Genome shuffling of *Saccharomyces cerevisiae* through recursive population mating to evolve tolerance to inhibitors of Spent Sulfite Liquor

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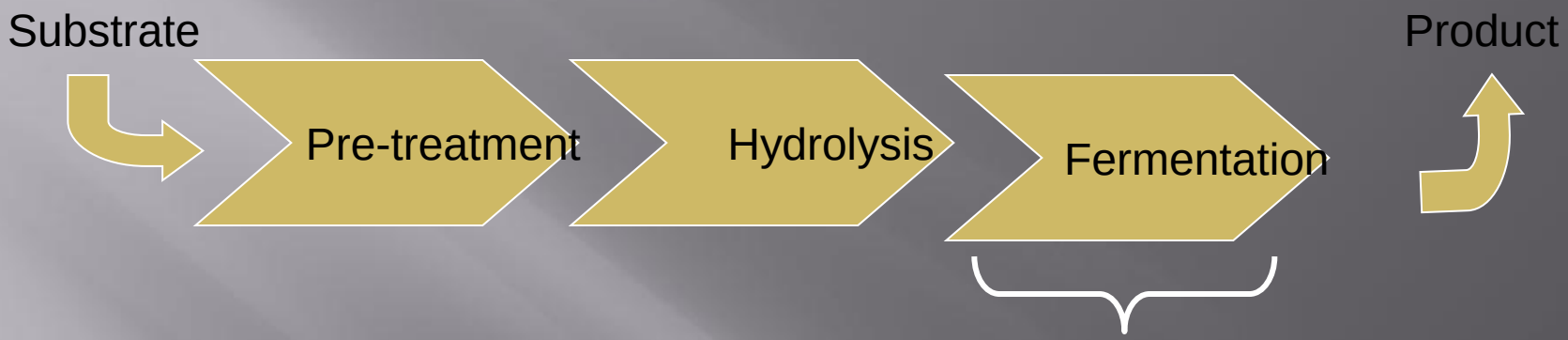
Biochemical Steps to Conversion of Cellulosics



Metabolic engineering
- Yeast, Clostridia
- C5/C6 fermentation
- Consolidated bioprocessing
- Resistance to inhibitors

CSFG
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NSERC BIOCONVERSION NETWORK

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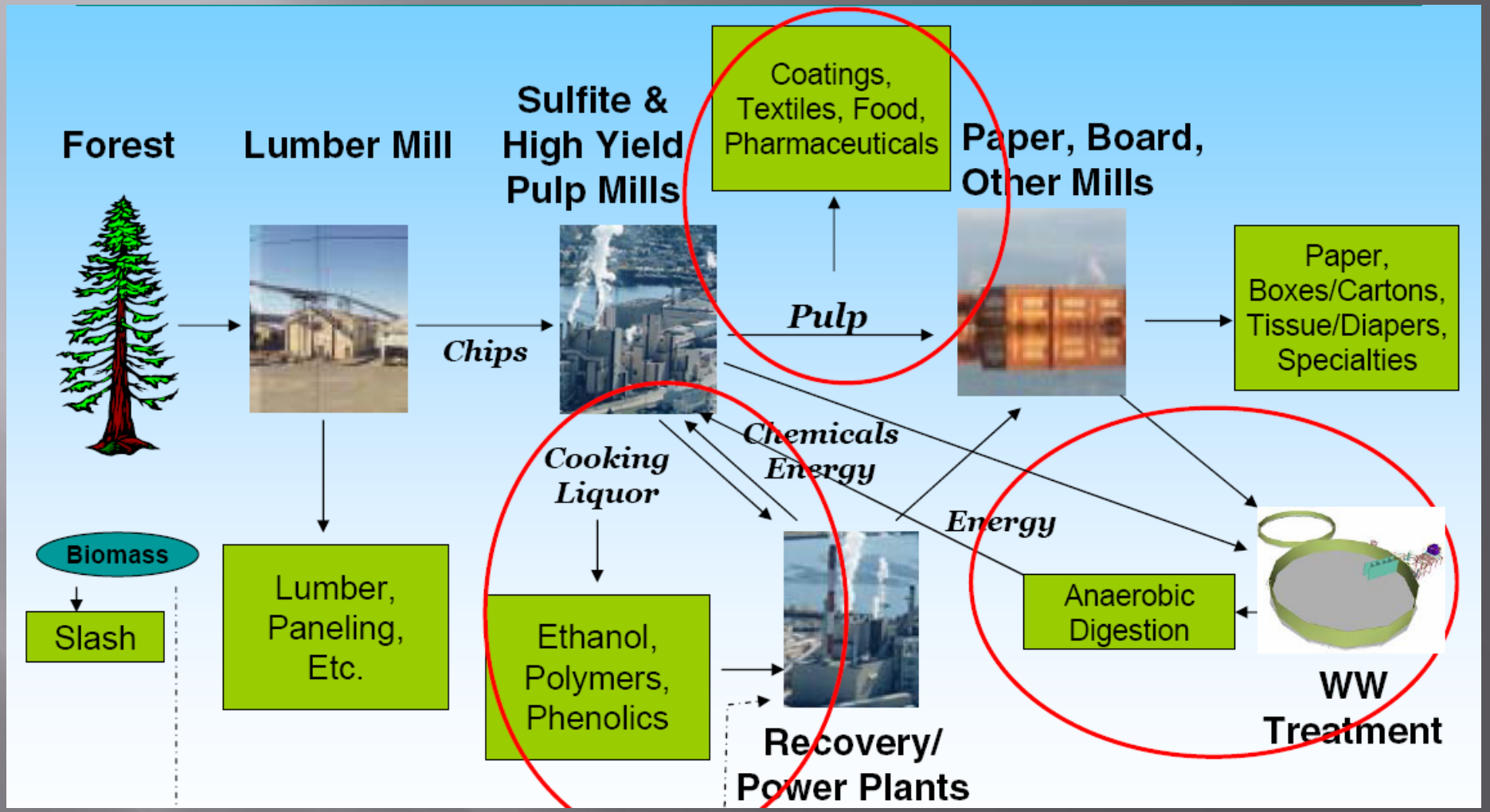
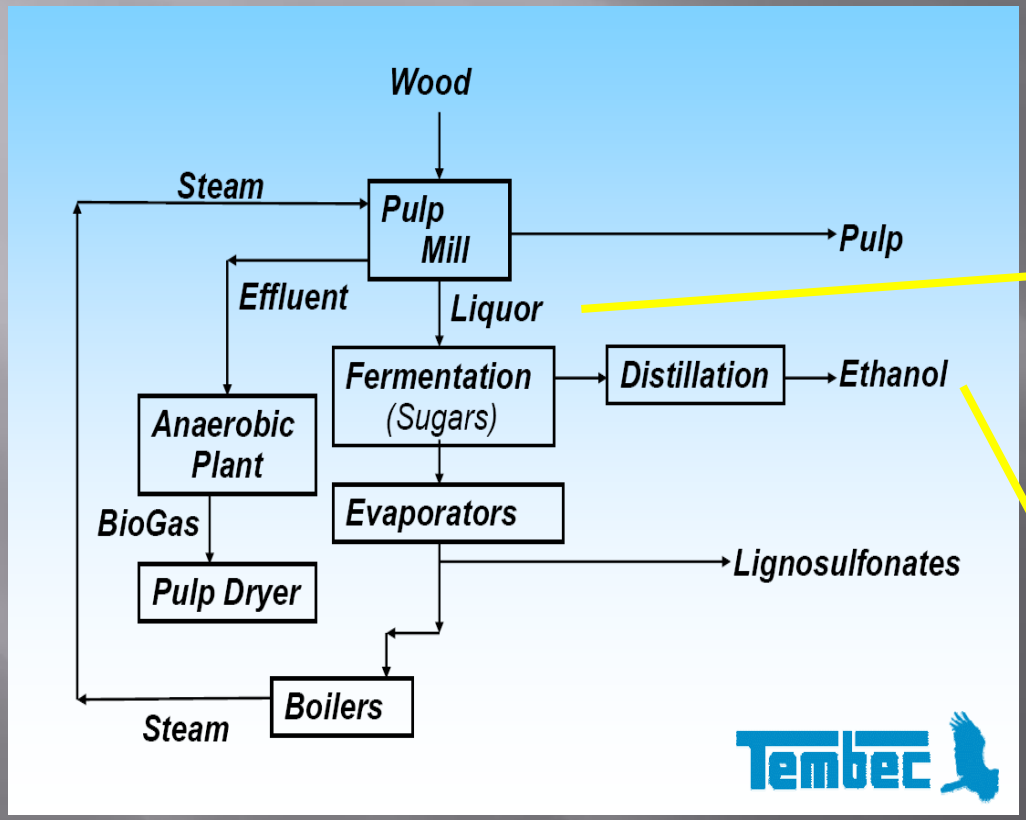
Bioproducts from Tembec's Temiscaming Biorefinery



