# YEAST ADAPTIVE EVOLUTION TOWARDS THE CELLULOSIC ETHANOL

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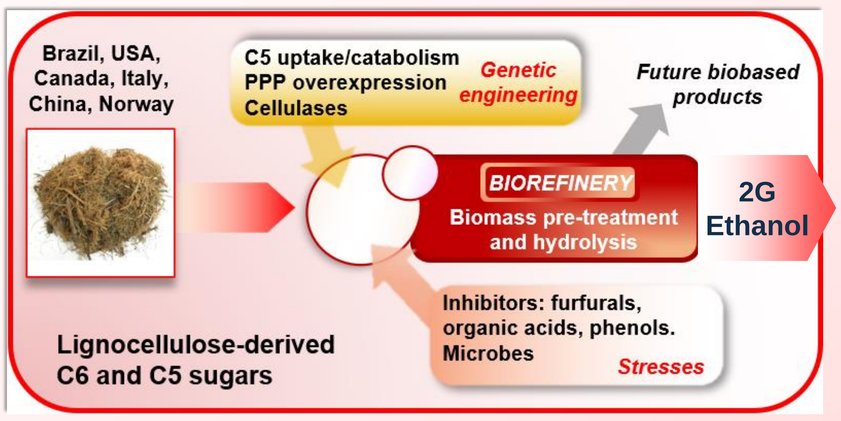
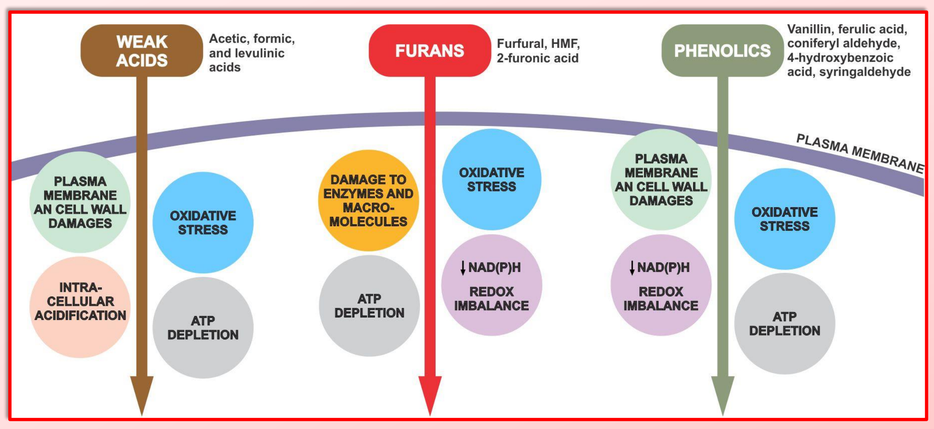
## INTRODUCTION

### *Cellulosic biorefinery*




*Cellulosic ethanol (second-generation, 2G, ethanol) is produced from biomass treated to release sugars from cellulose fibers. Yeast used for 2G ethanol production need specific genetic modifications to consume C5 sugars and require tolerance to lignocellulosic-derived inhibitors.*

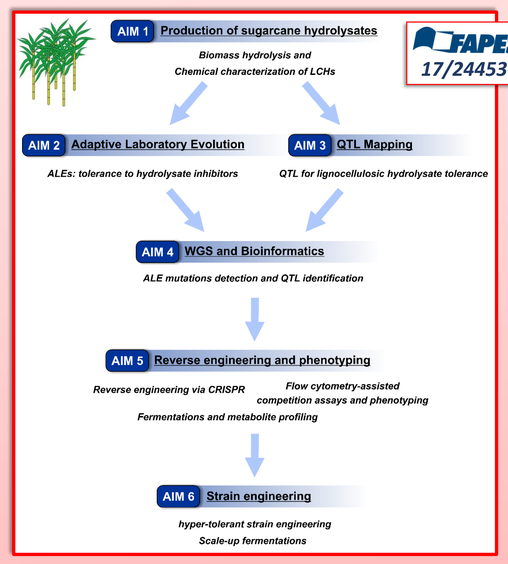
### *Lignocellulosic hydrolysates (LCH)*



