BioREFINE-2G: Utilisation of Waste Streams for Bioproducts and Bioenergy

Strain Development for Diacid Production

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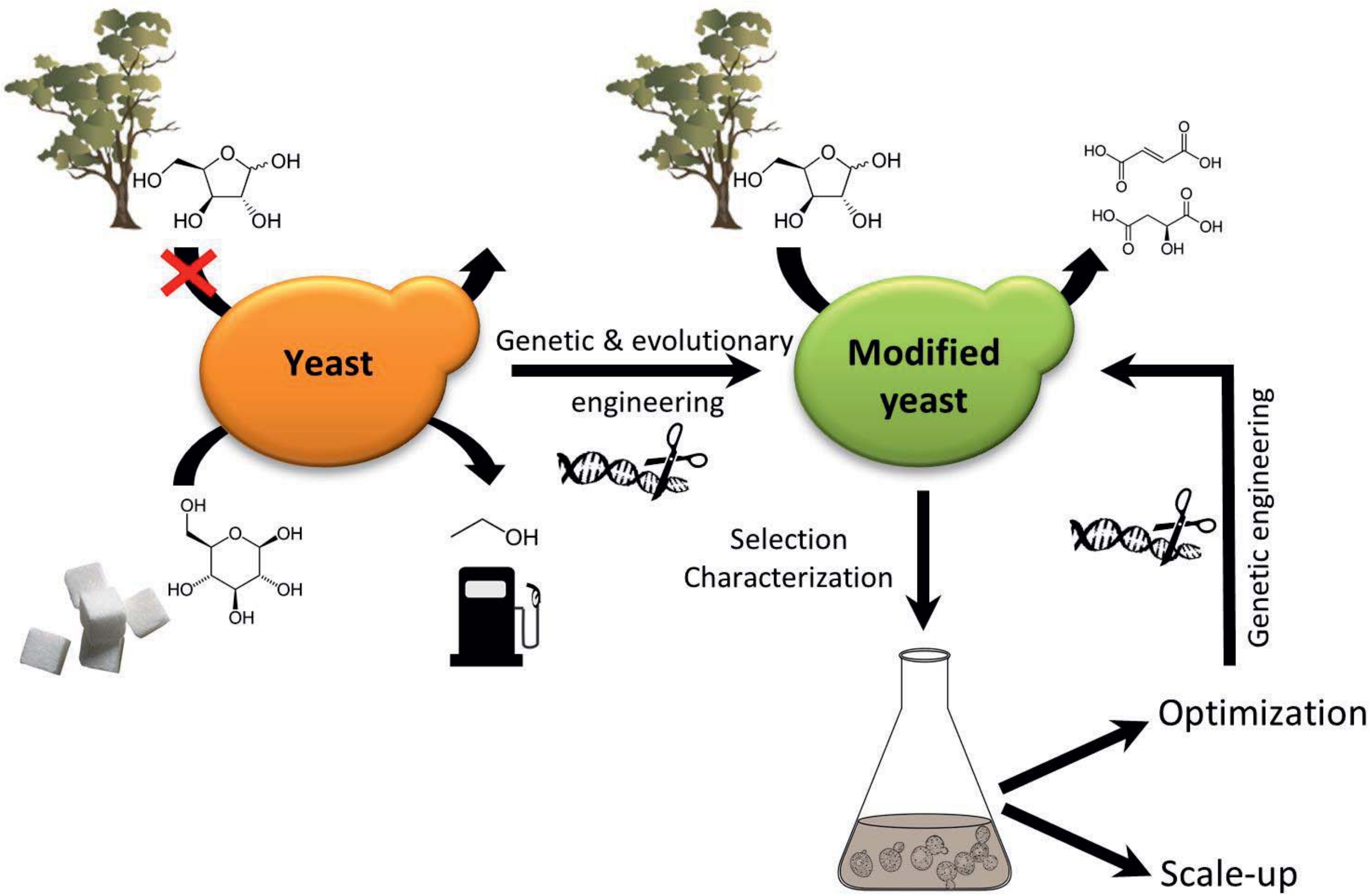
Overview of engineering strategies