Biochemical Flow Chart at the Tembec Biorefinery Plant



HWSSL SWSSL

- 3 x 1 million litres fermentors
- ~15 million litres per year industrial grade

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Fermenting The Residual Sugar in Spent Liquor

Mixed sugar stream:

Pentoses (0.076% arabinose, 2.2% xylose)

Hexoses (0.25% galactose, 0.33% glucose, 0.55% mannose)

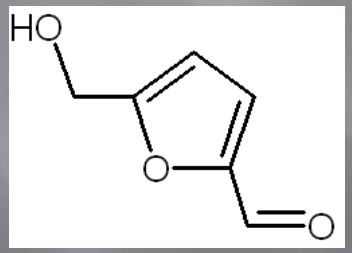
Inhibitors:

1% acetic acid

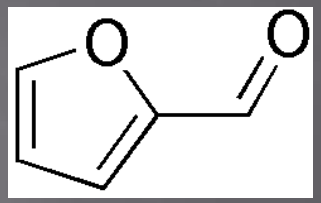
0.18% furfural and

0.11% 5-hydroxymethyl furfural (HMF)

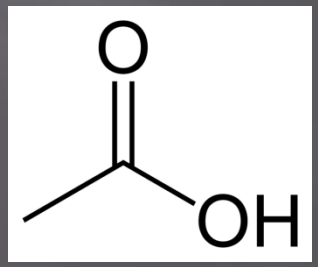
22% dissolved solids



Furfural



Hydroxymethylfurfural



Acetic Acid

Classical strain improvement

This is the process by which a reiterative sequence of mutate, grow, select is used to achieve a progressive improvement of a desired strain characteristic.

