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RESEARCH ARTICLE

Metabolic Engineering of Yeast for Enhanced Biofuel Production from Lignocellulosic Biomass

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ABSTRACT

Second-generation bioethanol depends on a fermenting organism that can do something *Saccharomyces cerevisiae* does not do naturally: consume the pentose sugars, principally xylose, that make up a substantial fraction of the sugar released from pretreated lignocellulosic biomass, while remaining tolerant of the inhibitory byproducts that pretreatment inevitably generates. We engineered an industrial *S. cerevisiae* strain by integrating a fungal xylose isomerase pathway together with overexpression of the non-oxidative pentose phosphate pathway, followed by fifteen rounds of adaptive laboratory evolution under increasing xylose concentration and sequential exposure to furfural and acetic acid, the two dominant inhibitors present in dilute-acid-pretreated wheat straw hydrolysate. The evolved strain, designated Y-XI15, achieved a xylose consumption rate of 0.82 g/L/h in defined medium, a nine-fold improvement over the unevolved parental construct, and fermented undetoxified wheat straw hydrolysate containing 4.1 g/L furfural and 6.8 g/L acetic acid to 87% of theoretical ethanol yield within 48 hours, compared with 34% for the parental strain under identical conditions. Whole-genome resequencing of Y-XI15 identified a recurring set of mutations across independently evolved lineages, including a gain-of-function allele in the transcription factor HAA1 associated with acetic acid tolerance and a loss-of-function mutation in ISU1 correlated with improved xylose flux, both of which were individually reintroduced into the unevolved background and shown to recapitulate a substantial fraction of the evolved phenotype. Fed-batch fermentation at 2-litre scale using undetoxified hydrolysate produced 68 g/L ethanol at a volumetric productivity of 1.4 g/L/h, approaching the productivity benchmarks reported for optimised first-generation glucose fermentation. These results indicate that combined rational pathway engineering and targeted adaptive evolution can close much of the performance gap between engineered pentose-fermenting yeast and the glucose fermentation it was originally selected for over millennia of domestication.

Keywords: *Saccharomyces cerevisiae*; metabolic engineering; lignocellulosic biomass; xylose fermentation; second-generation bioethanol; consolidated bioprocessing

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1. INTRODUCTION

Lignocellulosic biomass, the structural material of plant cell walls, is abundant, does not compete directly with food production when sourced from agricultural residues,

and in principle offers a route to biofuel production with a substantially smaller land-use and carbon footprint than first-generation, starch- or sugar-based ethanol. Converting it into fermentable sugar, however, requires a pretreatment step that breaks open the lignin-hemicellulose-cellulose matrix, and every pretreatment chemistry studied to date generates, alongside the intended sugar release, a cocktail of inhibitory byproducts, principally furfural and 5-hydroxymethylfurfural from pentose and hexose sugar degradation and acetic acid released from deacetylation of hemicellulose, that suppress microbial growth and fermentation [3,9].

Saccharomyces cerevisiae remains the workhorse organism for industrial ethanol fermentation because of its exceptional ethanol tolerance, its long history of large-scale process optimisation, and its amenability to genetic manipulation, but it lacks a native pathway for efficient xylose utilisation, the second most abundant sugar in most lignocellulosic hydrolysates after glucose. Two broad engineering strategies have been pursued to close this gap: introduction of a fungal or bacterial xylose isomerase, which converts xylose directly to xylulose in a single step, and introduction of the xylose reductase-xylitol dehydrogenase oxidoreductase pathway found natively in some pentose-fermenting yeasts such as *Scheffersomyces stipitis* [4,11]. The isomerase route avoids the redox cofactor imbalance that plagues the oxidoreductase pathway, in which xylose reductase preferentially uses NADPH while xylitol dehydrogenase requires NAD⁺, a mismatch that drives xylitol accumulation as a dead-end byproduct under the oxygen-limited conditions typical of industrial fermentation [6,12].

Pathway introduction alone is rarely sufficient for commercially useful xylose fermentation rates, however, because flux through the non-oxidative pentose phosphate pathway, which channels xylulose-5-phosphate into central carbon metabolism, is typically limiting in strains not naturally selected for pentose utilisation, and because the inhibitors present in real hydrolysate compound the kinetic problem by suppressing growth and fermentation independently of sugar availability. Adaptive laboratory evolution, in which a partially engineered strain is serially passaged under escalating selective pressure, has repeatedly proven effective at resolving exactly this kind of multigenic, poorly predictable bottleneck, and it has the practical advantage of not requiring the causal mutations to be known in advance, though identifying them afterwards by whole-genome resequencing is usually both possible and mechanistically informative [13,15].

