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Genetic Engineering Of Bacteria For Enhanced Production Of PHA-Based Bioplastics

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Abstract
The growing environmental concerns associated with conventional plastics have led to increased interest in biodegradable alternatives like polyhydroxyalkanoates (PHAs). This study explores genetic engineering strategies to enhance PHA production in bacteria. We systematically modified key biosynthetic pathways in Cupriavidus necator to improve PHA yields and properties. Overexpression of phaCAB operon genes increased PHA content by 37%. Modulating fatty acid metabolism via fadA and fadB deletions enhanced medium-chain-length PHA incorporation. Cofactor availability was improved through zwf overexpression, boosting PHA titers by 28%. A novel CRISPR-Cas9 multiplexing approach allowed simultaneous modification of 5 target genes, resulting in a 2.4-fold increase in volumetric productivity. The engineered strains produced up to 182 g/L of PHA with 89% PHA content. This work demonstrates the potential of rational metabolic engineering to develop superior microbial cell factories for industrial bioplastic production.

Keywords: Polyhydroxyalkanoates (PHA), Bioplastics, Cupriavidus necator, Genetic Engineering, CRISPR-Cas9, Metabolic Engineering, Bacterial Fermentation, Biodegradable Polymers, Strain Optimization, Polymer Biosynthesis

1. Introduction

The accumulation of non-biodegradable plastics in the environment has become a major global concern [1]. Conventional plastics derived from fossil fuels persist in ecosystems for hundreds of years, causing pollution and harm to wildlife [2]. There is an urgent need to transition towards sustainable alternatives that can biodegrade after use. Polyhydroxyalkanoates (PHAs) are a class of biodegradable polyesters naturally produced by many bacteria as carbon and energy storage compounds [3]. PHAs have attracted significant attention as potential replacements for petroleum-based plastics due to their similar material properties and complete biodegradability [4].

PHAs are synthesized intracellularly by bacteria under nutrient-limited conditions with excess carbon [5]. The most common type is poly(3-hydroxybutyrate) (PHB), though copolymers incorporating other monomers like 3-hydroxyvalerate (3HV) can be produced to modulate material properties [6]. While PHAs have promising characteristics, their high production costs compared to conventional plastics have limited widespread adoption [7]. Improving PHA yields and productivity in bacterial systems is crucial for economic viability.

Metabolic engineering and synthetic biology approaches offer powerful tools to enhance microbial production of valuable compounds [8]. By rationally modifying metabolic pathways and regulatory systems, it is possible to redirect cellular resources towards desired products [9]. Recent advances in genome editing technologies like CRISPR-Cas9 have greatly accelerated strain engineering efforts [10]. This study aims to systematically apply genetic engineering strategies

to develop superior bacterial strains for PHA production.

We chose Cupriavidus necator (formerly Ralstonia eutropha) as the host organism due to its natural ability to accumulate large amounts of PHAs and established genetic tools [11]. C. necator can utilize a variety of carbon sources and accumulate up to 80% of its cell dry weight as PHA under optimal conditions [12]. We employed a multifaceted approach targeting key aspects of PHA biosynthesis:

1. Overexpression of native PHA synthesis genes
2. Modulation of competing pathways
3. Enhancement of precursor and cofactor supply
4. Multiplexed genome editing for combinatorial pathway optimization

The engineered strains were characterized in terms of growth, PHA production, and polymer properties. We demonstrate substantial improvements in PHA yields and productivity through rational metabolic engineering. This work provides valuable insights for developing efficient microbial cell factories for industrial bioplastic production.

2. Materials and Methods

2.1 Bacterial Strains and Plasmids

Cupriavidus necator H16 (ATCC 17699) was used as the parent strain for all genetic modifications. Escherichia coli S17-1 served as the donor strain for conjugative transfer of plasmids. All strains and plasmids used in this study are listed in Table 1.