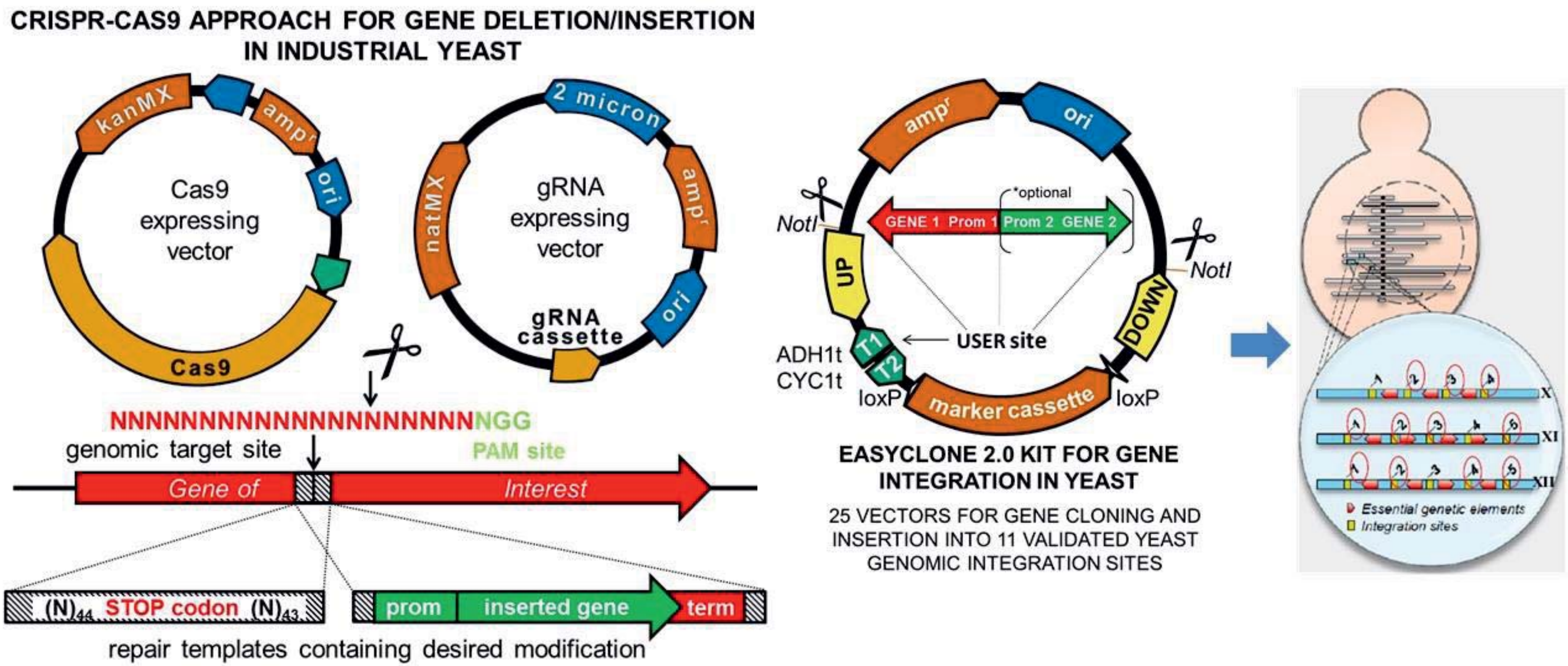
Activities

1) Host – a diploid industrial strain
    - a molecular toolbox based on CRISPR-Cas9
    - construction of vectors for stable heterologous gene insertions
    - fast and efficient strategy for gene disruption in industrial strains

2) Construction of xylose consuming industrial strain
    - improved xylose utilization properties
    - identification of causative mutations
    - strain tolerant to a biomass hydrolysate - uptake of industrial feedstock rich in C5 sugars