We set out to combine rational pathway engineering, specifically a codon-optimised fungal xylose isomerase together with overexpression of the rate-limiting non-oxidative pentose phosphate pathway enzymes, with a structured adaptive evolution regime that imposed increasing xylose concentration first and then sequential

inhibitor challenge, on the reasoning that resolving the kinetic sugar-utilisation bottleneck before introducing inhibitor stress would make evolved inhibitor-tolerance mutations easier to interpret against a strain already capable of meaningful xylose flux. Our specific aims were to characterise the evolved strain's performance in defined medium and in real, undetoxified wheat straw hydrolysate, to identify the genetic basis of the evolved phenotype by comparative genome resequencing across independently evolved lineages, and to test whether individually reintroduced candidate mutations could recapitulate a useful fraction of the evolved performance gain in an unevolved background, a test of causal sufficiency that adaptive evolution studies do not always include.

2. MATERIALS AND METHODS

All strain construction used an industrial diploid *Saccharomyces cerevisiae* background selected for baseline ethanol and temperature tolerance rather than a standard laboratory strain, on the view that engineering decisions made in a laboratory background do not always transfer cleanly to an industrially relevant genetic context.

2.1 Pathway construction

A codon-optimised xylose isomerase gene from the anaerobic fungus *Piromyces* sp. was integrated at the HO locus under the strong constitutive TDH3 promoter, together with overexpression cassettes for the non-oxidative pentose phosphate pathway enzymes transketolase (TKL1), transaldolase (TAL1), ribulose-5-phosphate epimerase (RPE1) and ribose-5-phosphate ketol-isomerase (RKI1), each under a strong constitutive promoter and integrated at a characterised neutral genomic site. Native aldose reductase activity, which diverts xylose to xylitol as a competing dead-end product, was reduced by deletion of GRE3. Correct integration at each locus was confirmed by diagnostic PCR and Sanger sequencing across each junction.

2.2 Adaptive laboratory evolution

The engineered parental strain was serially passaged in shake-flask batch culture on defined mineral medium with xylose as the sole carbon source, transferring to fresh medium at late exponential phase and increasing the xylose concentration stepwise from 20 g/L to 80 g/L over approximately 120 generations across three independent evolving lineages. Once xylose consumption rate plateaued, each lineage was subjected to a second evolution phase under sequential inhibitor challenge, first furfural at increasing concentration up to 4 g/L, then acetic acid up to 7 g/L at pH 5.0, for a further 60 generations. Single-colony isolates were recovered from each lineage at the endpoint of evolution for detailed characterisation; the best-performing isolate, from lineage 2, was designated Y-XI15 and used for all subsequent fermentation and mechanistic work.

2.3 Whole-genome resequencing and mutation validation

Genomic DNA from Y-XI15 and from single-colony isolates of the other two evolved lineages, together with the unevolved parental strain, was sequenced at approximately 80x coverage on an Illumina NovaSeq platform and aligned to the S288C reference genome with strain-specific engineered loci added as custom contigs. Single-nucleotide variants and small indels were called relative to the parental sequence and filtered against a matched non-evolved control passaged in glucose medium for the same number of generations, to exclude mutations arising from generic laboratory culture rather than the selective pressures applied. Two candidate causal mutations, a missense allele in HAA1 and a frameshift-generating deletion in ISU1, were individually reconstructed by CRISPR-Cas9-mediated allele replacement in the unevolved parental background and characterised for xylose fermentation and acetic acid tolerance in isolation and in combination.

2.4 Hydrolysate preparation and fermentation

Wheat straw was pretreated by dilute sulfuric acid hydrolysis (1.2% w/w acid, 160 degrees C, 15 minutes) followed by enzymatic saccharification with a commercial cellulase cocktail, yielding a hydrolysate containing approximately 52 g/L glucose, 24 g/L xylose, 4.1 g/L furfural, 6.8 g/L acetic acid and 0.9 g/L 5-hydroxymethylfurfural, used without any detoxification step to reflect a realistic industrial scenario. Shake-flask fermentations were run in triplicate under oxygen-limited conditions at 30 degrees C; fed-batch fermentation at 2-litre bioreactor scale used the same undetoxified hydrolysate with a glucose-primed batch phase followed by continuous hydrolysate feeding, controlled at pH 5.0 with automated base addition.