Start with wild-type (naïve) strain that produces the molecule (large or small) of interest

↓

Randomly mutate the strain using chemical or physical agents

↓

Screen for the strain that produces the highest titers above the naïve strain
First Generation (G1)

↓

Randomly mutate G1 using chemical or physical agents

↓

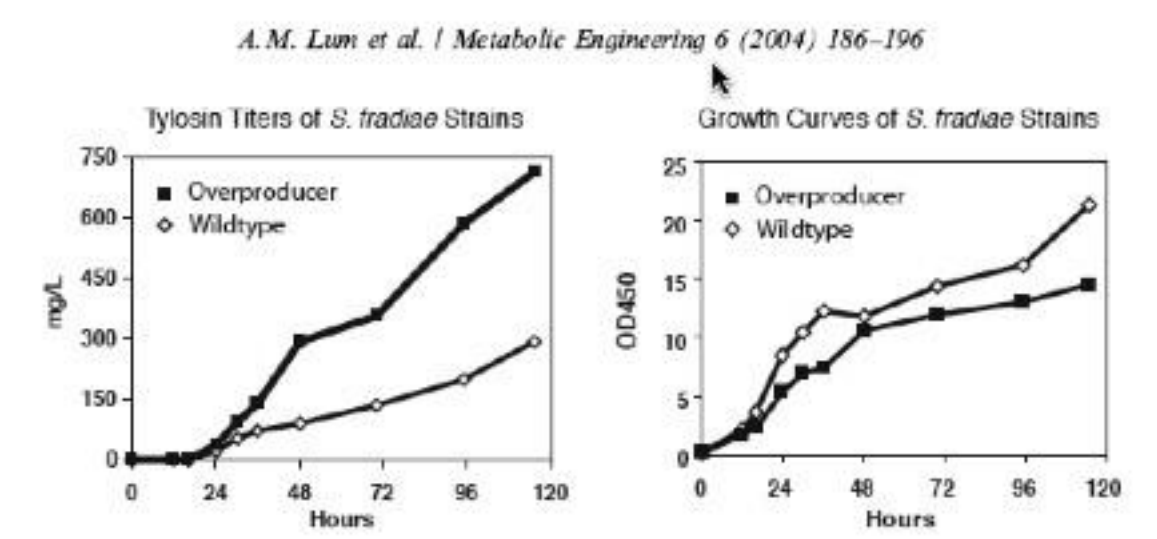
Screen for the strain that produces the highest titers above the naïve strain
Second Generation (G2)

↓

And so on

What are the problems the classical strain improvement approach ?

1. Unwanted background mutations are accumulated (you usually get more than a single mutation in a strain)



2. Most possible permutations of mutations cannot be explored
3. Time-consuming, labour-intensive and expensive >10^5 mutants may have to be screened per round of mutation

Recursive Breeding of……Saccharomyces cerevisiae

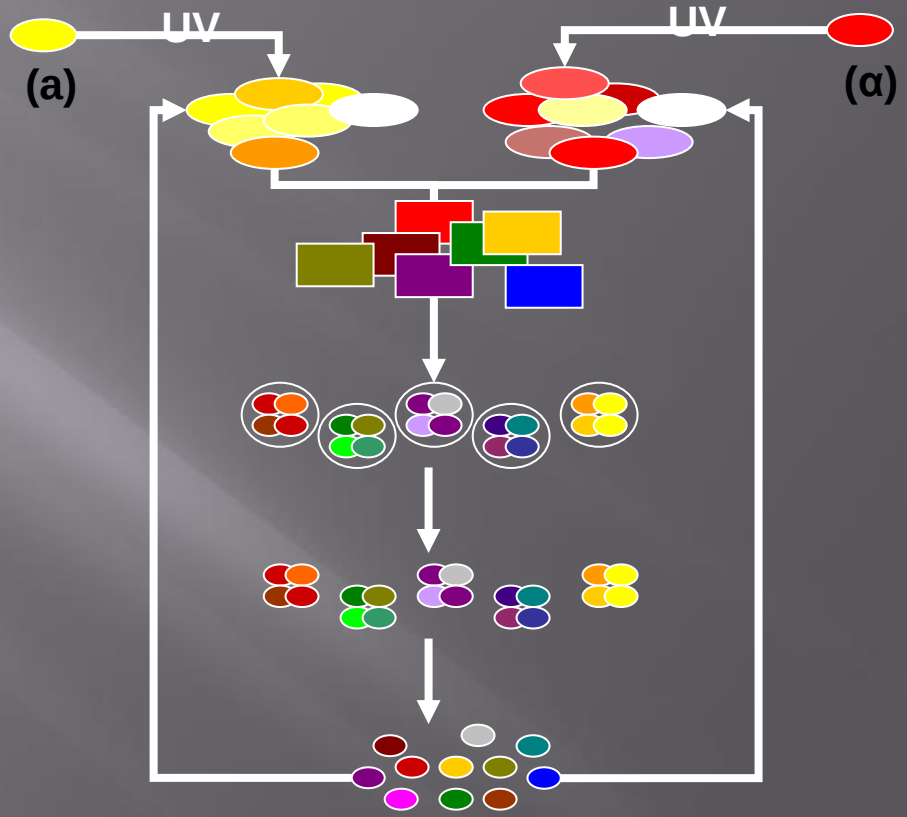
Best mom

X

Best dad

SELECT OFFSPRING FOR:

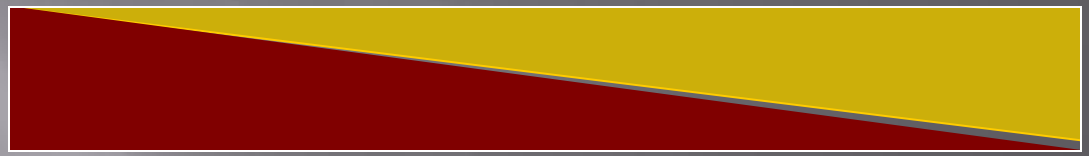
- Good feed conversion to milk production,
- Adaptability to local feeds,
- Fertility,
- Disease resistance and
- Lifespan