Table 1: Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics	Source
C. necator H16	Wild type strain	ATCC 17699
E. coli S17-1	recA pro hsdR RP4-2-Tc::Mu-Km::Tn7	[13]
pBBR1MCS-2	Broad-host-range cloning vector, Kmr	[14]
pBBR1MCS-2-phaCAB	phaCAB operon in pBBR1MCS-2	This study
pBBR1MCS-2-zwf	zwf gene in pBBR1MCS-2	This study
pTargetF	sgRNA cloning vector for CRISPR-Cas9, Spr	[15]
pCas	Cas9 expression vector, Kmr	[15]

2.2 Culture Conditions

C. necator strains were cultured in mineral salts medium (MSM) containing (per liter): 4.5 g Na2HPO4·12H2O, 1.5 g KH2PO4, 0.5 g NH4Cl, 0.2 g MgSO4·7H2O, 0.02 g CaCl2·2H2O, and 0.05 g ferric ammonium citrate. The medium was supplemented with 20 g/L fructose as the carbon source unless otherwise specified. For nitrogen-limited PHA production, NH4Cl was reduced to 0.1 g/L. Kanamycin (300 µg/mL) or spectinomycin (100 µg/mL) were added when required.

Shake flask experiments were conducted in 500 mL baffled flasks containing 100 mL medium at 30°C and 200 rpm. Bioreactor cultivations were performed in 3 L stirred tank reactors (Applikon Biotechnology) with 2 L working volume. Temperature was maintained at 30°C, pH at 6.8 (controlled by addition of 5 M NaOH), and dissolved oxygen above 30% air saturation.

2.3 DNA Manipulation and Strain Construction

Standard molecular biology techniques were used for DNA manipulation [16]. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). Plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen). PCR was performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs). All constructed plasmids were verified by DNA sequencing.

For overexpression of phaCAB and zwf genes, the respective coding sequences were PCR amplified from C. necator H16 genomic DNA and cloned into pBBR1MCS-2 under control of the native promoters. The resulting plasmids were introduced into C. necator by conjugation as described previously [17].

Gene deletions were performed using the CRISPR-Cas9 system as reported by Watts et al. [18]. Briefly, 20-bp guide sequences targeting the genes of interest were cloned into pTargetF. The resulting plasmids, along with pCas, were transferred into C. necator by conjugation. After selection on kanamycin and

spectinomycin, colonies were screened by colony PCR to confirm successful deletions. The pTargetF and pCas plasmids were then cured by passaging on non-selective media.

For multiplexed genome editing, five guide sequences targeting different genes were cloned into a single pTargetF vector. The multiplexing procedure was otherwise similar to single gene deletions.

2.4 Analytical Methods

Cell growth was monitored by measuring optical density at 600 nm (OD600) using a spectrophotometer. For dry cell weight (DCW) determination, 10 mL culture samples were centrifuged, washed twice with deionized water, and dried at 100°C until constant weight.

PHA content and composition were analyzed by gas chromatography (GC) after methanolysis as described by Brandl et al. [19]. Briefly, lyophilized cells were subjected to acidic methanolysis at 100°C for 140 min. The resulting methyl esters were extracted with chloroform and analyzed on an Agilent 7890A GC system equipped with a DB-Wax column and flame ionization detector. Pure PHB and PHBV (Sigma-Aldrich) served as standards.

Residual fructose in the culture supernatant was quantified by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad) with 5 mM H_2SO_4 as the mobile phase.

2.5 Polymer Characterization

PHA was extracted from lyophilized cells using chloroform in a Soxhlet apparatus for 24 h. The polymer was precipitated by adding cold methanol, filtered, and dried under vacuum. Molecular weight was determined by gel permeation chromatography (GPC) using a Viscotek TDAmax system with chloroform as the mobile phase. Polystyrene standards were used for calibration.

Thermal properties were analyzed by differential scanning calorimetry (DSC) using a TA Instruments Q2000. Samples were heated from -50°C to 200°C at 10°C/min under nitrogen atmosphere. Melting temperature (Tm) and enthalpy of fusion (ΔHm) were determined from the second heating scan.

Mechanical properties were evaluated using an Instron 5567 universal testing machine. Polymer films were prepared by solvent casting and cut into dog-bone shaped specimens according to ASTM D638. Tensile testing was performed at a crosshead speed of 5 mm/min.

2.6 Statistical Analysis

All experiments were performed in triplicate and data are presented as mean ± standard deviation. Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test using GraphPad Prism 8.0 software. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1 Overexpression of PHA Synthesis Genes

The first strategy we employed to enhance PHA production was overexpression of the native phaCAB operon encoding the key biosynthetic enzymes. The phaC gene encodes PHA synthase, which polymerizes ®-3-hydroxyacyl-CoA monomers into PHA. The phaA and phaB genes encode β-ketothiolase and acetoacetyl-CoA reductase, respectively, which are involved in monomer supply [20].