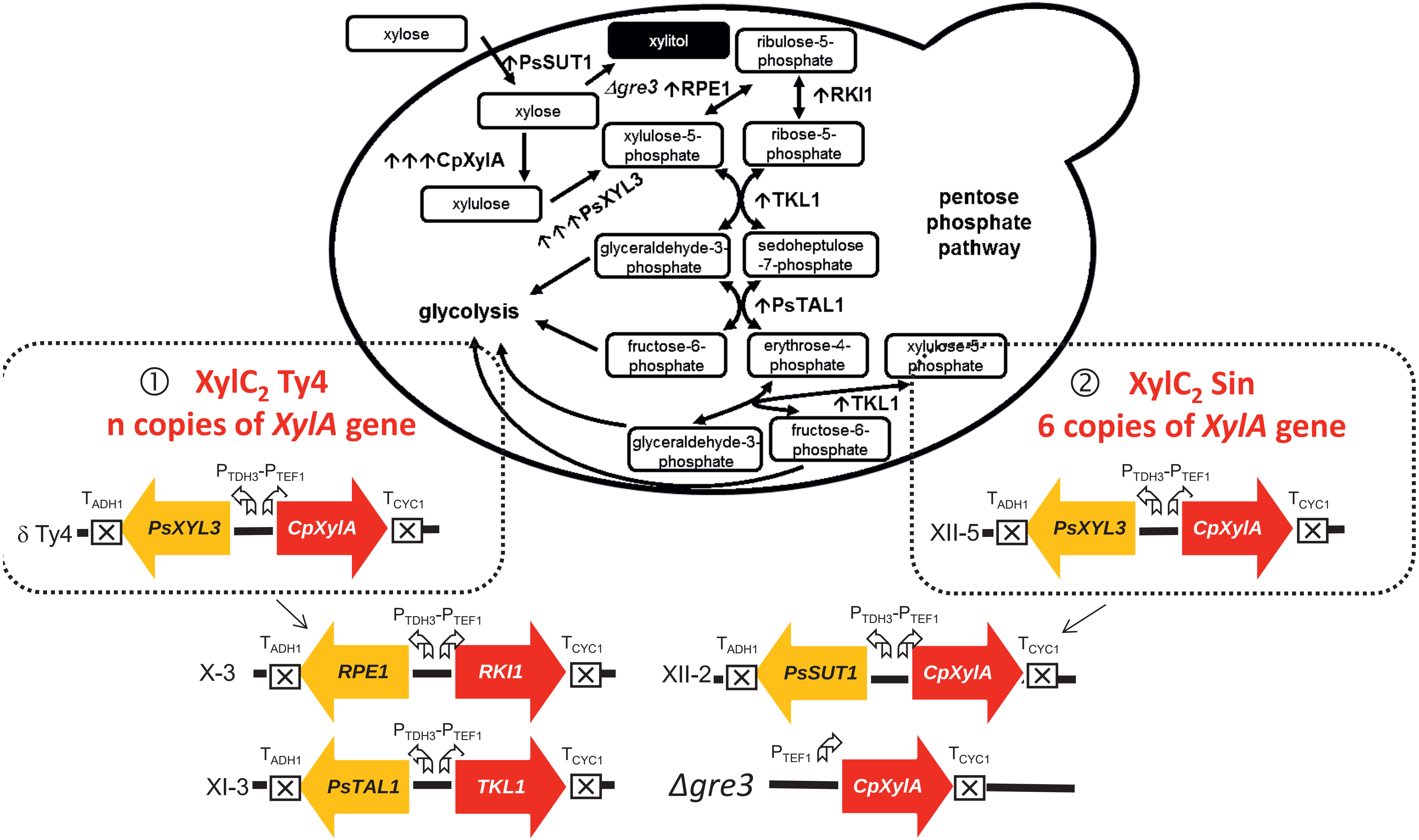
3) Strategies for production of diacids from xylose/C5-rich hardwood hydrolysate

1. Molecular toolbox based on CRISPR-Cas9



- Free for research purpose, licensing for commercial processes
- Distributed under MTA to 30+ academic and industrial laboratories
- 3 papers + 1 review cummulatively cited > 40 times

2. Construction of xylose utilizing strains



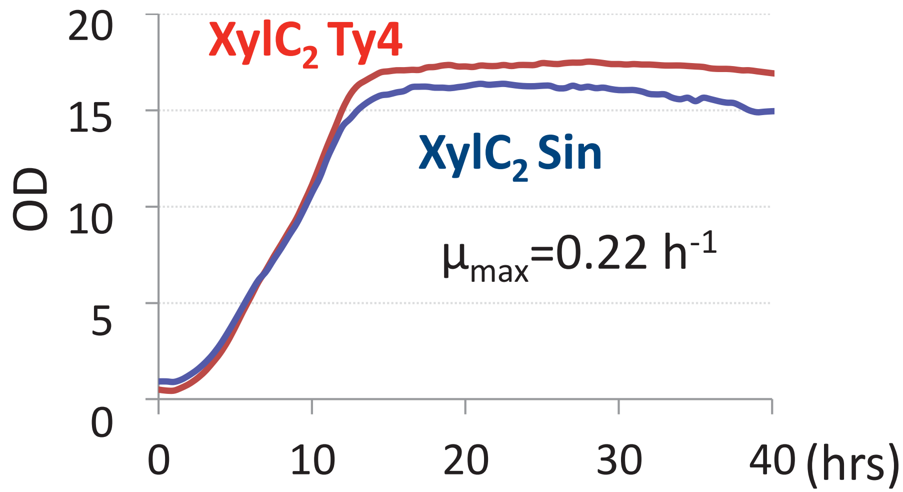
2. Initial conditions for adaptive evolution