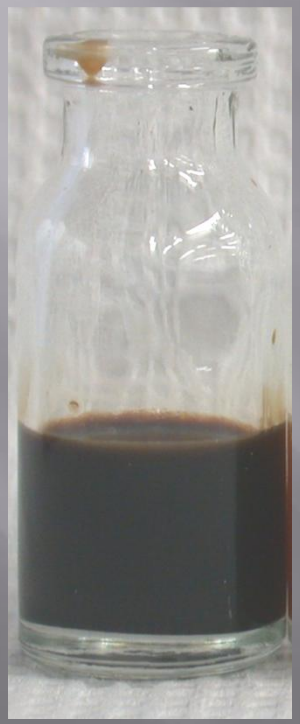


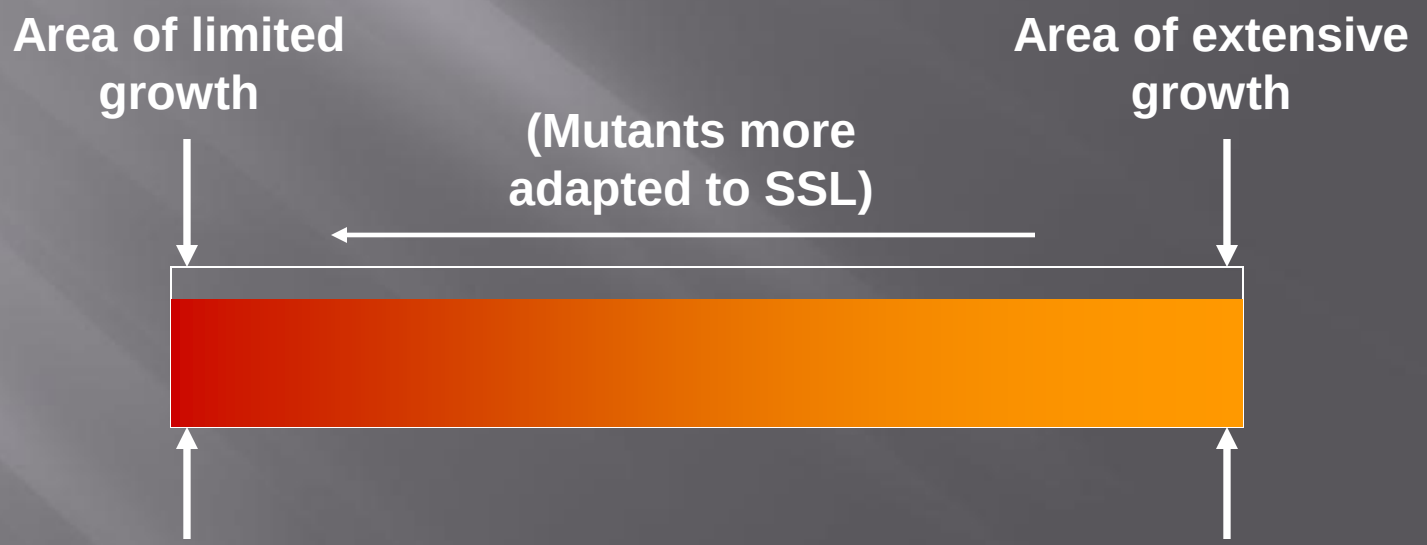
SELECT OFFSPRING FOR:

- Inhibitor resistance,
- Fermentation of pentoses
- Secretion of GH enzymes
- Etc……