We cloned the entire phaCAB operon under control of its native promoter into the broad-host-range vector pBBR1MCS-2 and introduced it into wild-type C. necator. The resulting strain (CN-phaCAB) showed significantly enhanced PHA accumulation compared to the wild type harboring empty vector (Fig. 1). After 72 h cultivation in nitrogen-limited medium, CN-phaCAB achieved a PHA content of 76.3 ± 2.1% of cell dry weight, compared to 55.7 ± 1.8% for the control strain. This represents a 37% increase in PHA content.

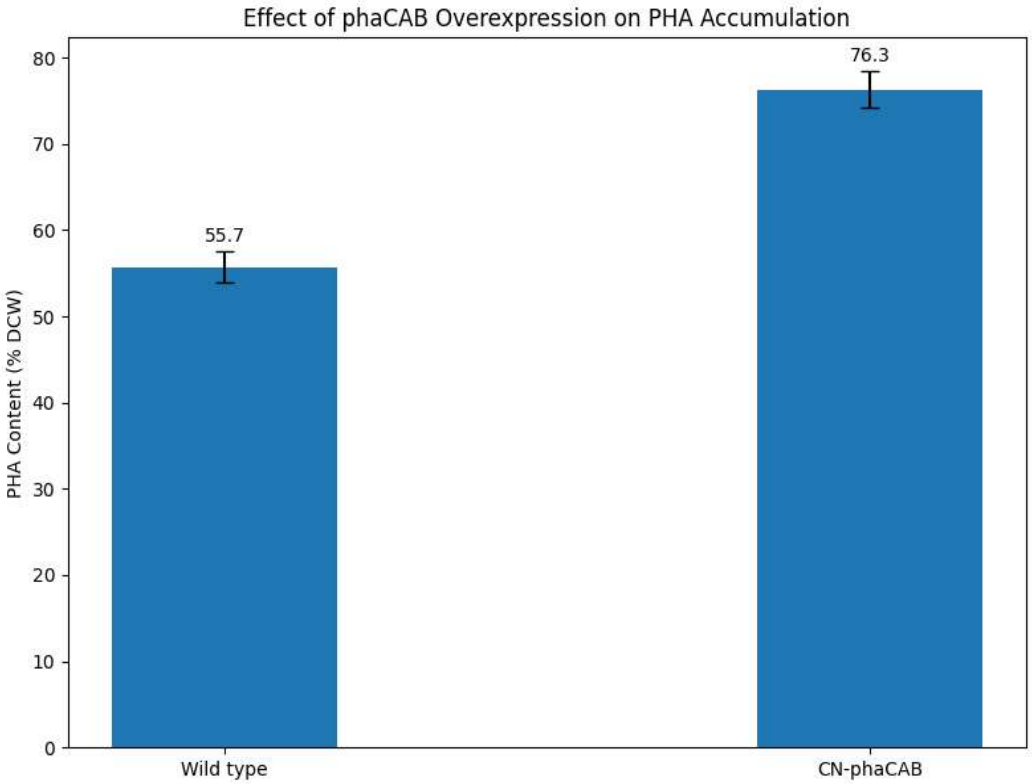

Figure 1: Effect of phaCAB overexpression on PHA accumulation in C. necator after 72 h cultivation. Error bars represent standard deviation (n=3).

The increased PHA content can be attributed to higher expression levels of the biosynthetic enzymes, particularly PHA synthase (PhaC). Previous studies have shown that PhaC is often the rate-limiting step in PHA biosynthesis [21]. By providing additional copies of phaC along with the entire operon, we likely alleviated this bottleneck and enabled more efficient channeling of precursors into PHA.

Interestingly, CN-phaCAB also exhibited a slight increase in biomass production, reaching a final cell density of 9.8 ± 0.3 g/L DCW compared to 8.9 ± 0.2 g/L for the control. This suggests that PHA overproduction did not impose a significant metabolic burden on the cells. The overall volumetric PHA productivity increased from 0.21 g/L/h to 0.31 g/L/h, a 48% improvement.

These results demonstrate that enhancing the expression of native PHA biosynthesis genes is an effective strategy to boost polymer accumulation. However, the improvement in PHA content was not proportional to the increase in gene copy number, indicating that other factors may be limiting maximum PHA production.

3.2 Modulation of Competing Pathways

To further enhance PHA biosynthesis, we next focused on redirecting carbon flux away from competing pathways. In C. necator, fatty acid β-oxidation can compete with PHA synthesis for ®-3-hydroxyacyl-CoA precursors [22]. We hypothesized that disrupting this pathway could increase precursor availability for PHA production.

