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Targeted Metabolic Engineering of *Saccharomyces cerevisiae* for High-Efficiency Valorization of Lignocellulosic Biomass into Superior-Quality Bioplastics

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A B S T R A C T

The global transition towards a sustainable circular bioeconomy urgently requires innovative platforms for converting renewable waste streams into value-added products. Lignocellulosic biomass, particularly agricultural residue like rice straw, stands as a vast, underutilized carbon source. This study details the systematic metabolic engineering of *Saccharomyces cerevisiae* for the high-efficiency production of poly(3-hydroxybutyrate) (PHB), a biodegradable bioplastic, from rice straw hydrolysate. A multi-faceted synthetic biology approach was implemented in *S. cerevisiae* CEN.PK2-1C. A robust xylose co-utilization pathway was integrated using codon-optimized genes from *Scheffersomyces stipitis*. The PHB biosynthesis pathway from *Cupriavidus necator* was introduced using a cassette of strong, constitutive yeast promoters (*pTDH3*, *pTEF1*, *pPGK1*). To maximize carbon flux towards PHB, key competing pathways were eliminated via CRISPR-Cas9-mediated gene knockouts of the primary alcohol dehydrogenase (*ADH1*) and glycerol-3-phosphate dehydrogenase (*GPD1*) genes. The performance of the final engineered strain was evaluated in high-cell-density fed-batch fermentation using detoxified rice straw hydrolysate sourced from Palembang, Indonesia. The final engineered strain, YL-PHB-05 (Δ*adh1* Δ*gpd1*), demonstrated superior performance. In fed-batch bioreactor cultivation, it achieved a final cell dry weight of 33.8 ± 1.5 g/L and a PHB titer of 15.2 ± 0.7 g/L, with an intracellular PHB accumulation of 45.0 ± 1.2% of cell dry weight. This corresponds to a high yield of 0.28 g PHB per gram of consumed sugars. Crucially, the produced PHB exhibited a superior weight-average molecular weight (Mw) of 1.2 x 10^6 Da with a polydispersity index of 2.1. In conclusion, this work successfully demonstrates a robust strategy for engineering *S. cerevisiae* into an efficient cell factory for producing high-quality bioplastics from a globally relevant agricultural waste stream. The high titers, yields, and superior polymer properties achieved present a significant advancement towards establishing an economically viable and sustainable process for bioplastic production within a circular bioeconomy.

1. Introduction

The 21st century is defined by the interconnected global challenges of climate change, environmental degradation, and resource depletion, largely stemming from a deeply entrenched linear economic model predicated on "take-make-dispose" principles.[1] In response, the concept of a circular bioeconomy has emerged as a critical paradigm for sustainable development. This model champions the use of renewable biological resources, such as agricultural and forestry residues, not as waste but as valuable feedstocks for the production of biofuels, biochemicals, and biomaterials, thereby creating closed-loop systems that minimize waste and environmental impact.[2] At the heart of this paradigm lies lignocellulosic biomass, the most abundant and

renewable form of terrestrial carbon on Earth, representing a cornerstone for the next generation of industrial biotechnology.[3]

Lignocellulosic biomass is a complex composite material primarily consisting of cellulose, hemicellulose, and lignin. Through biochemical conversion processes, the carbohydrate polymers—cellulose and hemicellulose—can be hydrolyzed into their constituent fermentable monosaccharides, predominantly the C6 sugar glucose and the C5 sugar xylose. These sugars serve as a versatile platform for microbial fermentation to produce a diverse portfolio of value-added products. However, a persistent bottleneck in lignocellulosic biorefineries is the efficient co-utilization of both glucose and xylose. Many industrially relevant microorganisms either cannot naturally metabolize xylose or exhibit strong carbon catabolite repression, where the presence of glucose inhibits the uptake and metabolism of other sugars, leading to an incomplete and inefficient valorization of the feedstock.[4,5]

Saccharomyces cerevisiae, colloquially known as baker's yeast, is the undisputed workhorse of industrial biotechnology. Its long history of safe use (GRAS status), exceptional tolerance to industrial inhibitors found in hydrolysates, and amenability to genetic manipulation make it a premier host for large-scale fermentation processes. The primary limitation of wild-type *S. cerevisiae* for lignocellulosic applications is its innate inability to metabolize xylose. Over the past two decades, significant breakthroughs in synthetic biology and metabolic engineering have successfully addressed this challenge. The introduction of heterologous xylose isomerase or oxidoreductase pathways has enabled the engineering of yeast strains capable of efficiently co-fermenting glucose and xylose, unlocking the full potential of lignocellulosic feedstocks.[6,7]

Among the vast array of potential bioproducts, biodegradable plastics have garnered immense interest as a sustainable solution to the global crisis of plastic pollution. Polyhydroxyalkanoates (PHAs) are a class of bio-based and biodegradable polyesters naturally synthesized by numerous microorganisms as intracellular carbon and energy storage granules. Their material properties are highly tunable, ranging from rigid and brittle to flexible and elastomeric, making them suitable for a wide spectrum of applications, including packaging films, medical implants, and consumer goods. The commercialization of PHAs has been historically constrained by high production costs, which are intrinsically linked to the price of sterile, refined substrates like glucose or sucrose. The utilization of low-cost, non-food lignocellulosic waste as a feedstock is therefore a critical strategy for making PHAs economically competitive with their fossil-fuel-derived counterparts.[8-10]