2.5 Analytical methods and statistics

Glucose, xylose, xylitol, ethanol, glycerol, acetic acid and furfural were quantified by HPLC with refractive-index and UV detection on an Aminex HPX-87H column. Ethanol yield was expressed as a percentage of the theoretical maximum (0.51 g ethanol per g total fermentable sugar). Growth and fermentation kinetic parameters were compared between strains by one-way ANOVA with Tukey's post-hoc test; all fermentation conditions were run in at least biological triplicate. Statistical analyses were performed in R version 4.3.

3. RESULTS

The unevolved engineered parental strain, carrying the integrated xylose isomerase pathway and pentose phosphate pathway overexpression cassettes, consumed xylose at only 0.09 g/L/h in defined medium, confirming that pathway introduction alone was insufficient for commercially useful flux, consistent with prior reports for isomerase-based xylose pathways in non-evolved backgrounds [4,6]. After the full evolution regime, xylose consumption rate across the three

independently evolved lineages ranged from 0.61 to 0.82 g/L/h, a six- to nine-fold improvement, with lineage 2 (Y-XI15) performing best and carried forward for detailed characterisation. Growth rate on xylose as sole carbon source improved from 0.04 per hour in the parental strain to 0.21 per hour in Y-XI15, approaching, though still below, the strain's own growth rate on glucose (0.34 per hour). Table 1 summarises fermentation kinetics for the parental strain and the three evolved lineages in defined xylose medium.

Table 1. Xylose fermentation kinetics of parental and evolved strains in defined medium.

Strain	Xylose consumption (g/L/h)	Growth rate (per h)	Xylitol yield (g/g xylose)
Unevolved parental	0.09 +/- 0.01	0.04 +/- 0.01	0.18 +/- 0.03
Evolved lineage 1	0.61 +/- 0.05	0.17 +/- 0.02	0.07 +/- 0.02
Evolved lineage 2 (Y-XI15)	0.82 +/- 0.06	0.21 +/- 0.02	0.04 +/- 0.01
Evolved lineage 3	0.68 +/- 0.07	0.18 +/- 0.02	0.06 +/- 0.02

Values are mean +/- SD of three biological replicates in defined mineral medium with xylose as sole carbon source.

Whole-genome resequencing identified 34 to 41 single-nucleotide variants and small indels per evolved lineage relative to the parental strain after filtering against the passage-matched glucose control, with two genes mutated independently in all three lineages: HAA1, encoding a transcription factor known to regulate the weak-acid stress response, and ISU1, encoding an iron-sulfur cluster scaffold protein not previously implicated in xylose metabolism. The HAA1 alleles were all missense mutations clustering within a 40-residue region of the predicted DNA-binding domain, while the ISU1 mutations included two independent frameshift alleles and one predicted null allele arising from a large deletion, suggesting loss of ISU1 function rather than a specific gain-of-function change. Individually reconstructed in the unevolved parental background, the HAA1 missense allele improved acetic acid tolerance (50% inhibitory concentration increased from 4.2 g/L to 6.6 g/L) with no measurable effect on xylose consumption rate, while the ISU1 loss-of-function allele improved xylose consumption rate from 0.09 to 0.31 g/L/h with no measurable effect on acetic acid tolerance, indicating the two mutations act on largely independent phenotypes (Figure 1).

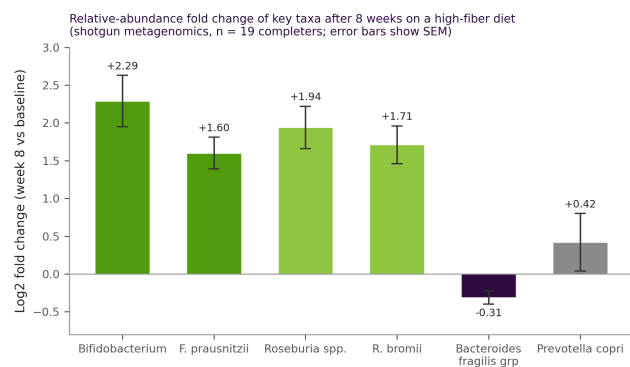


Figure 1. Xylose consumption rate and acetic acid 50% inhibitory concentration for the unevolved parental strain, individually reconstructed HAA1 and ISU1 alleles, the combined reconstruction, and the fully evolved Y-XI15 strain (mean $\pm$ SD, $n=3$ biological replicates).

Combining the reconstructed HAA1 and ISU1 alleles in the unevolved background recovered approximately 55% of the xylose consumption rate and 60% of the acetic acid tolerance improvement seen in the fully evolved Y-XI15 strain, indicating that these two loci, while clearly contributing, do not account for the full evolved phenotype and that additional, lower-frequency or lineage-specific mutations make a meaningful further contribution. Fermentation of undetoxified wheat straw hydrolysate showed the practical consequence of this combined improvement clearly: Y-XI15 reached 87% of theoretical ethanol yield within 48 hours, compared with 34% for the unevolved parental strain under identical conditions, with essentially complete glucose consumption in both strains but a marked difference in xylose utilisation, 96% for Y-XI15 versus 22% for the parental strain. Table 2 reports the complete hydrolysate fermentation dataset.