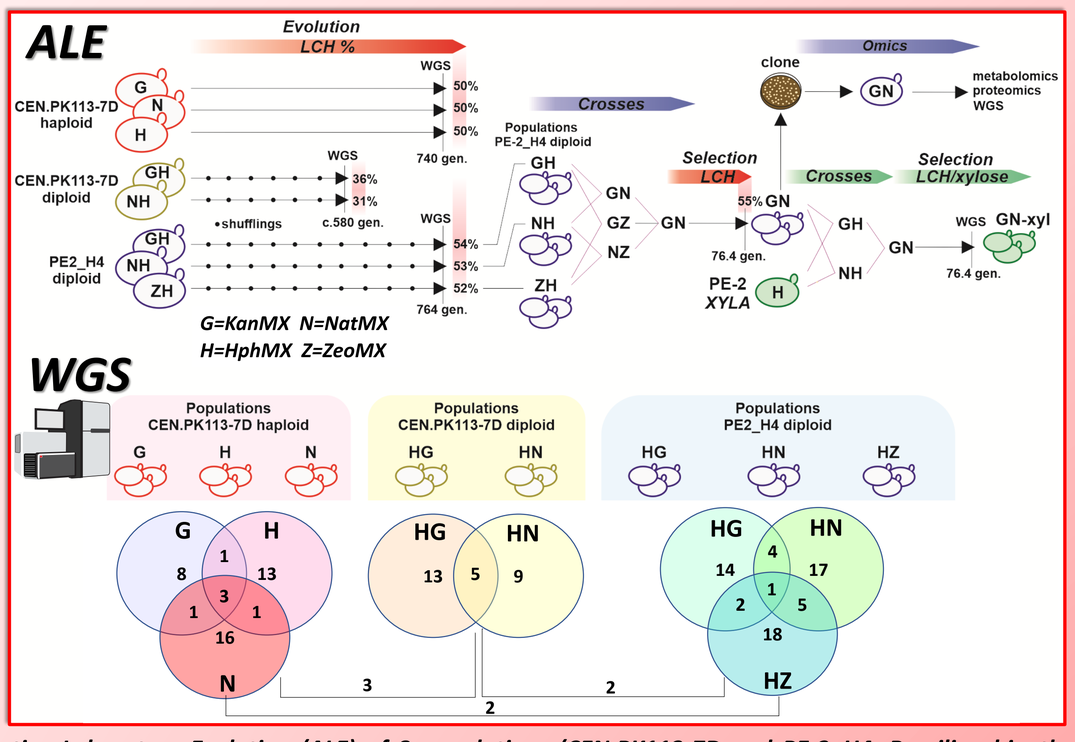

*Lignocellulosic hydrolysates (LCH) result from biomass pre-treatment and hydrolysis. This process also generates fermentations inhibitors (e.g., weak acids, furans, and phenolics) that are detrimental to yeast cell structures and physiology.* ***The goal of our project is to use modern genetic and evolution approaches to boost yeast tolerance to LCHs.***