Xylose Consumers ($XylC_2$)
Ethanol Red + XI
pathway+gre3Δ

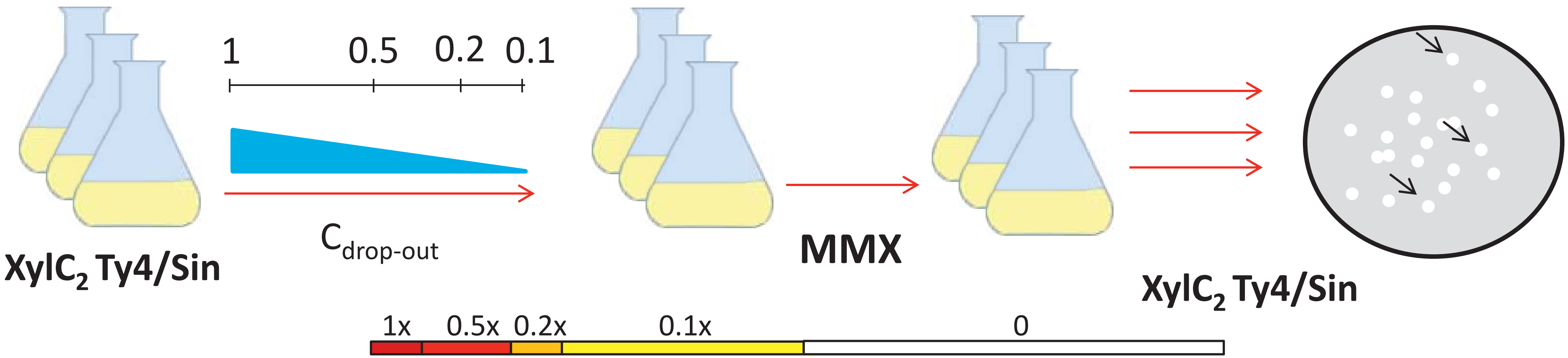
Growth profile in microtitre plates

No growth in defined mineral medium, only if supplemented with a mixture of aminoacids and other growth supplements

Growth in YPX (complex medium with 2% xylose)



Evolution of xylose consuming EthR (XylC2) in mineral medium with xylose as sole carbon source

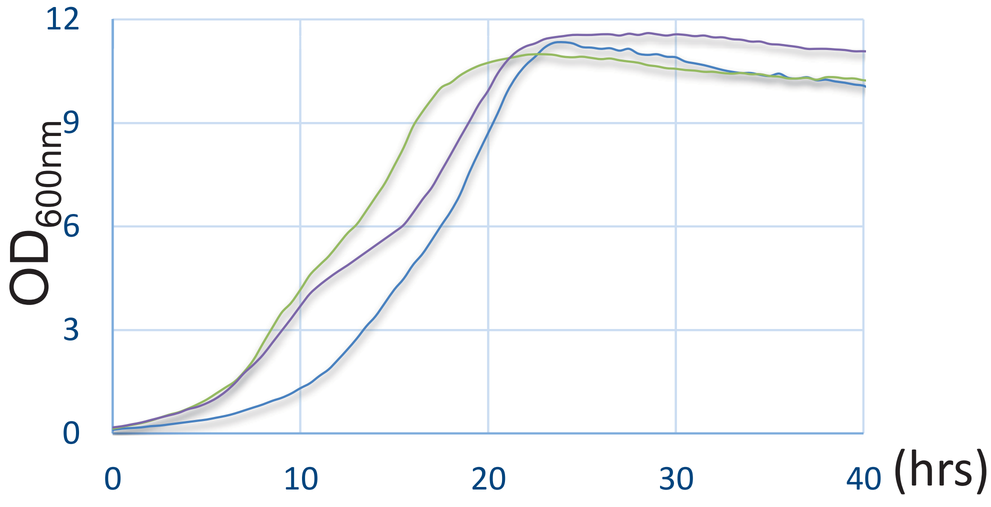


≈140 generations

Evolved strain performance in mineral medium with xylose

Growth parameters (Ty lines)