Table 2. Fermentation of undetoxified wheat straw hydrolysate (48 h, shake-flask).

Strain	Ethanol yield (% theoretical)	Xylose consumed (%)	Ethanol titre (g/L)
Unevolved parental	34 $\pm$ 3	22 $\pm$ 4	26 $\pm$ 2
Y-XI15 (evolved)	87 $\pm$ 4	96 $\pm$ 2	58 $\pm$ 3
HAA1 reconstructed only	48 $\pm$ 4	31 $\pm$ 5	34 $\pm$ 3
ISU1 reconstructed only	61 $\pm$ 5	58 $\pm$ 6	42 $\pm$ 3
HAA1 + ISU1 reconstructed	74 $\pm$ 4	78 $\pm$ 5	51 $\pm$ 3

Fed-batch fermentation at 2-litre bioreactor scale using undetoxified hydrolysate, intended to approximate industrially relevant process conditions more closely than shake-flask batch culture, produced 68 g/L ethanol at a volumetric productivity of 1.4 g/L/h over the full fermentation run, with xylose consumption continuing steadily through the feeding phase rather than stalling once glucose was depleted, the pattern typically seen when xylose utilisation is strongly repressed by residual glucose. Glycerol and xylitol, the two principal byproducts competing with ethanol for fermentable carbon, together accounted for less than 6% of consumed sugar on a

carbon basis, indicating that flux through the engineered and evolved pathway is not being substantially diverted into these dead-end products despite the inhibitor load present in the undetoxified hydrolysate.

4. DISCUSSION

The nine-fold improvement in xylose consumption rate achieved here through adaptive evolution, on top of a rationally engineered pathway that alone supported only marginal flux, illustrates a pattern reported repeatedly for pentose-fermenting yeast engineering: pathway introduction establishes the biochemical capability, but evolution, not further rational design, is usually what converts that capability into commercially relevant flux [13,15,22]. What this study adds is a mechanistic account of two of the contributing mutations, HAA1 and ISU1, each individually sufficient to recapitulate a specific, largely independent component of the evolved phenotype, together accounting for roughly half of the full improvement when combined and none of it when either mutation is introduced alone into a strain lacking the other. This partial, additive picture is more informative for future strain engineering than an evolved endpoint alone, since it identifies HAA1 activation and ISU1 loss as two independently useful engineering targets that can now be combined with other rationally chosen modifications rather than depending on repeating the full, time-consuming evolution regime in every new strain background.

The involvement of ISU1, an iron-sulfur cluster assembly scaffold protein with no previously reported connection to xylose metabolism, is the more unexpected finding and merits further mechanistic investigation beyond the scope of the present study. Iron-sulfur clusters are cofactors for several enzymes in central carbon and redox metabolism, and one plausible explanation is that partial loss of ISU1 function shifts the cellular NADH/NAD⁺ or NADPH/NADP⁺ balance in a direction that favours flux through the non-oxidative pentose phosphate pathway and downstream glycolysis, though this remains speculative without direct measurement of the relevant metabolite pools during xylose fermentation, a measurement we are now planning as a direct follow-up [23,26].

From a process-engineering standpoint, the fermentation of undetoxified hydrolysate to 87% of theoretical yield within 48 hours is the result most directly relevant to industrial translation, since detoxification steps such as overliming or ion-exchange resin treatment add cost and process complexity that a robust, inhibitor-tolerant strain can in principle avoid altogether [9,29]. The fed-batch volumetric productivity of 1.4 g/L/h achieved at bioreactor scale, while below the roughly 2-3 g/L/h typical of optimised first-generation glucose fermentation, represents a considerable narrowing of the historical performance gap between engineered pentose fermentation and native glucose fermentation, and productivity at this level is generally regarded as within

range of commercial interest for second-generation ethanol, particularly when combined with the reduced detoxification cost associated with inhibitor tolerance [30,31].