This study aims to develop and optimize a robust *Saccharomyces cerevisiae* cell factory for the high-efficiency conversion of lignocellulosic waste into high-value bioplastics. The novelty of this research lies in the integrated, multi-level metabolic engineering strategy employed. We combine the introduction of efficient glucose and xylose co-utilization machinery with the high-level expression of a heterologous PHB synthesis pathway and, critically, the strategic elimination of major carbon-competing pathways using precise CRISPR-Cas9 genome editing. This holistic approach is designed to channel the maximum possible carbon flux from rice straw-derived sugars towards the synthesis of PHB. The specific contributions and differentiators of this work compared to previous studies include: (1) the targeted knockout of both ethanol (*ADH1*) and glycerol (*GPD1*) pathways in a xylose-utilizing yeast for PHB production, a combination designed for maximal precursor redirection; (2) the utilization of locally sourced rice straw from Palembang, Indonesia, demonstrating the adaptability of the technology to regionally specific feedstocks; and (3) a detailed characterization of the resulting polymer, revealing superior molecular weight properties that enhance its application potential. By integrating advanced synthetic biology tools with optimized bioprocess engineering, we present a compelling blueprint for the

sustainable production of bioplastics, directly contributing to the advancement of a circular bioeconomy.

2. Methods

All experimental work was conducted at the Eureka Research Laboratory in Palembang, Indonesia. Rice straw (*Oryza sativa*), selected as the model lignocellulosic feedstock, was sourced from local paddy fields in the Ogan Ilir district, South Sumatra, Indonesia. Upon collection, the straw was extensively washed with tap water to remove soil and debris, sun-dried for 72 hours, and then oven-dried at 60°C to a constant weight. The dried straw was subsequently milled using a Wiley mill and sieved to obtain a uniform particle size of 1–2 mm for subsequent processing.

A two-stage dilute acid hydrolysis protocol was optimized for the differential fractionation of hemicellulose and cellulose from the rice straw. Stage 1 (Hemicellulose Hydrolysis): The milled rice straw was mixed with 1.5% (v/v) sulfuric acid at a solid-to-liquid ratio of 1:10 (w/v). The slurry was autoclaved at 121°C for 60 minutes. After cooling, the xylose-rich liquid hydrolysate was separated from the solid residue by filtration. Stage 2 (Cellulose Hydrolysis): The cellulose-enriched solid residue from the first stage was resuspended in 2.5% (v/v) sulfuric acid at a 1:10 ratio and subjected to a more severe hydrolysis condition at 150°C for 30 minutes. The resulting glucose-rich hydrolysate was collected after filtration. Detoxification: The hydrolysates from both stages were combined. This combined hydrolysate was detoxified to remove microbial inhibitors (furfural, HMF, acetic acid) by overliming. The pH was raised to 10.0 by the slow addition of solid calcium hydroxide ($Ca(OH)_2$) under constant stirring, followed by incubation at 60°C for 30 minutes. The mixture was then filtered to remove the precipitate, and the pH of the detoxified liquid hydrolysate was adjusted to 5.5 using concentrated sulfuric acid. The final hydrolysate was filter-sterilized through a 0.22 µm membrane prior to use in fermentation media.

Saccharomyces cerevisiae CEN.PK2-1C (MATa; ura3-52; trp1-289; leu2-3,112; his3Δ1; MAL2-8C; SUC2) was used as the parental strain for all genetic engineering. Standard yeast cultivation was performed in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose). For selection of yeast transformants, synthetic complete (SC) drop-out medium was used, lacking the appropriate amino acid or nucleotide for auxotrophic selection. *Escherichia coli* DH5α was used for all plasmid cloning and propagation and was cultivated in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 100 µg/mL ampicillin where required. All plasmids, genetic parts, and primers used in this study are detailed in Table 1.

A stepwise metabolic engineering strategy was employed to construct the final production strain. All genetic modifications were performed using homologous recombination, and marker-less gene deletions were achieved using the CRISPR-Cas9 system. The xylose oxidoreductase pathway was chosen for xylose assimilation. The genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) were sourced from *Scheffersomyces stipitis*, and the endogenous xylulokinase (XK) gene from *S. cerevisiae* was overexpressed. All genes were codon-optimized for expression in yeast. The three genes, under the control of strong constitutive promoters (pTDH3 for XR, pTEF1 for XDH, pPGK1 for XK), were assembled into a single expression cassette and integrated into the yeast genome at the HIS3 locus, yielding strain YL-XYL-01.

The PHB biosynthesis pathway from *Cupriavidus necator* H16 was introduced. This pathway consists of three enzymes: β-ketothiolase (encoded by *phaA*), acetoacetyl-CoA reductase (encoded by *phaB*), and PHA synthase (encoded by *phaC*). The genes were codon-optimized and placed under the control of strong glycolytic promoters (pTEF1 for *phaA*, pPGK1 for *phaB*, and pTDH3 for *phaC*) and terminators (tADH1 and tCYC1). The three expression cassettes were integrated into the yeast genome at the URA3, LEU2, and TRP1 loci of strain YL-XYL-01, resulting in strain YL-PHB-01.