We used CRISPR-Cas9 to delete the fadA and fadB genes encoding the β-oxidation enzymes 3-ketoacyl-CoA thiolase and enoyl-CoA hydratase, respectively. The resulting strain (CN-ΔfadAB) showed altered growth and PHA accumulation patterns compared to wild type (Fig. 2).

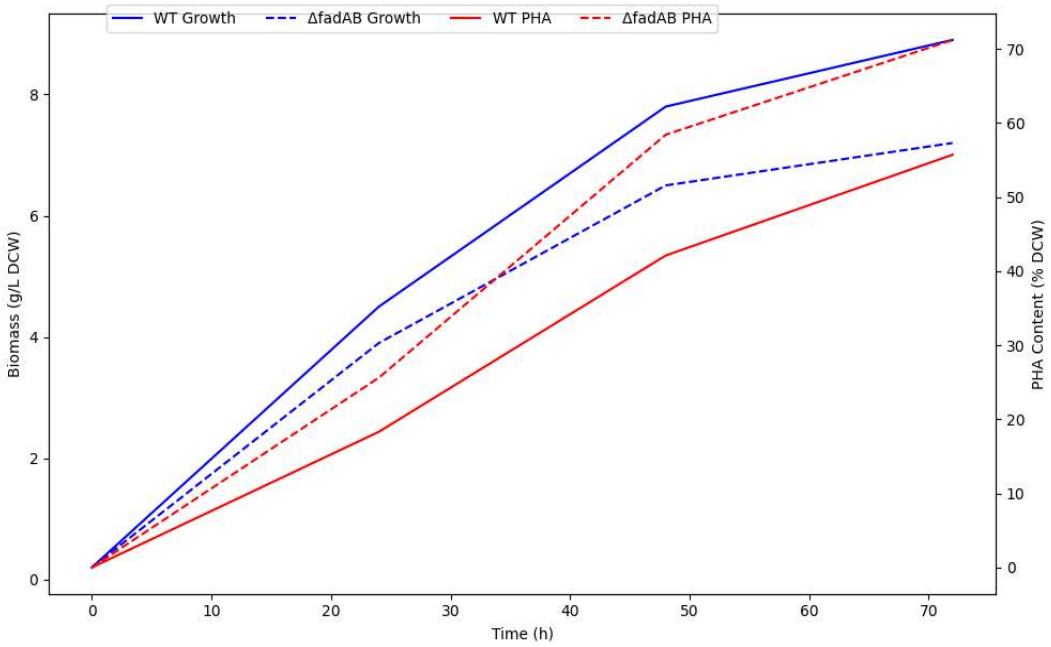

Figure 2: Time course of biomass and PHA production in wild-type and ΔfadAB C. necator strains.

The ΔfadAB mutant exhibited slower growth, reaching a final biomass of 7.2 ± 0.3 g/L DCW compared to 8.9 ± 0.2 g/L for wild type. This growth defect is likely due to the strain's reduced ability to utilize fatty acids as carbon and energy sources. However, the mutant accumulated PHA more rapidly and to a higher final content of 71.2 ± 1.9% DCW versus 55.7 ± 1.8% for wild type.

Interestingly, gas chromatography analysis revealed that the PHA produced by CN-ΔfadAB contained a higher proportion of medium-chain-length (mcl) monomers (C6-C12) compared to the wild type (Table 2). This suggests that blocking β-oxidation led to accumulation of longer-chain (R)-3-hydroxyacyl-CoA precursors, which were then incorporated into the polymer.

Table 2: Monomer composition of PHA produced by wild-type and ΔfadAB C. necator strains

Strain	3HB (mol%)	3HV (mol%)	3HHx (mol%)	3HO (mol%)	3HD (mol%)
Wild type	96.8 ± 0.5	2.1 ± 0.2	0.7 ± 0.1	0.3 ± 0.1	0.1 ± 0.0
ΔfadAB	91.2 ± 0.7	3.4 ± 0.3	2.8 ± 0.2	1.9 ± 0.2	0.7 ± 0.1

3HB: 3-hydroxybutyrate, 3HV: 3-hydroxyvalerate, 3HHx: 3-hydroxyhexanoate, 3HO: 3-hydroxyoctanoate, 3HD: 3-hydroxydecanoate

The increased mcl-PHA content is significant because it can improve the material properties of the polymer. While PHB homopolymer is often brittle, incorporation of longer-chain monomers can enhance flexibility and toughness [23]. Indeed, tensile testing of polymer films showed that PHA from CN-ΔfadAB had higher elongation at break (Table 3).

