanol production

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INDUSTRIAL BIOTECHNOLOGY

Background

Towards a fossil-free transport sector

80% reduction fossil fuels in the Swedish transport sector 2010-2030

60% reduction in CO_2 emissions

Lignocellulosic materials as a renewable source for **2nd generation bioethanol** production

***Saccharomyces cerevisiae* as a bioethanol production host**

Engineered *S. cerevisiae* is able to ferment glucose and xylose for ethanol production.

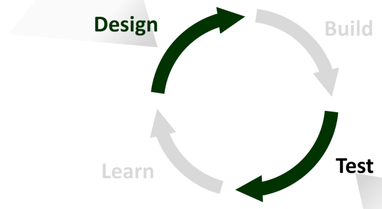
- Lignocellulosic hydrolysates contains a broad range of **inhibitors** for yeast
- Inhibitor **composition** depends on the type of raw material and pretreatment

An **efficient** genome editing strategy for engineering industrial yeast strains is needed

OBJECTIVE

To develop an efficient design-build-test-learn cycle for creating robust production strains

Use of CRISPR(i) based systems as a tool for studying the impact of genetic alterations



High-throughput analysis of yeast strains during fermentation in lignocellulosic hydrolysates

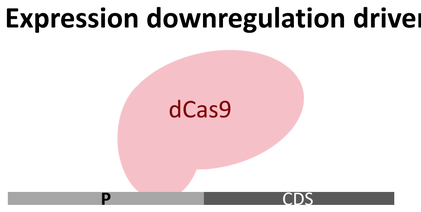
PHASE 1 – Genetic construction

In order to develop inhibitor tolerant strains, the expression of genes involved in tolerance will be systematically altered using **CRISPR interference (CRISPRi).** This methodology uses a catalytically inactive, dead Cas9 (dCas9).

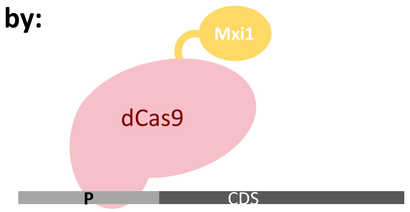
Vectors expressing dCas9+repressor/ activator constructs have been constructed using the Moclo Yeast Toolkit [1].

CRISPR will be used to stablish permanently gene modifications successfully tested with CRISPRi.

Expression downregulation driven by:

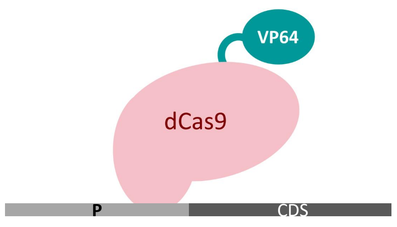


Targeting dCas9 to a promoter can lead to a repression in transcription by sterically blocking RNA polymerase binding.

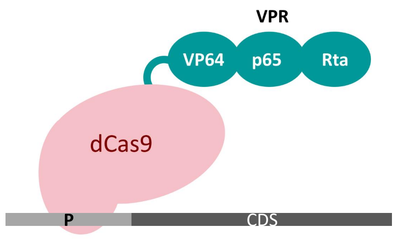


Co-expression of dCas9 + Mxi1 (mammalian transcriptional repressor) has been shown to improve CRISPRi based transcriptional repression [2].

Expression upregulation driven by:



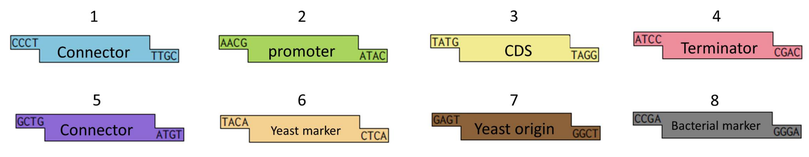
Co-expression of dCas9 + VP64 (4 tandem copies of Herpes Simplex Viral Protein 16) has been shown to improve CRISPRi based transcriptional activation [3].