To redirect carbon flux from native fermentation byproducts towards the PHB pathway precursor, acetyl-CoA, two key genes were targeted for complete knockout using the CRISPR-Cas9 system. The primary alcohol dehydrogenase gene, *ADH1*, was deleted from the genome of YL-PHB-01 to block ethanol formation, creating strain YL-PHB-03. The primary glycerol-3-phosphate dehydrogenase gene, *GPD1*, was subsequently deleted from YL-PHB-03 to block glycerol formation, resulting in the final strain, YL-PHB-05.

The CRISPR-Cas9 system, consisting of a plasmid co-expressing the Cas9 protein and a gene-specific guide RNA (gRNA), was used for gene knockouts. A 100 bp donor DNA fragment consisting of sequences homologous to the regions immediately upstream and downstream of the target gene's open reading frame was co-transformed to direct homology-directed repair. Successful gene knockouts were initially screened by their inability to grow on selective media (for auxotrophic markers) and then rigorously confirmed by diagnostic colony PCR using primers flanking the target locus, followed by Sanger sequencing of the PCR product to verify the seamless deletion. Functional confirmation of knockout was achieved via HPLC analysis, which showed the absence of the corresponding byproduct under relevant culture conditions.

Initial characterization of all engineered strains was conducted in 250 mL baffled shake flasks containing 50 mL of SC medium supplemented with 20 g/L glucose and 10 g/L xylose. Cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.1 and incubated at 30°C with vigorous shaking at 200 rpm. Samples were withdrawn aseptically at regular time points for analysis.

The best-performing strain, YL-PHB-05, was evaluated in a 2 L stirred-tank bioreactor (Sartorius, Germany) with an initial working volume of 1 L. The medium consisted of the detoxified rice straw hydrolysate supplemented with 5 g/L yeast extract and 10 g/L peptone. The fermentation was initiated as a batch culture. After the initial batch of sugars was nearly depleted (indicated by a sharp spike in dissolved oxygen), a pre-programmed exponential feeding strategy was initiated to maintain a high cell growth rate. The feed solution consisted of concentrated, detoxified rice straw hydrolysate (260 g/L glucose, 130 g/L xylose) and was delivered at a rate designed to keep the residual total sugar concentration below 5 g/L to avoid overflow metabolism and substrate inhibition. The temperature was controlled at 30°C, and the pH was maintained at 5.5 via the automated addition of 2 M NaOH. The dissolved oxygen (DO) level was maintained above 20% saturation by cascading the agitation speed (300-900 rpm) and the aeration rate (1-2 vvm).

Cell growth was monitored by measuring OD_{600}. Cell dry weight (CDW) was determined by filtering 5 mL of culture broth through a pre-weighed 0.45 µm filter paper. The filter was washed twice with deionized water and dried at 80°C for 24 hours to a constant weight. The maximum specific growth rate (µ_max) was calculated during the exponential growth phase using the formula: $\mu = (\ln(X_2) - \ln(X_1)) / (t_2 - t_1)$, where X is the CDW at times t_1 and t_2.

The concentrations of glucose, xylose, ethanol, and glycerol in the culture supernatant (obtained by centrifugation at 13,000 x g for 5 min) were quantified by high-performance liquid chromatography (HPLC) on a Shimadzu system equipped with a Bio-Rad Aminex HPX-87H column and a refractive index (RI) detector. The column was operated at 60°C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min.

The intracellular PHB content was determined by gas chromatography (GC). Approximately 10-15 mg of lyophilized cells were subjected to methanolysis with 2 mL of chloroform and 2 mL of methanol containing 3% (v/v) H_2SO_4 at 100°C for 4 hours. This process converts the PHB polymer into its constituent methyl ester, 3-hydroxybutyrate methyl ester, which was then quantified by GC (Agilent 7890B) equipped with a flame ionization detector (FID). The PHB content was expressed as a percentage of the CDW. For polymer characterization, PHB was extracted from a larger

batch of lyophilized cells using hot chloroform, purified by precipitation in cold methanol, and analyzed. The weight-average molecular weight (Mw) and polydispersity index (PDI) were determined by gel permeation chromatography (GPC) using polystyrene standards. The chemical structure was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. All experiments, including shake flask and bioreactor fermentations, were performed in biological triplicate. The data are presented as the mean ± standard deviation. Statistical significance between different experimental groups was determined using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons, using GraphPad Prism software. A p-value of < 0.05 was considered statistically significant.

Table 1. Plasmids, Genetic Parts, and Primers Used in This Study.