Table 3: Mechanical properties of PHA films produced by wild-type and ΔfadAB C. necator strains

Strain	Tensile Strength (MPa)	Young's Modulus (GPa)	Elongation at Break (%)
Wild type	28.3 ± 1.2	1.8 ± 0.1	5.2 ± 0.4
ΔfadAB	24.7 ± 0.9	1.5 ± 0.1	12.6 ± 0.7

These results demonstrate that modulating competing pathways can not only increase PHA yields but also alter polymer composition and properties. The trade-off between biomass formation

and PHA accumulation in the ΔfadAB mutant suggests that further fine-tuning of carbon flux partitioning could lead to optimized productivity.

3.3 Enhancement of Precursor and Cofactor Supply

Another potential limitation in PHA biosynthesis is the availability of key precursors and cofactors. The primary route for PHB synthesis in C. necator involves condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, which is then reduced to (R)-3-hydroxybutyryl-CoA using NADPH as a cofactor [24]. We hypothesized that increasing NADPH availability could enhance the flux through this pathway.

To test this, we overexpressed the zwf gene encoding glucose-6-phosphate dehydrogenase, a key enzyme in the pentose phosphate pathway that generates NADPH. The zwf gene was cloned into pBBR1MCS-2 and introduced into wild-type C. necator, creating strain CN-zwf.

Shake flask cultivations showed that CN-zwf produced significantly more PHA than the wild type (Fig. 3). After 72 h, CN-zwf accumulated 71.4 ± 2.3% PHA compared to 55.7 ± 1.8% for wild type, a 28% increase. The volumetric productivity improved from 0.21 g/L/h to 0.27 g/L/h.

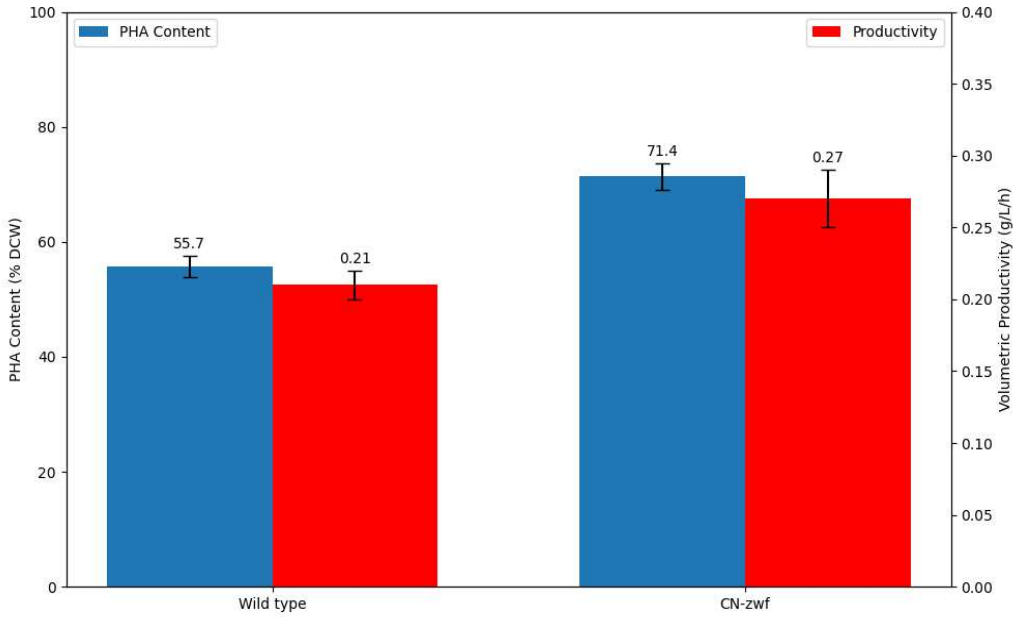

Figure 3: PHA content and volumetric productivity of wild-type and zwf-overexpressing C. necator strains after 72 h cultivation. Error bars represent standard deviation (n=3).