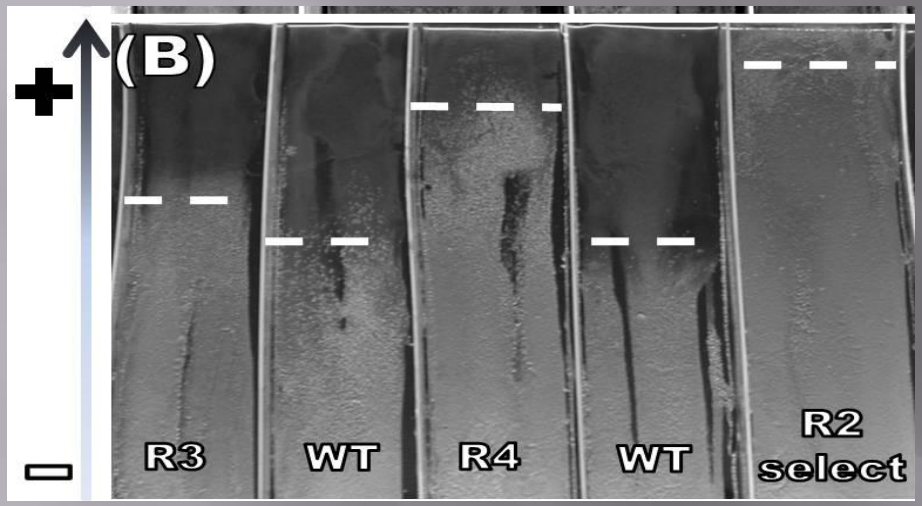
SSL Plates for Selection of Mutants

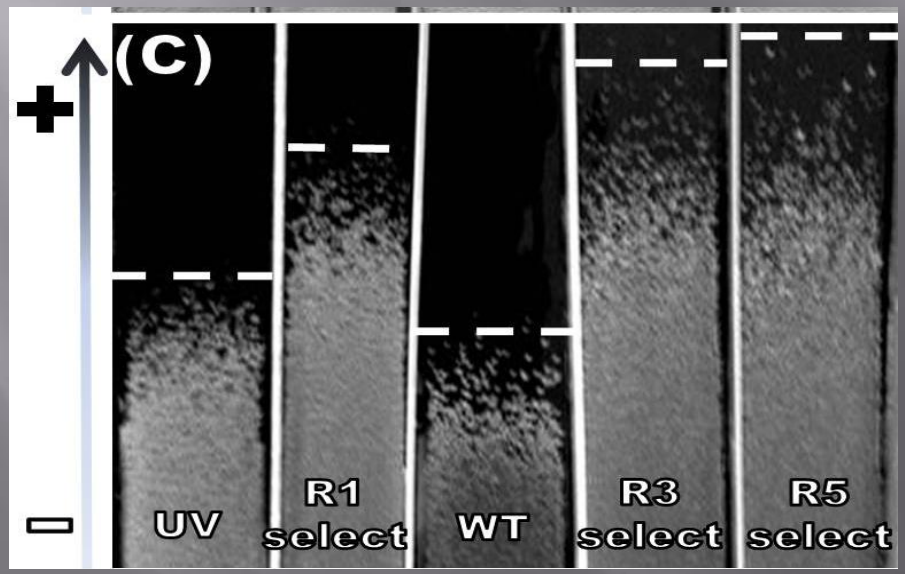




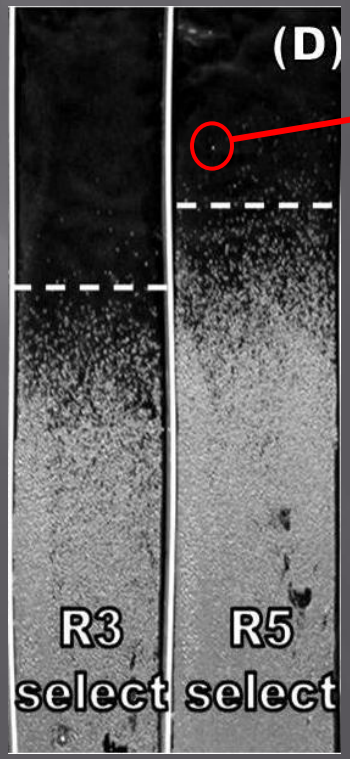


Selection of UV Mutants and Genome Shuffled Strains





Strain selection between breeding rounds speeds up strain evolution



Pick colonies and characterize in detail

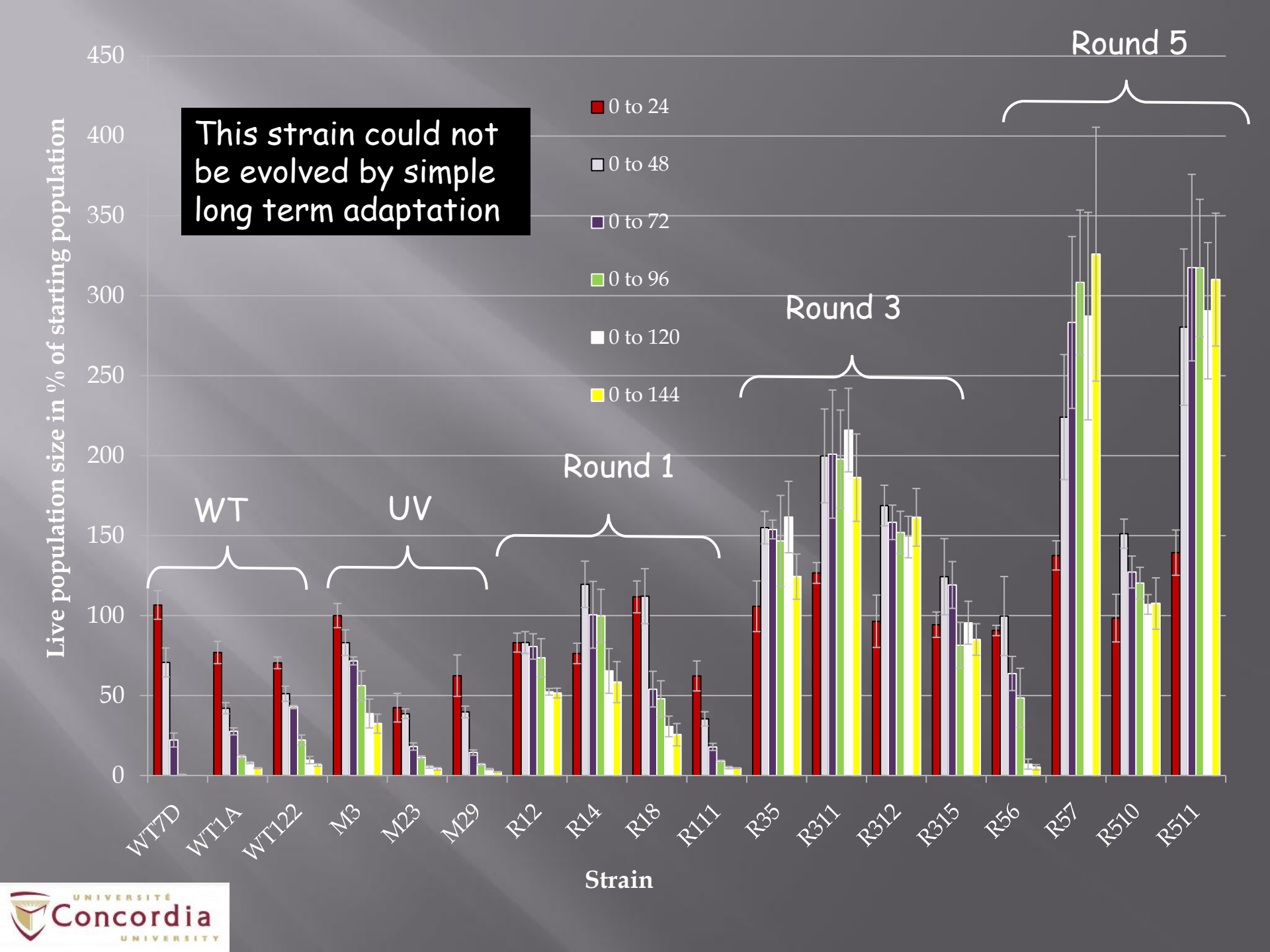
Screening of Mutants and Shuffled Strains