μ_{max}=0.11-0.16 h^{-1}

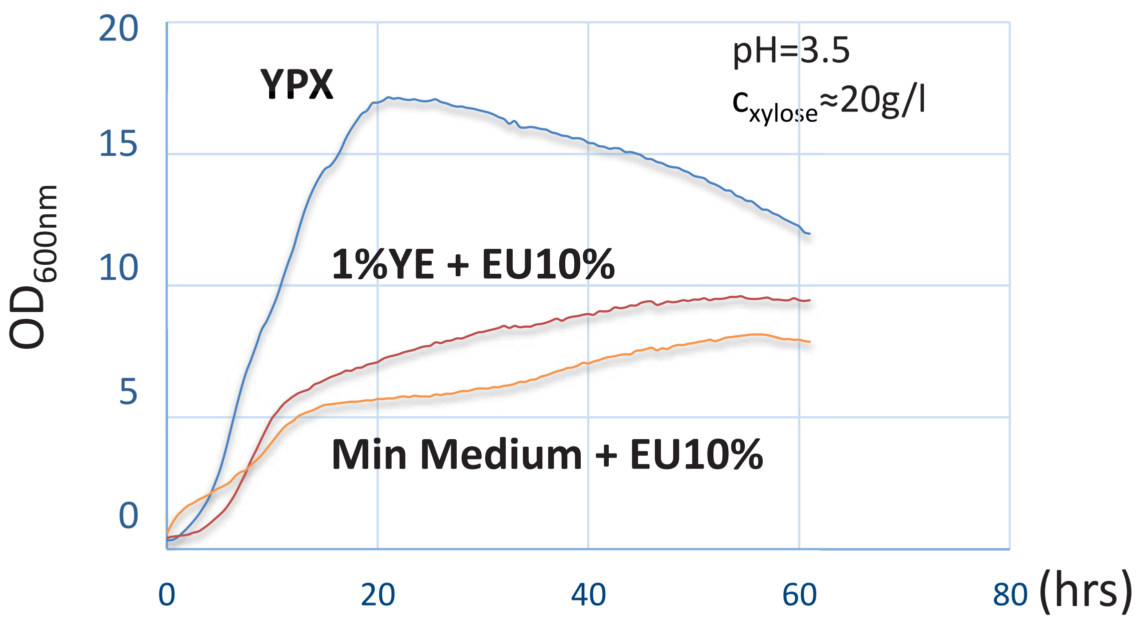


- Identification of (a) nutrient(s) (AA) needed for growth of the non-evolved strains in mineral medium
- NGS analysis – identification of putative driver mutations (nutrient sensing regulation, metabolism of aromatic compounds → reverse engineering ongoing

Adaptive evolution for strain performance in Eucalyptus SSL
Initial conditions for adaptive evolution

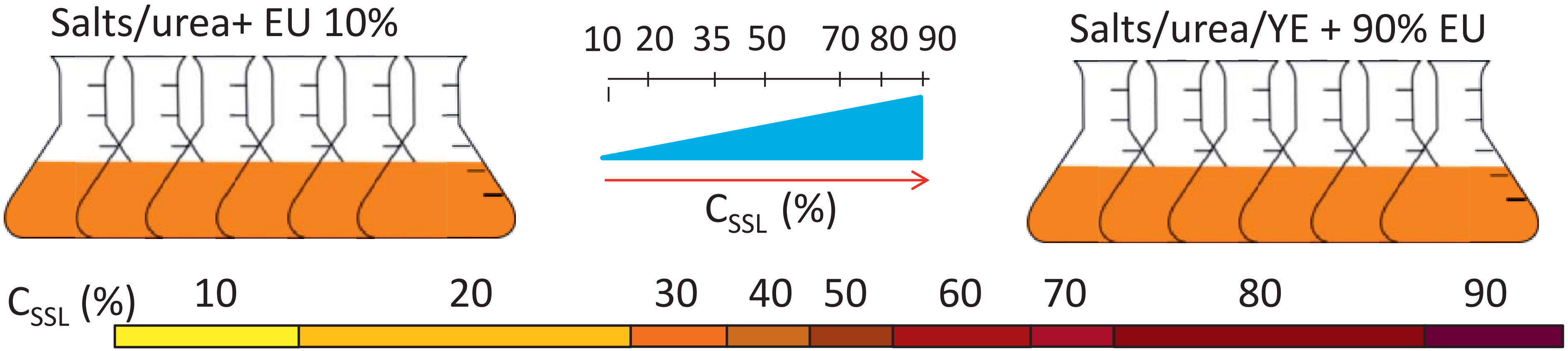
Ethanol Red
$XylC_2$ (Ty4) – Sin derivative not displayed (similar profile)

Growth in hardwood SSL hydrolyzate - EU (supplemented with yeast extract/salts+urea)



✘ No growth in >20% Hardwood SSL

Adaptive evolution of the engineered xylose consumers in a biomass (C5-rich) feedstock