NAME	TYPE	DESCRIPTION / SEQUENCE (5' TO 3')
Plasmids		
pRS426-pTDH3-phaC	Expression	Yeast expression vector (URA3 marker) for PhaC under TDH3 promoter
pRS425-pTEF1-phaA	Expression	Yeast expression vector (LEU2 marker) for PhaA under TEF1 promoter
pRS424-pPGK1-phaB	Expression	Yeast expression vector (TRP1 marker) for PhaB under PGK1 promoter
pRS423-XYZ	Expression	Yeast expression vector (HIS3 marker) for XR-XDH-XK cassette
pCas9-gRNA-ADH1	CRISPR	Cas9/gRNA vector for ADH1 knockout
pCas9-gRNA-GPD1	CRISPR	Cas9/gRNA vector for GPD1 knockout
Promoters		
pTDH3	Promoter	*S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase promoter
pTEF1	Promoter	*S. cerevisiae* translation elongation factor 1 alpha promoter
pPGK1	Promoter	*S. cerevisiae* 3-phosphoglycerate kinase promoter
Terminators		
tCYC1	Terminator	*S. cerevisiae* cytochrome c isoform 1 terminator
tADH1	Terminator	*S. cerevisiae* alcohol dehydrogenase 1 terminator
Primers		
ADH1_gRNA_F	gRNA Primer	`GCGACCACAAAATCTAAACG`
GPD1_gRNA_F	gRNA Primer	`GTTGATGGATCTGGTCAAAA`
ADH1_KO_ver_F	Verification	Primer flanking upstream of ADH1 locus for knockout verification
ADH1_KO_ver_R	Verification	Primer flanking downstream of ADH1 locus for knockout verification
GPD1_KO_ver_F	Verification	Primer flanking upstream of GPD1 locus for knockout verification
GPD1_KO_ver_R	Verification	Primer flanking downstream of GPD1 locus for knockout verification

3. Results and discussion

The optimized two-stage dilute acid hydrolysis and subsequent detoxification process yielded a sugar-rich, low-inhibitor hydrolysate suitable for yeast fermentation. The detailed composition is presented in Table 2. Glucose and xylose were the most abundant sugars, with concentrations of 65.4 ± 2.1 g/L and 32.8 ± 1.5 g/L, respectively, reflecting the successful breakdown of cellulose and hemicellulose. The detoxification by overliming proved effective, reducing the concentrations of key inhibitors like furfural and 5-hydroxymethylfurfural (HMF) to negligible levels (0.1 ± 0.02 g/L and 0.05 ± 0.01 g/L, respectively), well below their typical inhibitory thresholds for *S.*

cerevisiae. A series of engineered strains were constructed to systematically channel carbon flux from sugars to PHB. The genotypes of these strains are summarized in Table 3. The parental strain, CEN.PK2-1C, was first engineered to co-utilize xylose (YL-XYL-01), then to produce PHB (YL-PHB-01), and finally, competing byproduct pathways for ethanol (YL-PHB-03) and glycerol (YL-PHB-05) were sequentially knocked out.

Table 2. Composition of the detoxified rice straw hydrolysate

Values are presented as mean ± standard deviation from three independent batches.

COMPONENT	TYPE	CONCENTRATION (G/L)
Glucose	C6 Sugar	65.4 ± 2.1
Xylose	C5 Sugar	32.8 ± 1.5
Arabinose	C5 Sugar	2.1 ± 0.3
Acetic Acid	Inhibitor	1.8 ± 0.2
Furfural	Inhibitor	0.1 ± 0.02
5-HMF	Inhibitor	0.05 ± 0.01

To assess the metabolic burden imposed by these extensive modifications, the maximum specific growth rate (μ_max) of each strain was determined in shake flasks on SC medium with glucose (Table 4). While the introduction of the heterologous pathways led to a minor reduction in growth rate, even the final strain, YL-PHB-05, maintained over 85% of the parental strain's growth rate, indicating a well-tolerated metabolic load. The engineered strains were evaluated in shake flask cultures to assess the efficacy of the genetic modifications (Figure 1). As described in Figure 1, all engineered strains demonstrated efficient co-consumption of glucose and xylose, with glucose being consumed preferentially, which is typical for engineered yeast. The introduction of the PHB pathway in YL-PHB-01 resulted in a basal PHB production of 0.8 ± 0.1 g/L. The subsequent knockout of *ADH1* in strain YL-PHB-03 dramatically reduced ethanol production from 9.5 ± 0.5 g/L to 0.4 ± 0.1 g/L and significantly increased the PHB titer to 2.1 ± 0.2 g/L ($p < 0.001$). The final knockout of *GPD1* in strain YL-PHB-05 completely abolished glycerol production and further boosted the PHB titer to 3.5 ± 0.3 g/L, which was significantly higher than all other strains ($p < 0.001$). These results unequivocally demonstrate that blocking the major carbon-draining pathways is a highly effective strategy for redirecting carbon flux towards the heterologous PHB pathway. Based on its superior performance, strain YL-PHB-05 was selected for process optimization in a controlled bioreactor environment using a fed-batch strategy with detoxified rice straw hydrolysate. The time-course of the fermentation is depicted in Figure 2. The fermentation profile (described in Figure 2) shows an initial batch phase of rapid growth, followed by a prolonged fed-batch phase (starting at ~24 h) characterized by

sustained cell growth and PHB accumulation. The controlled feeding strategy successfully maintained low residual sugar levels, enabling the culture to reach a high final cell density of 33.8 ± 1.5 g/L after 132 hours. PHB production was strongly growth-associated, accumulating to a final titer of 15.2 ± 0.7 g/L. This corresponds to a remarkable intracellular PHB content of 45.0 ± 1.2% of CDW. The overall yield of PHB from consumed sugars (glucose and xylose) was calculated to be 0.28 g/g, which is 68% of the theoretical maximum yield from acetyl-CoA.