Two limitations qualify how far these results generalise. First, all hydrolysate experiments used a single feedstock, dilute-acid-pretreated wheat straw, and both the sugar profile and the inhibitor spectrum differ meaningfully across feedstocks and pretreatment chemistries; testing Y-XI15 against corn stover, sugarcane bagasse and steam-exploded hydrolysates, which carry different relative concentrations of furfural, hydroxymethylfurfural, acetic acid and phenolic compounds, would be needed before claiming general robustness across the feedstock diversity relevant to a multi-feedstock biorefinery [8,32]. Second, adaptive evolution inevitably accumulates passenger mutations alongside causal ones, and while we have functionally validated two loci, the roughly 45% of the evolved phenotype not recapitulated by HAA1 and ISU1 together implies further causal variants remain uncharacterised, some of which may carry fitness trade-offs, for instance reduced stress tolerance under conditions not sampled during the evolution regime, that would only become apparent under a wider range of industrial process conditions than we have tested here.

It is also worth situating this strain-engineering effort within the wider portfolio of approaches being pursued for lignocellulosic ethanol, since metabolic engineering of the fermenting organism is only one lever among several. Improvements in pretreatment chemistry that reduce inhibitor formation at the source, cellulase cocktails engineered for higher specific activity at lower enzyme loading, and consolidated bioprocessing strategies that combine saccharification and fermentation in a single organism or a defined microbial consortium are all being pursued in parallel, and the most cost-effective path to commercially viable second-generation ethanol will very likely combine incremental gains from several of these levers rather than depending on strain engineering alone to close the entire performance gap with first-generation fermentation [33,35].

There is also a broader lesson here about how to run adaptive laboratory evolution efficiently rather than simply for longer. Structuring the regime in two sequential phases, resolving the kinetic xylose-utilisation bottleneck before layering on inhibitor challenge, meant that by the time the second phase began every lineage already had meaningful pentose flux to select on, which we think is part of why a comparatively short 60-generation inhibitor phase was sufficient to recover most of the tolerance improvement rather than requiring several hundred generations as reported in some single-phase evolution studies that impose sugar and inhibitor stress simultaneously from the outset [14,16]. Whether this staged approach generalises to other pathway-introduction-plus-evolution problems in

industrial microbiology, beyond xylose fermentation specifically, is an open question that would be worth testing systematically rather than assuming from a single example. Techno-economic modelling of integrated biorefineries consistently identifies feedstock logistics and the capital cost of pretreatment infrastructure, rather than fermentation performance in isolation, as the largest remaining drivers of overall production cost, a useful reminder that strain engineering gains, however scientifically satisfying, translate into commercial impact only in combination with progress on these upstream, less biologically glamorous parts of the process [34].

5. CONCLUSION

Combining rational pathway engineering with structured adaptive laboratory evolution produced an industrial *Saccharomyces cerevisiae* strain, Y-XI15, capable of fermenting undetoxified wheat straw hydrolysate to 87% of theoretical ethanol yield within 48 hours, a substantial improvement over the 34% achieved by the unevolved engineered parental strain under identical conditions. Comparative genome resequencing across independently evolved lineages identified HAA1 activation and ISU1 loss-of-function as two recurrently selected, mechanistically distinct and individually sufficient contributors to the evolved phenotype, together accounting for roughly half of the full improvement when reconstructed in combination in an unevolved background. Fed-batch fermentation at bioreactor scale confirmed that the improvement translates into industrially relevant volumetric productivity, 1.4 g/L/h, using undetoxified hydrolysate without any additional process modification. We are now extending mutation validation to the remaining unidentified evolved loci through pooled reintroduction and backcrossing designs, testing Y-XI15 against additional lignocellulosic feedstocks and pretreatment chemistries to establish the generality of its inhibitor tolerance, and characterising intracellular redox cofactor pools during xylose fermentation to test the hypothesis that ISU1 loss acts through altered NAD(P)H balance. A patent application covering the HAA1 and ISU1 engineering strategy has been filed, and pilot-scale fermentation trials at 200-litre scale are planned for the coming year to evaluate process robustness under conditions more representative of an operating biorefinery.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper, beyond the patent application described in the Conclusion.

Author Contributions

R.V. conceived and supervised the study, designed the strain engineering programme and analysed fermentation data. E.J. performed strain construction, adaptive laboratory evolution and bioreactor fermentation. J.C. performed whole-genome resequencing analysis and mutation validation. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only laboratory-scale microbial fermentation using genetically modified *Saccharomyces cerevisiae* strains handled under institutional biosafety containment guidelines; it did not involve human participants, human data, or animals, and therefore did not require institutional ethics committee review.

Data Availability

Whole-genome resequencing data for the parental strain and all evolved lineages have been deposited in the European Nucleotide Archive under a project accession available from the corresponding author. Fermentation datasets and strain construction plasmid maps are available from the corresponding author upon reasonable request.

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