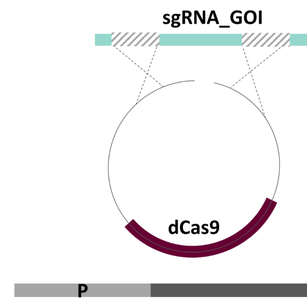
Co-expression of dCas9 + VPR (3 activator domains) is a promising alternative approach to improve CRISPRi based transcriptional activation [4].

Vector construction

1. Assembly of parts to obtain vectors expressing a CDS or a domain of interest (dCas9 or activator/repressor, respectively).



2.Assembly of a vector containing multiple transcription units, expressing dCas9 + activator/repressor and a cassette for sgRNA expression. The sgRNA is assembly into the vector through *in vivo* homologous recombination.



Expression of the sgRNA under the tRNA Phe promoter and *SNR52* terminator

PHASE 2 – Establishment of a high-throughput screening platform

In order to analyse a large number of strains a rapid, high-throughput system to characterise the performance of new strains in lignocellulosic hydrolysates is essential.

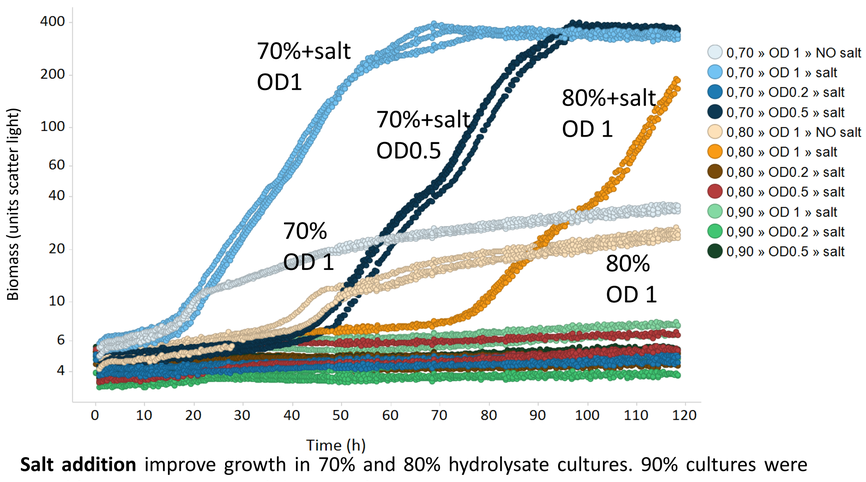
Various culture conditions and parameters have been tested in the Biolector® microbioreactor platform:

- Lignocellulose hydrolysate concentration (% v/v)
- Size of inoculum
- Addition of salt to the medium
- Preculture adaptation
- Aerobic / anaerobic conditions

MATERIALS AND METHODS

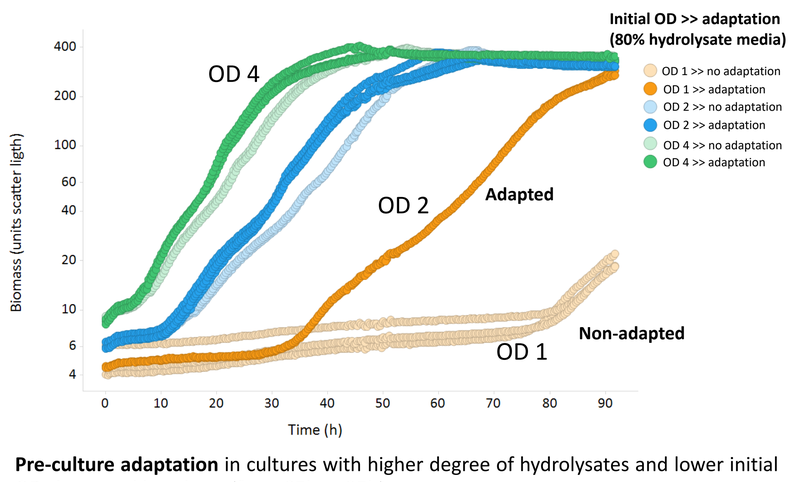
- Culture volume: 1 mL
- Polyploid industrial strain
- Media: Wheat straw hydrolysate with YPD supplementation (final sugar concentration: 60 g/L glucose + 30 g/L xylose), pH 5.5
- Adaptation in anaerobic conditions was performed by addition of different lignocellulosic hydrolysate concentration (0%, 14% and 40%) in the YPD preculture media, with an overnight incubation