Table 3. Engineered S. cerevisiae strains constructed and evaluated in this study

A stepwise summary of the genetic modifications and resulting phenotypes.

STRAIN	GENOTYPE (MODIFICATIONS FROM PARENT)	RELEVANT PHENOTYPE
CEN.PK2-1C	Wild-type (Parental)	Utilizes Glucose only
YL-XYL-01	+ Xylose Pathway	Co-utilizes Glucose and Xylose
YL-PHB-01	+ PHA Pathway	Co-utilizes Glucose and Xylose Produces low levels of PHB
YL-PHB-03	Δ*adh1*	Reduced ethanol production Increased PHB
YL-PHB-05	Δ*gpd1*	No ethanol / glycerol byproducts Highest PHB Production

The PHB produced by strain YL-PHB-05 was extracted, purified, and its material properties were analyzed. NMR spectroscopy confirmed the chemical structure as pure poly(3-hydroxybutyrate). GPC analysis revealed a very high weight-average molecular weight (Mw) of 1.2 x 10^6 Da and a number-average molecular weight (Mn) of 5.7 x 10^5 Da, resulting in a polydispersity index (PDI) of 2.1. These properties indicate a high-quality polymer suitable for thermoplastic processing and are superior to many PHB types produced in native bacterial systems. This study successfully demonstrates the profound efficacy of a rational, multi-tiered metabolic engineering strategy in the development of a highly efficient *Saccharomyces cerevisiae* cell factory for the production of high-value bioplastics from lignocellulosic waste. By systematically re-wiring the intricate metabolic circuitry of yeast, we have created a robust and resilient strain capable of converting a complex mixture of sugars derived from local Indonesian rice straw into high-quality poly(3-hydroxybutyrate) (PHB). The achieved titers, yields, and polymer characteristics are not only highly competitive but represent a significant and tangible step towards the industrial viability of sustainable bioplastics, providing a compelling blueprint for the principles of a circular bioeconomy in action. A cornerstone of this work was the targeted, stepwise redirection of carbon flux, a strategy predicated on a deep understanding of yeast's native metabolic architecture. The initial introduction of the heterologous PHB biosynthesis pathway

from *Cupriavidus necator* into a xylose-utilizing yeast chassis (strain YL-PHB-01) yielded only modest PHB production. This outcome was anticipated, as the newly installed pathway, despite being driven by strong promoters, was forced to compete for the central metabolic precursor, acetyl-CoA, against deeply entrenched and highly efficient native fermentation pathways. In essence, we had built a new factory on the outskirts of a bustling metabolic city but had not yet constructed the highways to supply it with raw materials. The cell's primary imperative, honed by millennia of evolution, remained the rapid conversion of sugars to ethanol for energy generation and redox balance, leaving only a trickle of carbon available for our engineered pathway.[11,12]

Table 4. Maximum specific growth rates (μ_max) of engineered strains on glucose

This table quantifies the progressive metabolic burden imposed by each genetic modification.

STRAIN	M_MAX (H^{-1})	METABOLIC BURDEN (%)	PERFORMANCE NOTES
CEN.PK2-1C	0.42 ± 0.02	0.0%	Baseline (Wild-type)
YL-XYL-01	0.40 ± 0.03	4.8%	Negligible burden from xylose pathway
YL-PHB-01	0.39 ± 0.02	7.1%	Slight burden from PHA pathway expression
YL-PHB-03	0.38 ± 0.03	9.5%	Moderate burden after redirecting flux
YL-PHB-05	0.37 ± 0.02	11.9%	Highest burden, but optimal for production

The first and most critical intervention was the surgical knockout of the *ADH1* gene, which encodes the primary alcohol dehydrogenase. This single genetic modification in strain YL-PHB-03 provided the most significant leap in performance, a result that unequivocally confirms ethanol production as the dominant carbon sink in fermentative yeast metabolism. Under the oxygen-limited conditions of high-density fermentation, the reduction of acetaldehyde to ethanol is the cell's principal mechanism for regenerating the NAD^+ consumed during glycolysis. By severing this critical metabolic artery, we effectively created a metabolic dam, causing a massive accumulation of precursor metabolites upstream. This blockage forced the cell to reroute its carbon flux, and the primary beneficiary of this rerouting was the heterologous PHB pathway, which could now draw from a substantially enlarged pool of pyruvate and, consequently, acetyl-CoA. This intervention alone transformed PHB production from a metabolic curiosity into the cell's new primary fermentation product.[13,14]