## OBJECTIVES

### *Project workflow*

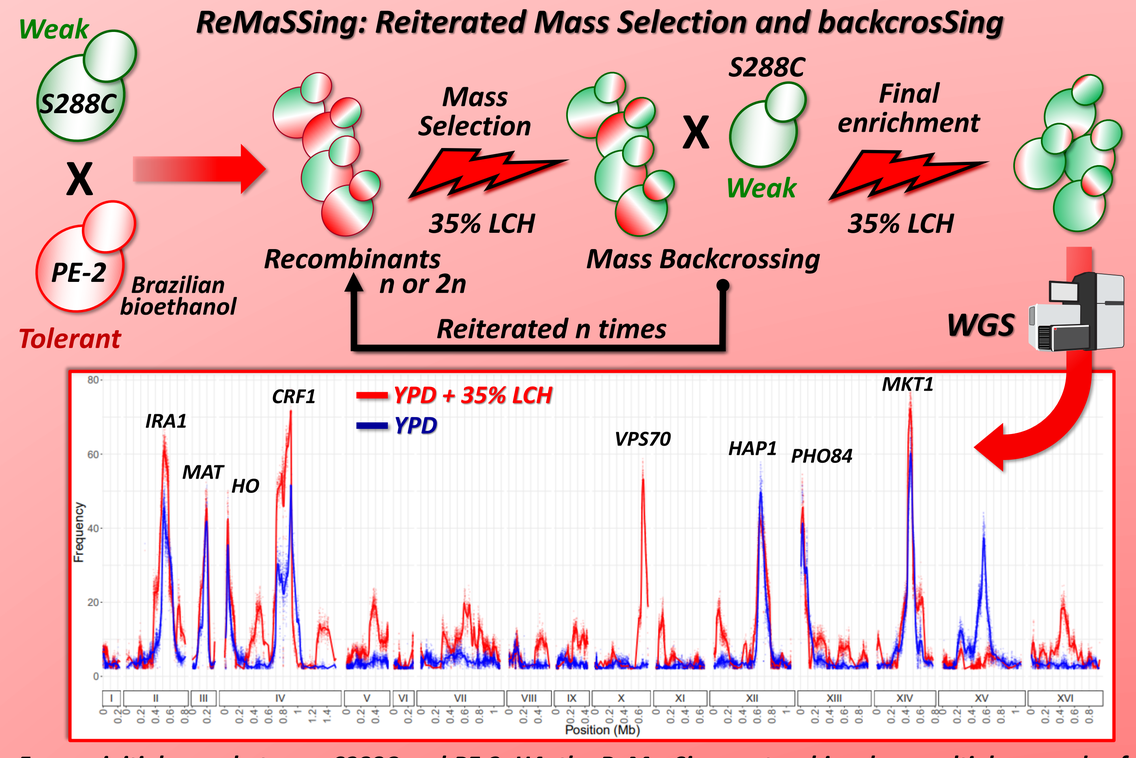



## ADAPTIVE EVOLUTION TO SUGARCANE LCH



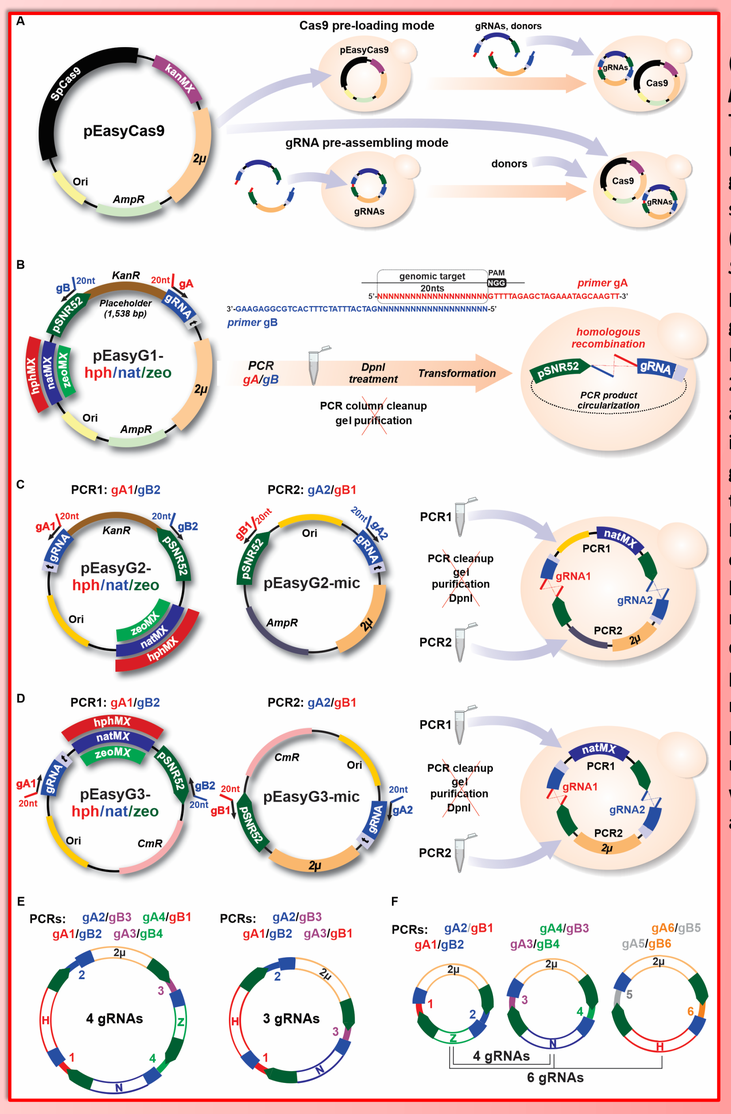

*Adaptive Laboratory Evolution (ALE) of 8 populations (CEN.PK113-7D and PE-2_H4, Brazilian bioethanol) under increasing amounts of sugarcane bagasse LCH. Diploid evolved populations PE-2_H4 were intercrossed, selected, and crossed with a yeast strain expressing xylose isomerase (XYLA). Further selection for 76.4 generations in xylose and LCH yielded a hyper-tolerant yeast for 2G ethanol production. A selected clone from PE-2_H4 intercrosses will be targeted for multiple analyses. Whole-genome sequencing (WGS) of ALE populations revealed unique and shared mutations between populations.*

## QTL MAPPING: TOLERANCE TO SUGARCANE LCH




*From a initial cross between S288C and PE-2_H4, the ReMaSSing protocol involves multiples rounds of bulk selection and mass backcrossing to generate a recombinant population enriched in QTL for tolerance to sugarcane LCH. After 5 ReMaSSing cycles, recombinants selected under 35% HLC exposure and without HLC treatment (only YPD) were targeted for WGS. Plotting PE-2 SNP frequencies over the S288C chromosomal coordinates generated a QTL map. Identified QTL are under analysis (below).*

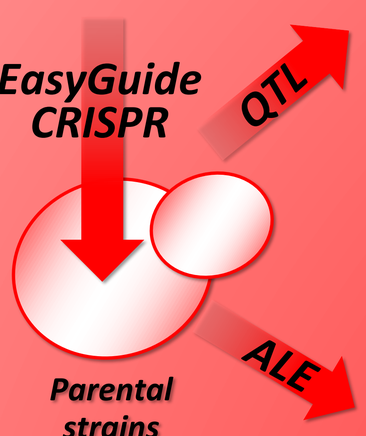
## REVERSE ENGINEERING: CRISPR/Cas9 *EasyGuide* APPROACH