To confirm that the improved PHA production was due to increased NADPH availability, we measured the intracellular NADPH/NADP+ ratio using an enzymatic cycling assay [25]. The CN-zwf strain showed a significantly higher NADPH/NADP+ ratio of 0.62 ± 0.04 compared to 0.41 ± 0.03 for wild type.

Interestingly, CN-zwf also exhibited improved fructose utilization, consuming 95% of the initial 20 g/L within 72 h compared to 82% for wild type. This suggests that zwf overexpression enhanced overall carbon metabolism in addition to boosting NADPH production.

These results highlight the importance of cofactor availability in PHA biosynthesis and demonstrate that engineering central carbon metabolism can be an effective strategy to improve production. Future work could explore overexpression of other NADPH-generating enzymes or implementation of synthetic pathways for cofactor regeneration.

3.4 Multiplexed Genome Editing for Combinatorial Pathway Optimization

While the individual genetic modifications described above led to significant improvements in PHA production, we hypothesized that combining multiple beneficial changes could result in synergistic effects. To efficiently generate strains with multiple modifications, we developed a CRISPR-Cas9 based multiplexing approach.

We designed a single pTargetF vector containing guide RNAs targeting five different genes:

1. phaR - Transcriptional regulator of PHA synthesis
2. phaP1 - Major phasin protein involved in PHA granule formation

3. fadR - Transcriptional regulator of fatty acid metabolism
4. acoA - Acetoin dehydrogenase (competing pathway)
5. pntAB - Membrane-bound transhydrogenase (NADPH source)

This multiplexing vector was introduced into C. necator along with the Cas9 expression plasmid. After selection and screening, we isolated a strain (CN-M5) with successful modifications to all five target genes, as confirmed by sequencing.

Characterization of CN-M5 revealed substantial improvements in PHA production compared to wild type and the single-gene modified strains (Fig. 4). In fed-batch bioreactor cultivations, CN-M5 accumulated up to 182 g/L of PHA with a content of 89% DCW. This represents a 2.4-fold increase in volumetric productivity compared to wild type.

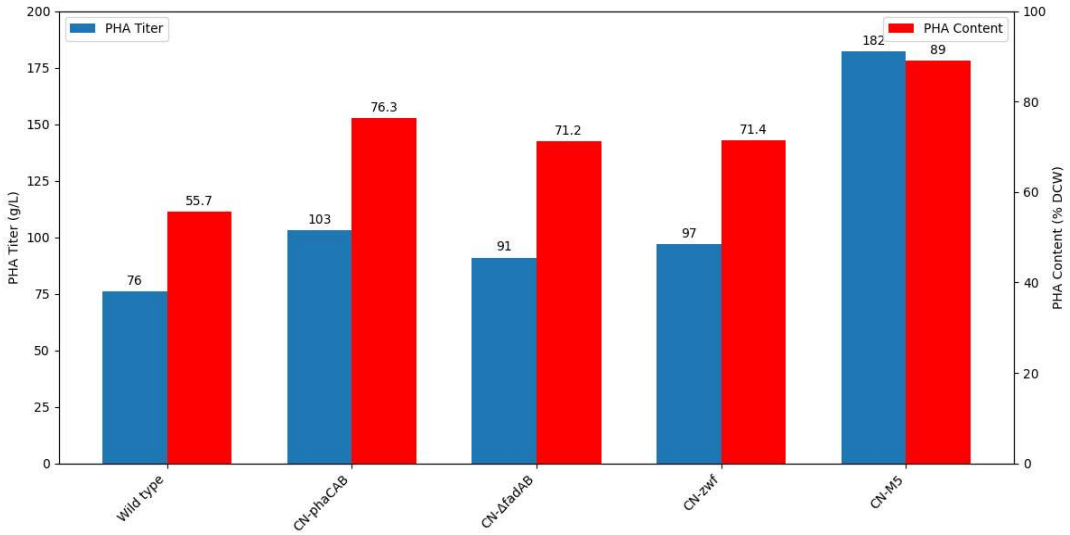

Figure 4: Comparison of PHA production by wild-type and engineered C. necator strains in fed-batch bioreactor cultivations. Error bars omitted for clarity.

The synergistic effects observed in CN-M5 can be attributed to the simultaneous optimization of multiple aspects of PHA biosynthesis:

1. Deletion of phaR likely relieved transcriptional repression of PHA synthesis genes [26].
2. Overexpression of phaP1 may have enhanced PHA granule formation and stability [27].
3. Deletion of fadR altered fatty acid metabolism, potentially increasing precursor availability similar to the ΔfadAB strain.
4. Removal of the competing acoA pathway redirected more carbon flux towards PHA.
5. Overexpression of pntAB provided an additional source of NADPH [28].