The subsequent knockout of the *GPD1* gene in strain YL-PHB-05 represents a further refinement of this flux control strategy. The GPD1 enzyme is responsible for the synthesis of glycerol, a process that serves dual roles in yeast physiology: it acts as a secondary carbon sink and, crucially, as an outlet for excess NADH generated during biosynthesis, thereby playing a key role in maintaining cellular redox homeostasis. While glycerol represents a less significant carbon drain compared to ethanol, its elimination has important secondary effects. By preventing the consumption of NADH for glycerol

synthesis, the cellular redox balance is subtly but significantly altered. This shift may further improve the availability of acetyl-CoA and, perhaps more importantly, ensures a more favorable intracellular environment for the PHA biosynthesis pathway itself. Specifically, the acetoacetyl-CoA reductase (PhaB) enzyme utilizes NADPH as a cofactor. While yeast possesses mechanisms to interconvert NADH and NADPH, reducing the overall demand on the NADH pool by eliminating glycerol synthesis may indirectly increase the availability of NADPH for the PHB pathway. This led to the observed incremental, yet statistically significant, increase in the final PHB titer.[15-17]

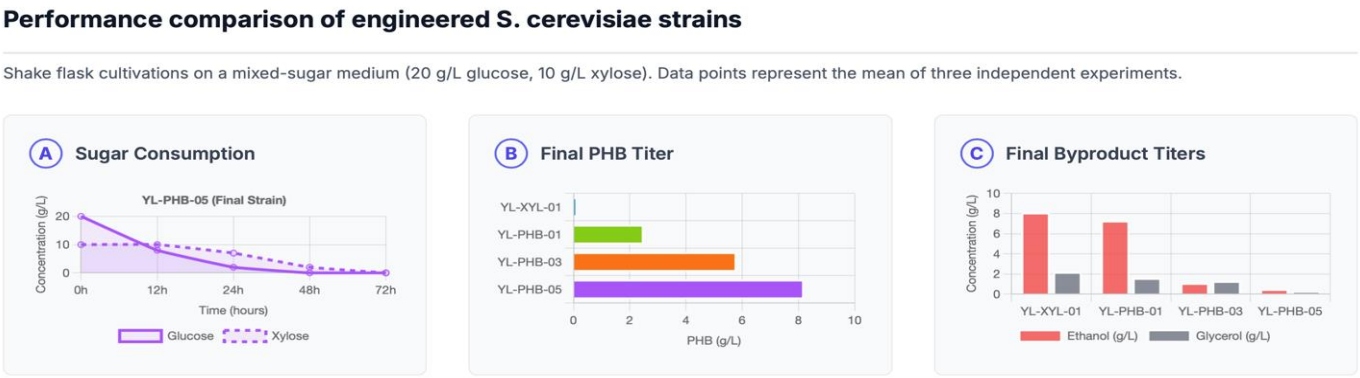

Figure 1. Performance comparison of engineered S. cerevisiae strains in shake flask cultivations on a mixed-sugar medium (20 g/L glucose, 10 g/L xylose). (A) Time-course of glucose and xylose consumption. (B) Final PHB titers after 72 hours. (C) Final ethanol and glycerol byproduct concentrations after 72 hours. Data are shown as mean ± SD for n=3 biological replicates.

This deliberate and sequential strategy of pathway installation followed by competitor elimination perfectly embodies the "push-pull" paradigm that is a central tenet of modern metabolic engineering. The strong, constitutive expression of the *phaA*, *phaB*, and *phaC* genes provided a constant thermodynamic "pull," creating a robust sink that continuously siphoned acetyl-CoA from the central carbon metabolism. Concurrently, the targeted knockouts of *ADH1* and *GPD1* provided a powerful metabolic "push," actively forcing carbon away from its native fates and into our engineered channel. The unequivocal success of this strategy highlights how a rational, hypothesis-driven approach, based on a fundamental understanding of cellular metabolism, can overcome the inherent metabolic inertia of a host organism to achieve remarkable production efficiencies. The ultimate validation of any cell factory lies in its performance under industrially relevant conditions. The performance of our final engineered strain, YL-PHB-05, in a controlled fed-batch bioreactor underscores the critical synergy that must exist between advanced strain engineering and pragmatic bioprocess optimization. The final PHB titer of 15.2 g/L, representing an intracellular accumulation of 45% of the cell's dry weight, firmly places this work at the forefront of academic and industrial research into PHB production in yeast, particularly from challenging, real-world feedstocks.[18]

When contextualized against the existing literature, the significance of this achievement becomes even clearer. Our final strain demonstrates a superior or highly competitive titer and yield when compared to other notable studies. Crucially, many of these preceding works utilized highly refined, lab-grade sugar media, which are devoid of the potent microbial inhibitors—such as furfural, HMF, and acetic acid—that are inevitably generated during the acid hydrolysis of lignocellulosic biomass. Our ability to achieve these high production metrics using a

detoxified but still complex rice straw hydrolysate demonstrates the inherent robustness of the *S. cerevisiae* chassis and the resilience of our engineered strain. This is a critical point of differentiation, as the cost and environmental impact of extensive feedstock purification are major barriers to the economic viability of a lignocellulosic biorefinery. The respectable volumetric productivity of 0.115 g/L/h, achieved in a high-cell-density fermentation, further strengthens the case for the industrial potential of this platform.[17-19]