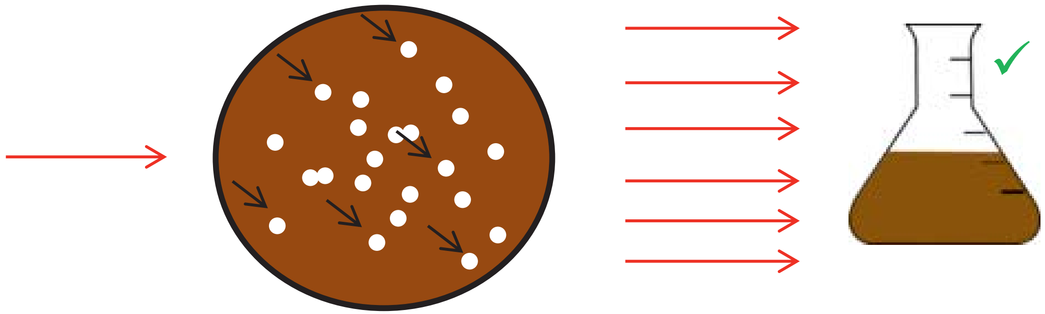


≈300 generations

Time (days)

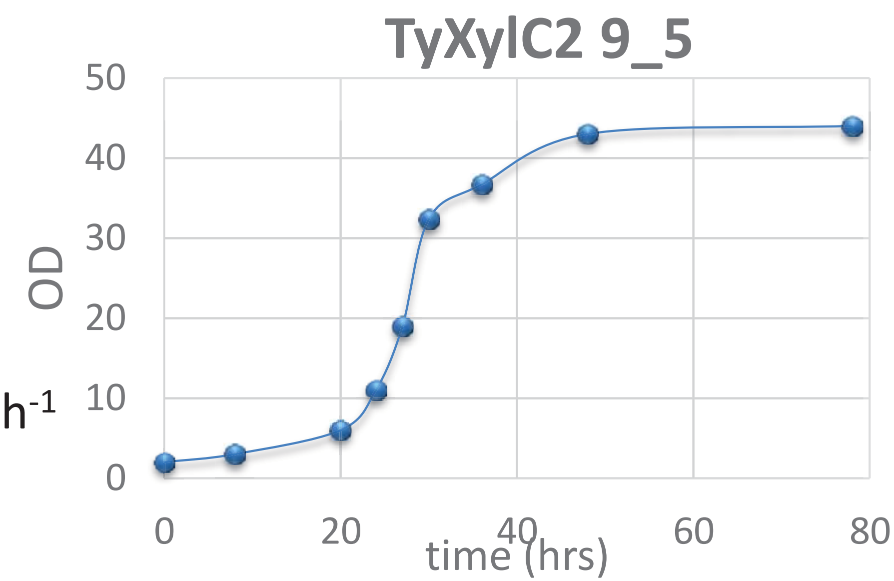
EU SSL evolved strains engineered to produce diacids - characterization

YE/MM + 90% EU



Strains (single clone isolates) can perform in up to 90% Hardwood SSL (both YE, Min M - supplemented) at pH=3.5