1) AEROBIC CONDITIONS + SALT ADDITION



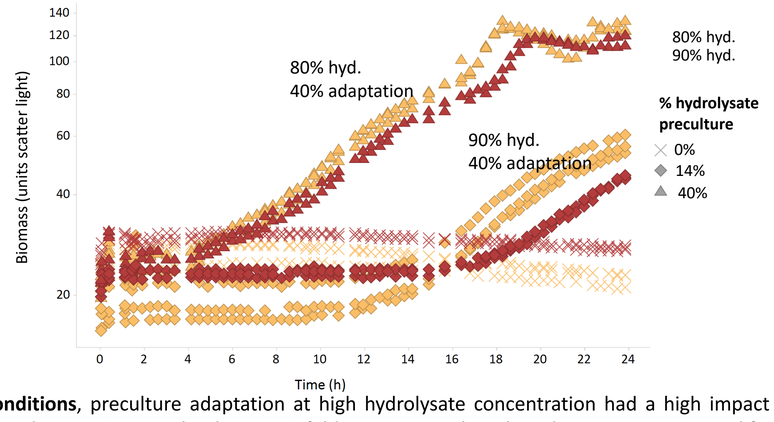
Salt addition improve growth in 70% and 80% hydrolysate cultures. 90% cultures were not able to grow in any condition tested.

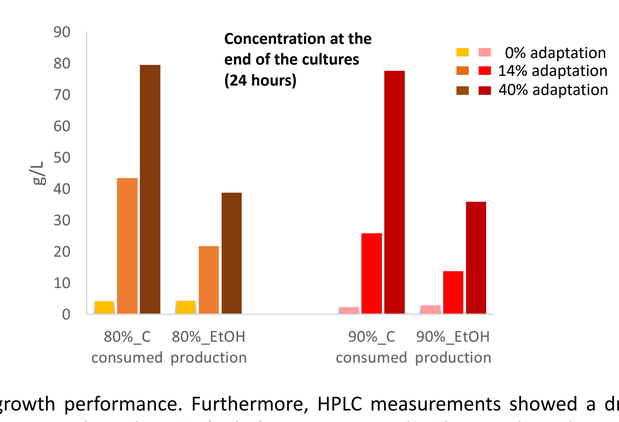
2) AEROBIC CONDITIONS + ADAPTATION



Pre-culture adaptation in cultures with higher degree of hydrolysates and lower initial OD shortened lag phase (from 85h to 35h).

3) ANAEROBIC CONDITIONS + ADAPTATION LEVELS





Under **anaerobic conditions**, preculture adaptation at high hydrolysate concentration had a high impact in growth performance. Furthermore, HPLC measurements showed a drastic increase in ethanol production. Concretely, about a 10-fold increase in ethanol production was measured for the strains adapted in 40% hydrolysates compared with non-adapted strains.

Conclusions

- ❒ An efficient platform for high-throughput screening of yeast directly in lignocellulosic hydrolysates was established
- ❒ Preliminary results showed a reliable correlation in terms of growth with shake flask cultures

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2. Gilbert, L. A. *et al.* CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154,** 442–451 (2013).
3. Naranjo, S. *et al*. Dissecting the Genetic Basis of a Complex cis - Regulatory Adaptation. *Plos* 1–19 (2015).
4. Chavez, A. *et al.* Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods* **12,** 326–328 (2015).