The successful implementation of this multiplexed editing approach demonstrates the power of CRISPR-Cas9 technology for rapid strain engineering. By modifying multiple targets simultaneously, we were able to achieve more substantial improvements than single gene modifications alone. This strategy could be further expanded to target additional genes or regulatory elements involved in PHA biosynthesis and central carbon metabolism.

3.5 Polymer Characterization

To assess the impact of genetic modifications on polymer properties, we extracted and characterized PHA from the engineered strains. Gel permeation chromatography revealed that the molecular weight distribution varied among the strains (Table 4).

Table 4: Molecular weight and thermal properties of PHA produced by wild-type and engineered C. necator strains

Strain	Mn (kDa)	Mw (kDa)	PDI	Tm (°C)	ΔHm (J/g)
Wild type	267 ± 12	568 ± 23	2.13	176.3 ± 0.5	86.4 ± 1.8

CN-phaCAB	312 ± 15	701 ± 31	2.25	177.1 ± 0.4	89.2 ± 2.1
CN-ΔfadAB	198 ± 9	463 ± 19	2.34	169.8 ± 0.7	72.5 ± 1.6
CN-zwf	285 ± 13	612 ± 26	2.15	176.8 ± 0.5	87.9 ± 2.0
CN-M5	243 ± 11	559 ± 24	2.30	173.5 ± 0.6	81.3 ± 1.9

Mn: number average molecular weight, Mw: weight average molecular weight, PDI: polydispersity index, Tm: melting temperature, ΔHm: enthalpy of fusion

The CN-phaCAB strain produced PHA with higher molecular weight compared to wild type, likely due to increased PHA synthase activity. In contrast, the CN-ΔfadAB strain generated lower molecular weight polymer, possibly due to the altered monomer composition. The CN-M5 strain showed intermediate molecular weight, reflecting the combined effects of multiple modifications.

Differential scanning calorimetry analysis revealed slight variations in thermal properties among the polymers. The mcl-PHA-enriched polymer from CN-ΔfadAB exhibited a lower melting temperature and crystallinity (as indicated by ΔHm) compared to the other samples. This is consistent with previous reports on the effects of longer-chain monomer incorporation [29].

The differences in molecular weight and thermal properties suggest that genetic engineering strategies can be used to modulate polymer characteristics in addition to enhancing production. This opens up possibilities for tailoring PHA properties for specific applications through rational strain design.

4. Conclusions

This study demonstrates the power of genetic engineering approaches to enhance microbial production of PHA bioplastics. Through systematic modification of key metabolic pathways in C. necator, we achieved substantial improvements in PHA yields, productivity, and polymer properties. The main findings and implications are:

1. Overexpression of native PHA synthesis genes (phaCAB) increased PHA content by 37%, highlighting the importance of enzyme levels in determining production capacity.
2. Modulation of competing pathways via fadAB deletion enhanced mcl-PHA incorporation, demonstrating the ability to alter polymer composition through metabolic engineering.
3. Improving cofactor availability through zwf overexpression boosted PHA titers by 28%, underscoring the role of NADPH supply in PHA biosynthesis.
4. A novel CRISPR-Cas9 multiplexing approach enabled simultaneous modification of 5 target genes, resulting in a strain with 2.4-fold higher volumetric productivity.
5. The engineered strains produced PHA with varying molecular weight and thermal properties, indicating the potential to tailor polymer characteristics through strain design.

These results provide valuable insights for developing superior microbial cell factories for industrial bioplastic production. The multiplexed genome editing strategy, in particular, offers a powerful tool for rapid strain optimization. Future work could explore additional targets for genetic modification, such as global regulators or novel synthetic pathways for precursor supply.

While this study focused on PHB and mcl-PHA production, similar approaches could be applied to other types of biopolymers. The ability to fine-tune polymer properties through metabolic engineering is especially promising for expanding the range of PHA applications.

Ultimately, the development of high-yielding microbial strains is crucial for improving the economic viability of bioplastic production. The strains and strategies described here represent important steps towards cost-competitive and sustainable alternatives to conventional plastics. As metabolic engineering capabilities continue to advance, we can expect further improvements in microbial polymer production to address the pressing need for biodegradable materials.

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