80% YE SSL

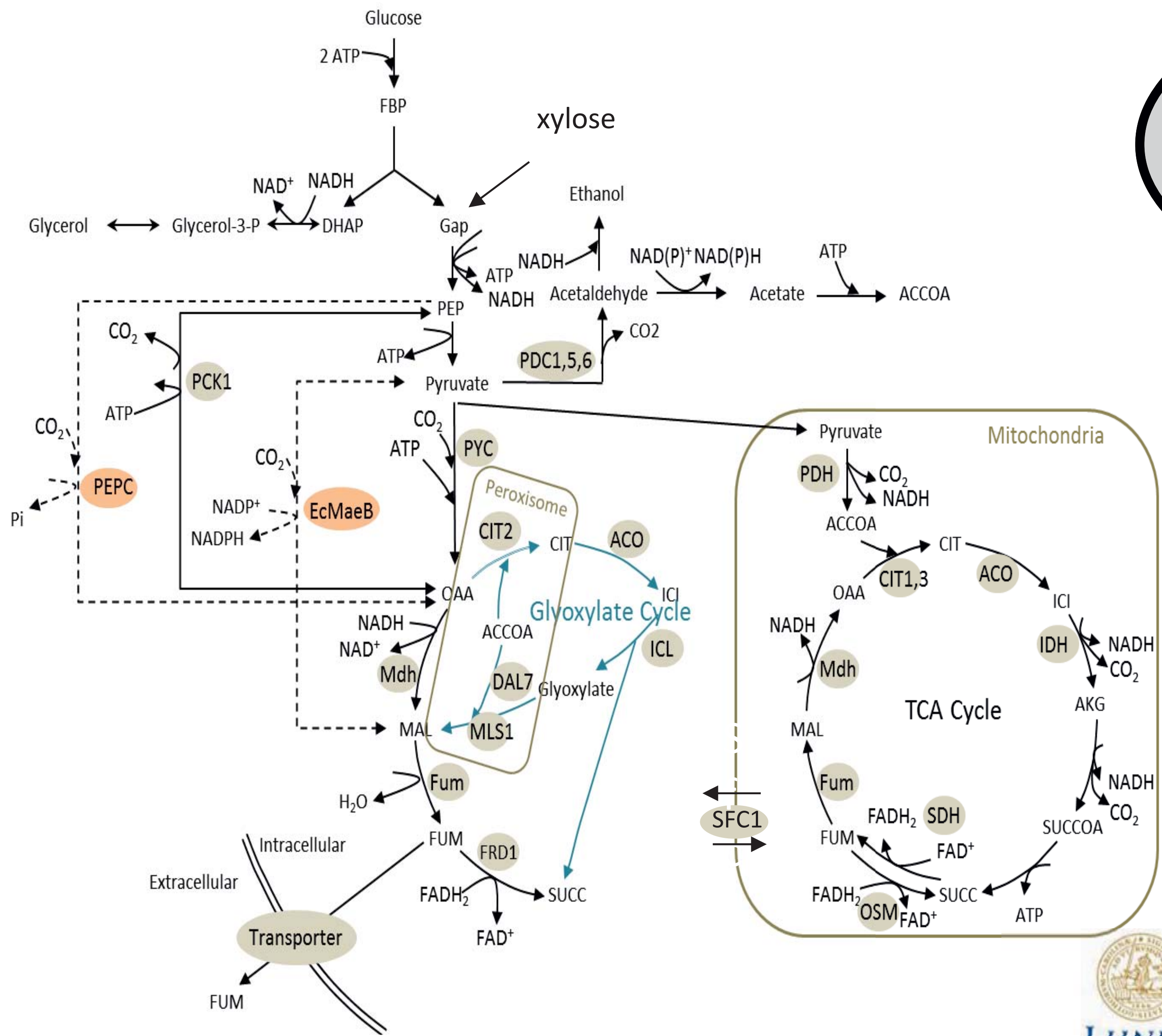


EVO EU SSL XylC2
Evolution in Eucalyptus hydrolysate
MM/YE

$\mu_{max} = 0{,}0556\ h^{-1}$

NGS analysis – identification of causative mutations – stress tolerance mechanisms