Preliminary screen

- 30 UV mutant strains
- 15 strains each from rounds 1,3, and 5

Detailed characterization

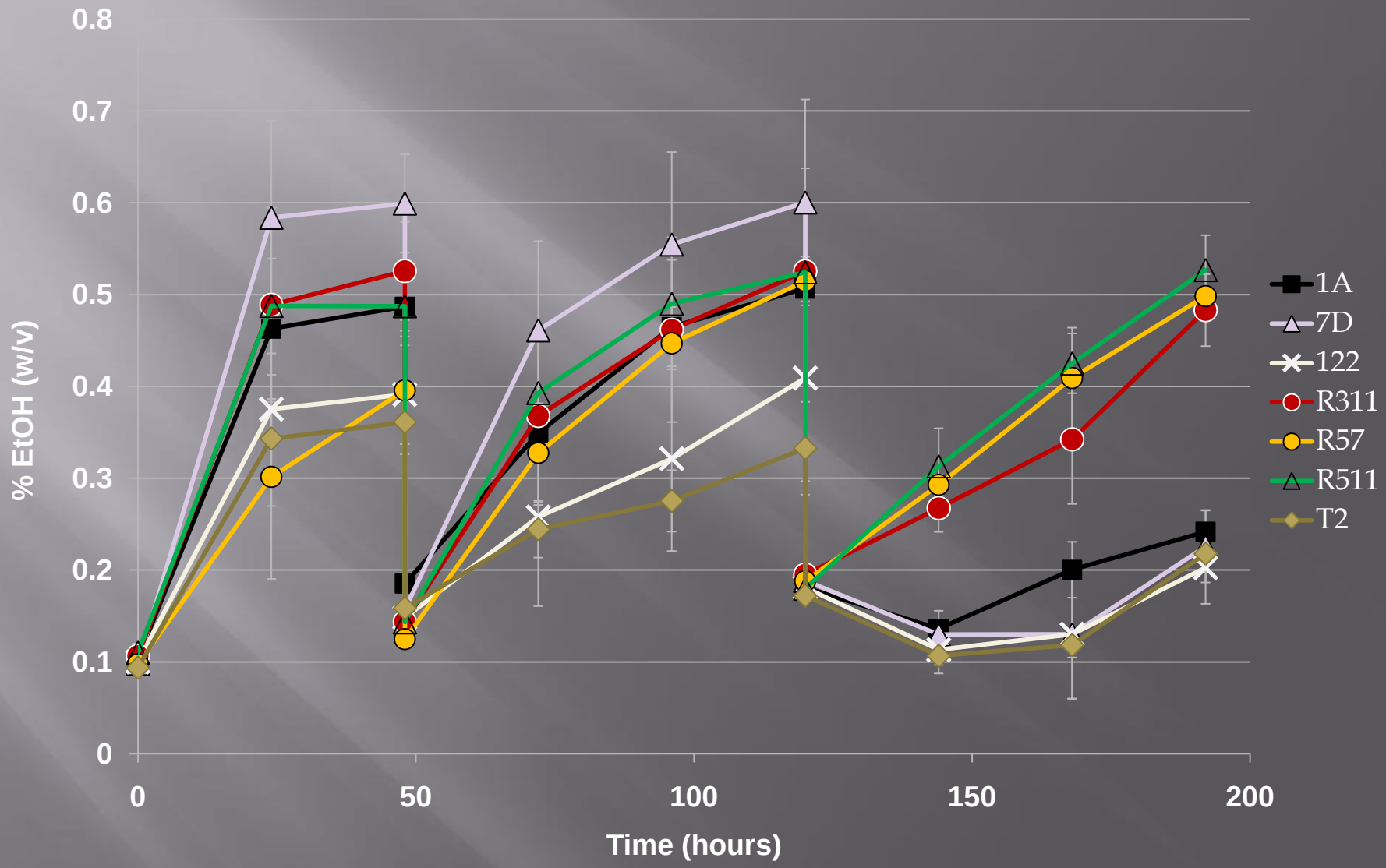
- 3 wild type strains,
- 3 UV mutant
- 4 from each from rounds 1, 3, and 5
- **Growth/survival assays:** low cell density ~10^5 cells into 100% HW SSL and sampled daily from 0 to 6 days to assess tolerance to SSL



Fermentation in HWSSL

- To confirm that tolerant strains are able to maintain ethanol productivity the most robust strains from growth/survivability experiments were selected.
- Pre-grown in minimal media (YNB 0.67%, Glucose 2%) and inoculated at ~2 g/l DCW into 100% HW SSL pH 5.5
- Samples were taken daily and analysed for ethanol content on GC
- Cultures were recycled after 48 h and 120 h for continuous exposure to HW SSL