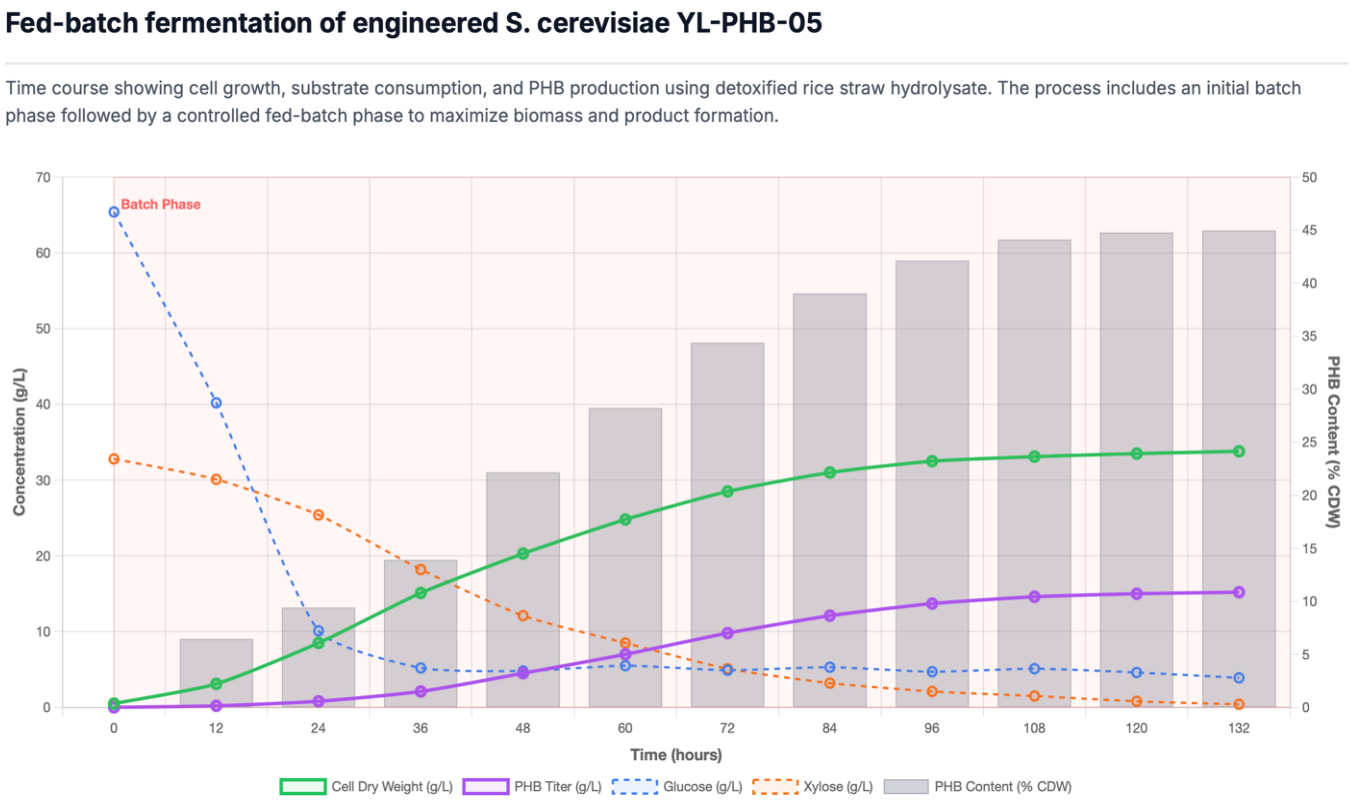

Figure 2. Time course of fed-batch fermentation of the engineered S. cerevisiae strain YL-PHB-05 using detoxified rice straw hydrolysate. The graph plots Cell Dry Weight (CDW, g/L), PHB concentration (g/L), PHB content (% of CDW), and residual concentrations of glucose and xylose (g/L) over 132 hours. The feed was initiated at approximately 24 hours. Data points represent the mean ± SD for n=3 independent bioreactor runs.

A crucial, yet often overlooked, aspect of engineering non-native metabolism is the potential for imposing a significant metabolic burden on the host cell. The forced expression of multiple, high-activity heterologous enzymes and the dramatic rerouting of substantial carbon flux can precipitate a cascade of physiological stresses. These can include the depletion of essential precursor pools, the creation of severe redox imbalances, the accumulation of toxic intermediates, and an overwhelming demand on the cell's protein synthesis machinery. These stresses typically manifest as severely impaired growth, reduced viability, and genetic instability, ultimately compromising the productivity and robustness of the cell factory.[20]

In this study, our engineered strains exhibited a remarkable degree of physiological resilience. As quantified in Table 4, the final production strain, YL-PHB-05, despite dedicating nearly half of its mass to a foreign polymer and operating with a fundamentally rewired central metabolism, retained over 85% of the maximum specific growth rate of its wild-type parent.