Production of diacids in the industrial strain evolved for tolerance to the SSL

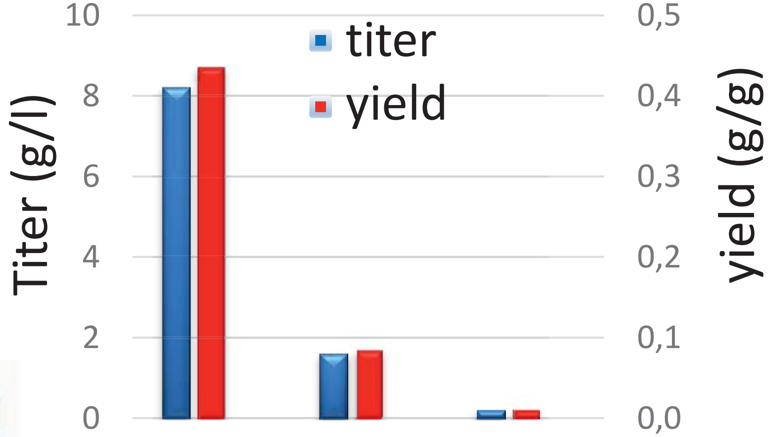


TyXYLC2 9_5 MA2

•↑*PYC2*↑*MDH3*$^{\Delta SKL}$

↑*SpMAE*

Nitrogen limitation
(C/N ratio=10)
20 g/l xylose



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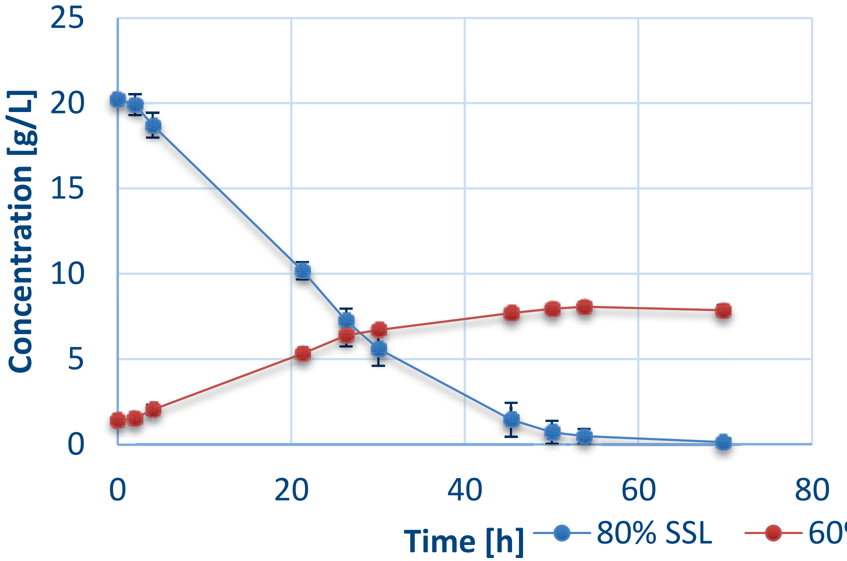
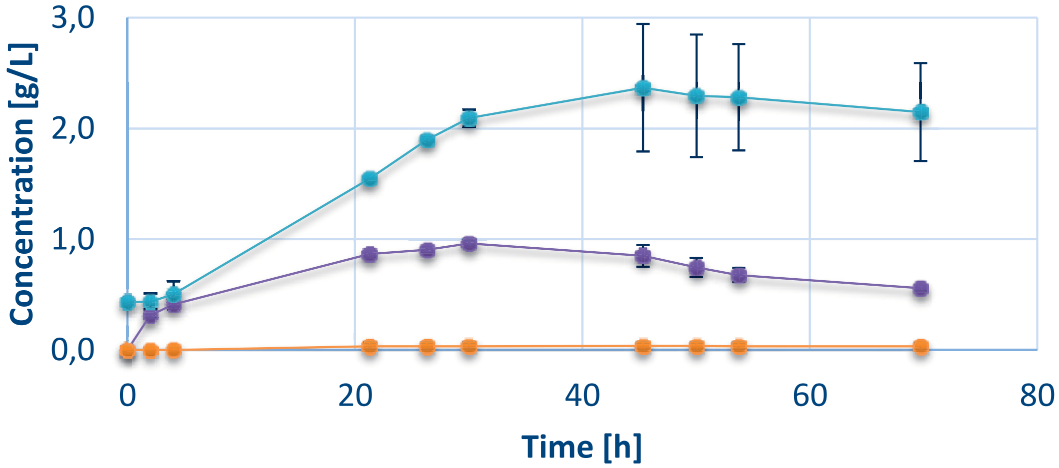
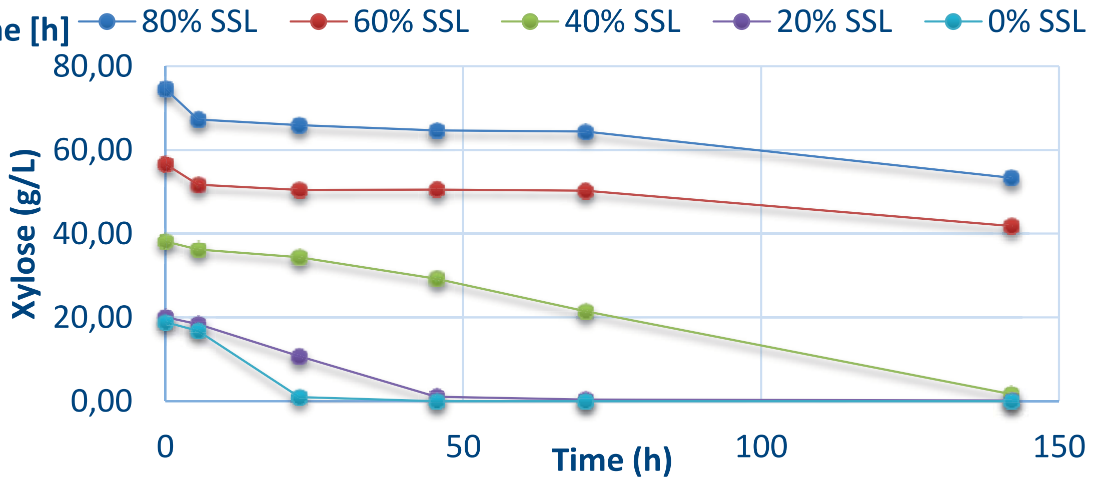
Strain characterization in larger scale

1 l bioreactor (90% air/10% CO_2, pH=4)







More concentrated SSL – demo scale-up

Determination of the production in the SSL – ongoing

Scale up process – ongoing

Highlights & Conclusions

- **Development of a molecular toolbox based on CRISPR-Cas9 for fast engineering of industrial yeast strains**
- **Construction of xylose utilizing industrial strains**
- **Improved xylose utilization properties via adaptive evolution – platform strain**
- **Tolerance of the industrial xylose consumers to a C5-rich SSL (both YE, MM supplemented) at low pH – platform strain**
- **Industrial strain tolerant to the SSL engineered for production of dicarboxylic acids converts up to 50% of the xylose content to a mixture of diacids with low proportion of fumaric acid**

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