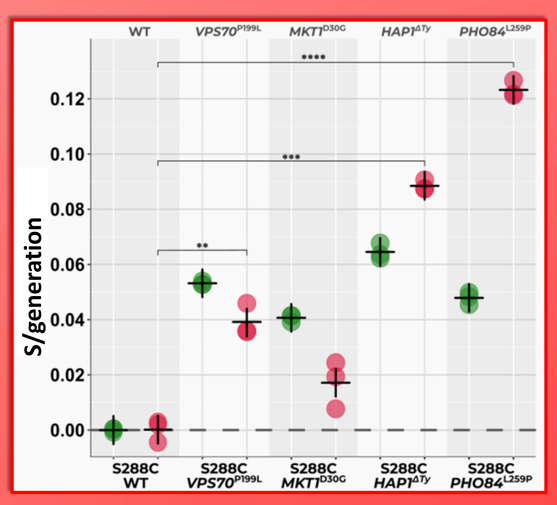

### *EasyGuide plasmids*

(A) The pEasyCas9 has a 2µ origin and encodes the *S. pyogenes* Cas9 and a geneticin resistance mark (kanMX). The pEasyCas9 can be transformed into a host strain that is used for genome editing experiments. Alternatively, a gRNA(s) may be pre-assembled into a yeast strain for subsequent co-transformation of pEasyCas9 and donor(s). (B) The pEasyG1 plasmids contain a gRNA scaffold (blue), a *SUP4* terminator (t) and is preceded by the the Pol III promoter *pSNR52* (green), which is separated from the gRNA by a placeholder. pEasyG1 is a template to amplify PCR fragments with primers gA and gB. Both oligos specify 20-nts spacers that provide recombination sites for in vivo assembly of a functional gRNA. The spacer sequence of gA is the 20-nts upstream of the PAM sequence on the yeast genome. PCR fragments can be directly transformed from the PCR reaction into yeast cells, dispensing (red crossed) DNA purification steps. (C) The pEasyG2-zeo/nat/hph encode antibiotic resistance marks (zeoMX, natMX, or hphMX), and pEasyG2-mic contains the 2µ origin. In vivo recombination between amplicons (PCR1 and PCR2) derived from these templates generates a functional plasmid expressing two gRNAs. (D) Similarly, in vivo recombination between amplicons PCR1 and PCR2 from pEasyG3-zeo/nat/hph and pEasyG3-mic, respectively, results in a plasmid expressing two gRNAs. (E, F) Alternative ways to assemble three, four, or six gRNAs from pEasyG3(2) amplicons. PCR primer combinations are indicated.

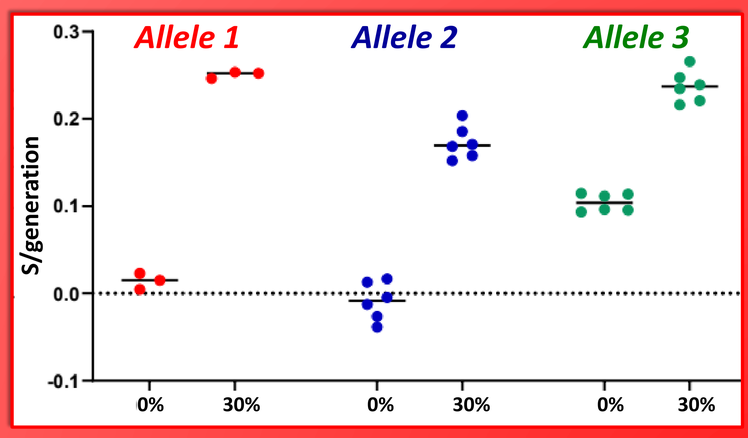
***ALE , QTL alleles***




## PHENOTYPING: COMPETITIONS




***Competition assays between S288C-GFP and S288C with reverse engineered PE-2 QTL. Selection coefficients (S) were calculated and normalized per cell doubling during the competition (one round of growth with 30% LCH or without LCH). Values derive from triplicate competitions. Tested alleles significantly improved fitness on LCH and normal medium.***




***Competition assays between CEN.PK113-7D-GFP and CEN.PK113-7D with 3 ALE alleles reverse engineered. Completions were conducted in triplicates in 30% sugarcane HLC and normal YPD medium. Reverse engineered alleles conferred growth advantages under LCH. Selection coefficients (S) were normalized per cell doubling.***

### *Key references*

Jacobus AP, Barreto JA, de Bem LS, Menegon YA, Fier I, Bueno JGR, dos Santos LV, Gross J. EasyGuide plasmids support in vivo assembly of gRNAs for CRISPR/Cas9 applications in Saccharomyces cerevisiae.

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