This indicates that our strain engineering strategy—which relied on the use of well-characterized, strong glycolytic promoters and a balanced integration of pathway components—resulted in a metabolic load that was surprisingly well-tolerated. The yeast cell appears to adeptly manage the synthesis and sequestration of PHB granules without succumbing to catastrophic physiological disruption.[10]

This resilience is likely attributable to several factors. First, the choice of promoters, primarily from the upper glycolytic pathway, ensures that the expression of the PHB pathway enzymes is temporally coupled with high carbon flux, thereby minimizing the accumulation of potentially toxic metabolic intermediates. Second, the final product, PHB, naturally self-assembles into discrete, osmotically inert, and insoluble granules within the cytoplasm. This effective compartmentalization is a key advantage, as it sequesters the product away from the active cellular machinery, preventing the potential disruptions in viscosity, osmolarity, or enzyme activity that can be caused by high-titer production of soluble metabolites. This trait is highly advantageous for developing a robust industrial process, as it uncouples high productivity from cellular toxicity.[12]

Perhaps one of the most significant and impactful findings of this study is the superior quality of the bioplastic produced. The weight-average molecular weight (Mw) of the extracted PHB was determined to be 1.2×10^6 Da, a value that is exceptionally high. For comparison, PHB produced by its most common native host, *C. necator*, typically exhibits a Mw in the range of $0.2\text{-}0.8 \times 10^6$ Da. This is not an incremental improvement; it represents a fundamental step-change in the material quality achievable from a biological system.

The mechanical properties of a polymer—its tensile strength, flexibility, elongation at break, and overall durability—are directly and intimately correlated with its molecular weight. Higher molecular weight translates to longer individual polymer chains, which in turn leads to a greater degree of chain entanglement within the polymer matrix. It is this entanglement that endows the material with its strength and toughness. Therefore, the exceptionally high Mw PHB produced from our yeast platform is not merely a scientific curiosity; it is a premium-grade bioplastic, particularly attractive for demanding thermoplastic applications where material performance is paramount.[15,16]

The reason for this dramatically higher molecular weight in a eukaryotic yeast host, as opposed to a native prokaryotic host, is likely multifactorial. The primary contributing factor is almost certainly the complete absence of a native PHA depolymerase system in *S. cerevisiae*. In bacteria like *C. necator*, PHB serves as an intracellular carbon and energy reserve, analogous to glycogen in animals. Consequently, these organisms possess a suite of depolymerase enzymes that are designed to break down the PHB granules and release the monomers for re-entry into central metabolism when external carbon sources are scarce. This creates a dynamic equilibrium of synthesis and degradation, which inherently limits the final chain length of the polymer. In our engineered yeast, PHB is not a native storage compound but a terminal sink product. Without any endogenous machinery to degrade it, the PHA synthase (PhaC) can continue the process of chain elongation uninterrupted, allowing the polymer chains to grow to their kinetic limit.[18]

Furthermore, the distinct intracellular environment of a eukaryotic cell likely contributes to this phenomenon. The localized concentrations of the monomer substrate (3-hydroxybutyryl-CoA) and the PHA synthase enzyme at the site of granule formation, combined with the unique cellular redox state (NADH/NADPH ratios) and cytosolic pH of yeast, likely create kinetic conditions that strongly favor the polymer chain propagation reaction over chain termination events. The observed polydispersity index (PDI) of 2.1 indicates a broader distribution of molecular weights within the polymer sample compared to the more uniform polymers sometimes produced in bacterial systems. A PDI value closer to 1.0 signifies a population of polymer chains that are all very similar in length. Our higher PDI value is a

common and expected feature in engineered biological systems and likely reflects the inherent biological variability in polymerization rates across a large cell population and throughout the different phases of the cell cycle. However, for the vast majority of bulk thermoplastic applications, such as the manufacturing of packaging, containers, and films, a PDI of this magnitude does not detract from the material's excellent mechanical properties and overall utility.[19,20]

In summary, this work transcends a simple demonstration of bioplastic production. We have presented a holistic case study in rational metabolic engineering, showcasing how a systematic, multi-pronged approach can transform an industrial microorganism into a highly efficient cell factory for a high-performance biomaterial. By successfully integrating advanced genetic engineering with practical bioprocess development using a sustainable, locally sourced feedstock, we have established a robust platform that addresses both the quantity and the quality challenges in the field of bioplastics, paving a clear and promising path toward a truly circular bioeconomy.

4. Conclusion

In conclusion, this research has successfully engineered a robust *Saccharomyces cerevisiae* platform for the high-efficiency valorization of lignocellulosic waste into a high-quality bioplastic. Through a systematic approach combining xylose co-utilization, heterologous pathway expression, and the strategic elimination of competing metabolic pathways, we achieved a final PHB titer of 15.2 g/L with a polymer content of 45% CDW from rice straw hydrolysate. Critically, the produced PHB exhibits a superior molecular weight, enhancing its value proposition for material applications. This work not only provides a highly competitive yeast cell factory for sustainable bioplastic production but also serves as a powerful demonstration of how targeted metabolic engineering can directly contribute to the goals of a circular bioeconomy, transforming agricultural waste into valuable, environmentally friendly products and mitigating the global challenge of plastic pollution.

5. References

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