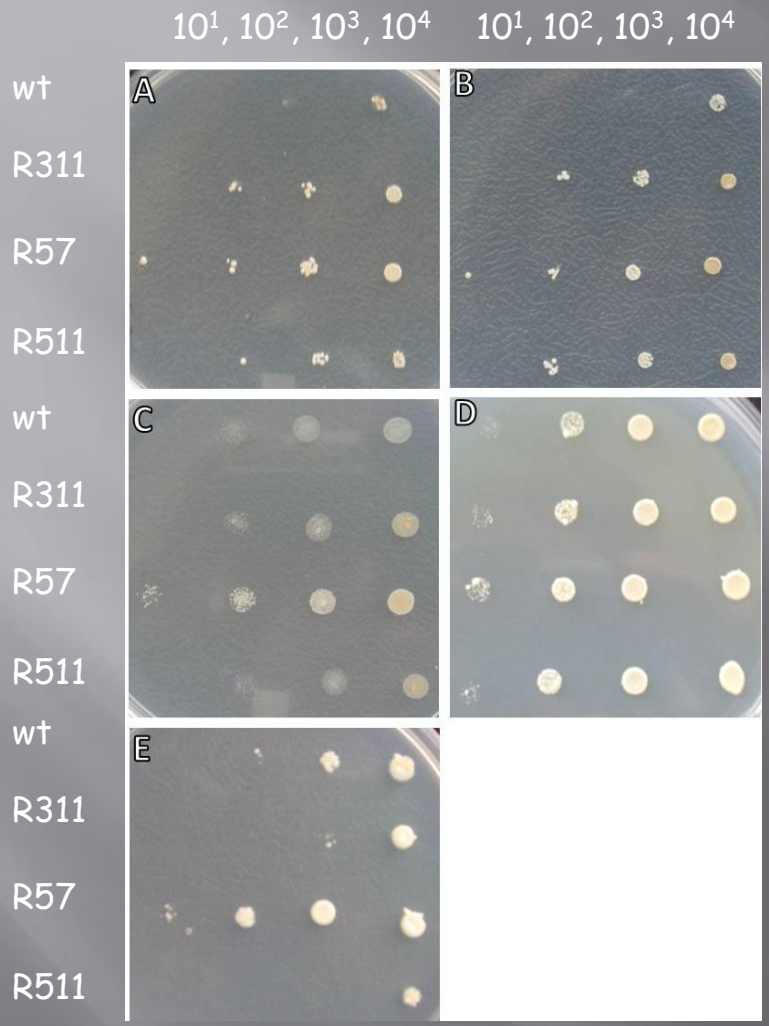
Fermentation of HWSSL



Single Inhibitor Studies

- Compared strains of interest R311, R57, and R511 on separate inhibitors to try to understand the SSL tolerant phenotype has been evolved
- Inhibitors tested: furfural, hydroxymethylfurfural, acetic acid, phenolic compound (4-HBA), osmotic stress (NaCl), hydrogen peroxide, ethanol, ammonium sulfite.
- Compounds incorporated into agar with glucose 2% w/v and various cell concentrations were spotted onto them (10, 100, 1000, 10000)

Single Inhibitor Studies



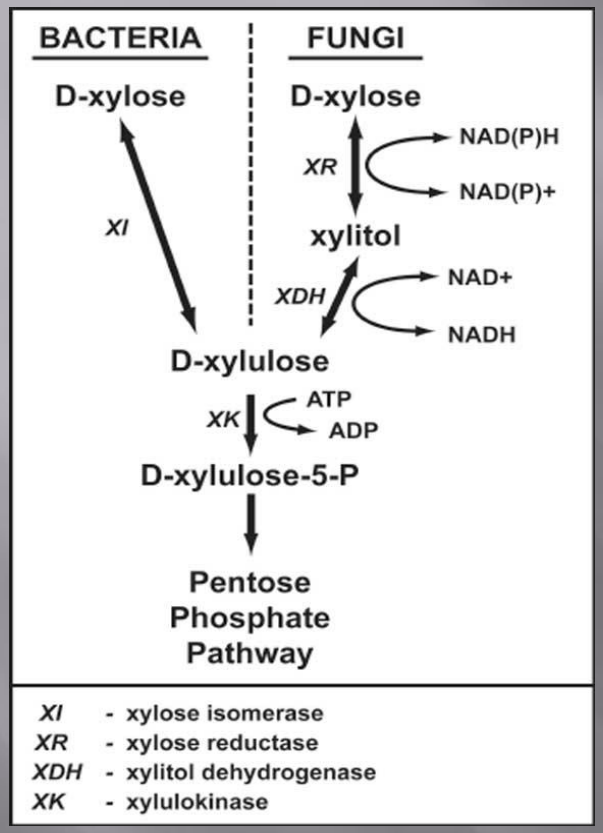
A) acetic acid pH 5.5 1 %
B) acetic acid pH3 0.5 %

C) NaCl 7 %
D) HMF 0.5 %

E) H_2O_2 1mM

Genome shuffling was able to engineer a strain with a very complex phenotype in a relatively short period

What now?



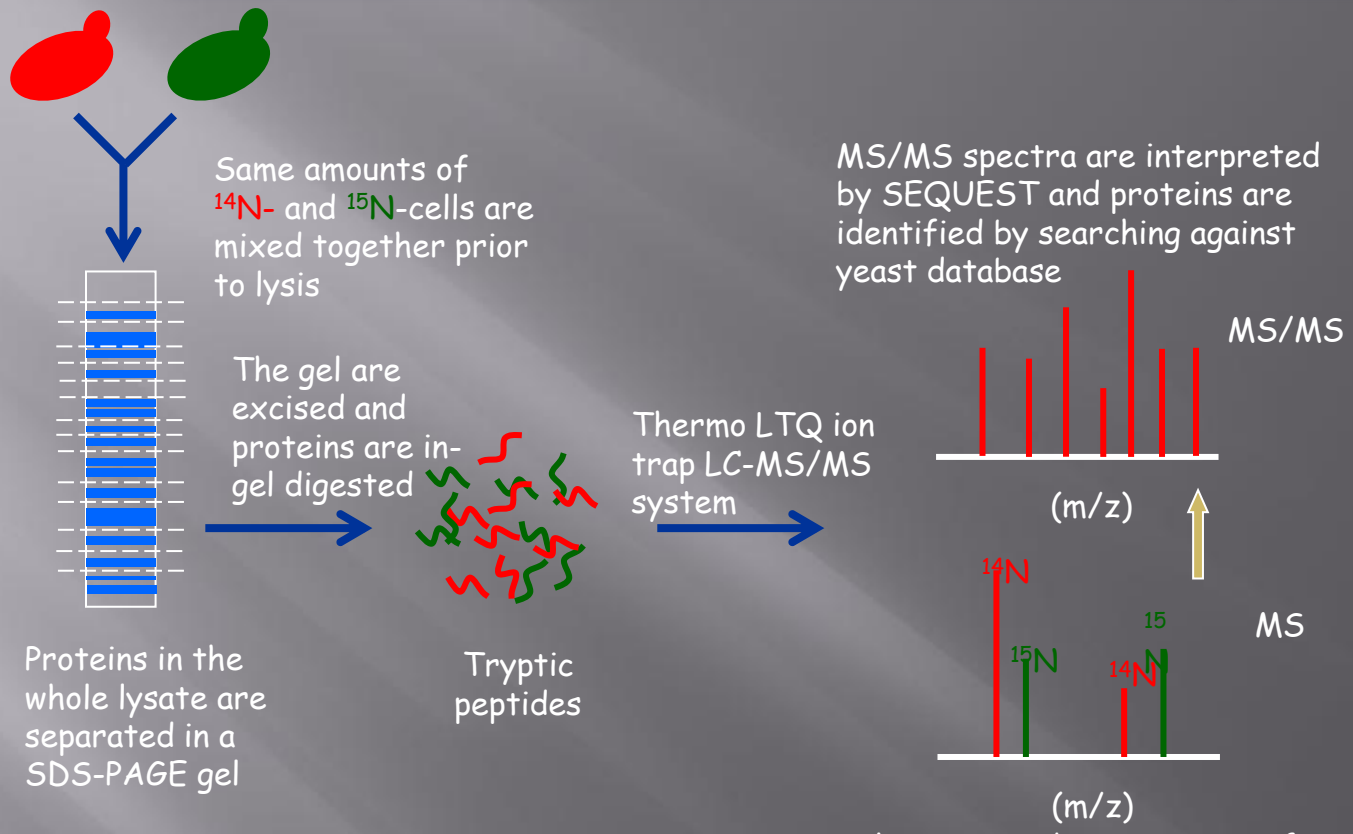
- Breeding in the xylose utilization pathway by genome shuffling

- Testing the phenotype on other substrates

Omics analysis (genotype to phenotype)

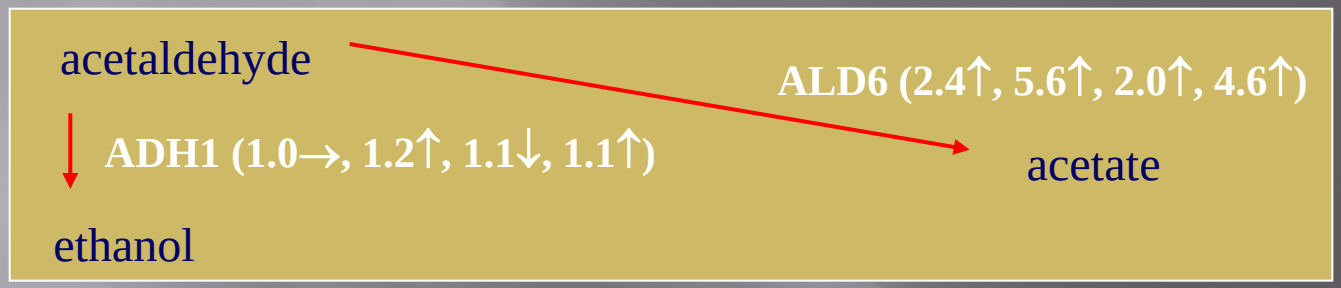
- Genome sequencing to determine what mutations/genes are involved with SSL tolerance
- RNA-seq to study transcriptome
- Proteomics

Quantitative Proteomic Analysis



R57-YNB *vs.* wild type-YNB	R57-SSL *vs.* wild type-SSL	wild type-SSL wild type-YNB	R57-SSL R57-YNB

Cultures		# identified proteins	# identified peptides
wild type	YNB	392	877
R57	YNB	451	981
wild type	SSL	549	1184
R57	SSL	569	1302



Changes in protein expression are constitutive

		R57-YNB *vs.* WT-YNB	R57-SSL *vs.* WT-SSL	WT-SSL *vs.* WT-YNB	R57-SSL *vs.* R57-YNB
SSA2	Protein folding and vacuolar import of proteins	4.4 ↑	3.1 ↑	1.2 ↑	1.1 ↓
CDC48	Protein degradation	2.3 ↑	2.0 ↑	1.2 ↑	1.0 →
HSC82	Protein folding and stress	2.1 ↑	2.5 ↑	1.0 →	1.2 ↑
HS104	Stress	1.5 ↑	1.4 ↑	1.3 ↓	1.3 ↓
HSP26	Stress	2.2 ↓	2.2 ↓	1.1 ↑	1.1 ↑
SSA1	Stress	2.5 ↓	2.1 ↓	1.1 ↓	1.1 ↑
CPR1	Stress	1.3 ↓	1.8 ↓	1.1 ↑	1.2 ↑
PNC1	Redox reactions and energy metabolism	1.7 ↓	2.0 ↓	1.2 ↓	1.4 ↓
YNK1	DNA/RNA metabolism	1.5 ↓	1.8 ↓	1.1 ↓	1.4 ↓

Future research

- Continue genomics and transcriptomics research
- Identify target genes associated with SSL tolerance
- Use this information for engineering further strain improvement

Thank you!