

Synergistic experimental and computational approach identifies novel strategies for polyhydroxybutyrate overproduction

Adil Alsiyabi ^{a,1}, Brandi Brown ^{b,1}, Cheryl Immethun ^a, Dianna Long ^b, Mark Wilkins ^{b,c,d}, Rajib Saha ^{a,*}

^a Department of Chemical and Biomolecular Engineering, University of Nebraska-Lincoln, Lincoln, NE, 68588, USA

^b Department of Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, NE, 68583, USA

^c Industrial Agricultural Products Center, University of Nebraska-Lincoln, Lincoln, NE, 68583, USA

^d Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE, 68588, USA



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ABSTRACT

Polyhydroxybutyrate (PHB) is a sustainable bioplastic produced by bacteria that is a potential replacement for conventional plastics. This study delivers an integrated experimental and computational modeling approach to decipher metabolic factors controlling PHB production and offers engineering design strategies to boost production. In the metabolically robust *Rhodopseudomonas palustris* CGA009, PHB production significantly increased when grown on the carbon- and electron-rich lignin breakdown product *p*-coumaric acid ($C_9H_8O_3$) compared to virtually no PHB titer from acetate ($C_2H_3NaO_2$). The maximum yield did not improve further when grown on coniferyl alcohol ($C_{10}H_{12}O_3$), but comparison of the PHB profiles showed that coniferyl alcohol's higher carbon content resulted in a higher rate of PHB production. Combined experimental results revealed that cytoplasmic space may be a limiting factor for maximum PHB titer. In order to obtain a systems-level understanding of factors driving PHB yield, a model-driven investigation was performed. The model yielded several engineering design strategies including utilizing reduced, high molecular weight substrates that bypass the thiolase reaction (*phaA*). Based on these strategies, utilization of butyrate was predicted and subsequently validated to produce PHB. Model analysis also explained why nitrogen starvation was not essential for PHB production and revealed that renewable and abundant lignin aromatics are ideal candidates for PHB production. Most importantly, the generality of the derived design rules allows them to be applied to any PHB-producing microbe with similar metabolic features.

1. Introduction

Due to the global plastic waste crisis, there has been extensive research on polyhydroxyalkanoates (PHAs) as potential replacements for petroleum-derived plastics. PHAs are biopolymers produced by bacteria that have similar thermomechanical properties as conventional plastics, but are also biodegradable, have shown biocompatibility in therapeutics, and can be produced from a wide array of sustainable carbon sources (Brown et al., 2020; Medeiros Garcia Alcântara et al., 2020). These bioplastics are generally produced by microbes under stressful conditions (e.g. nutrient starvation) and stored as granules inside the cytoplasm for carbon balance, stress mitigation, and as a redox sink (Kleiner et al., 2012). PHAs are considered to be the largest group of

natural polyesters with over 150 monomers reported, and offer a unique platform for easily manipulating the thermomechanical properties of the bioplastic produced (Li et al., 2016). Due to these characteristics, PHAs have been used in a wide array of applications ranging from medical implants to biodegradable packaging (Medeiros Garcia Alcântara et al., 2020; Chen, 2009; Chen and Zhang, 2018). However, the widespread adoption of PHAs is hampered by high production costs, which is largely attributed to feedstock costs (Chen et al., 2020). Therefore, engineering a microbe that can overproduce the most common form of PHA, called poly-3-hydroxybutyrate (PHB), from inexpensive renewable feedstocks is needed.

However, choosing the right engineering strategy to maximize PHB production can be tricky due to the complexity in metabolism across

* Corresponding author.

E-mail address: rsaha2@unl.edu (R. Saha).

¹ First author. These authors contributed equally to this work.

PHB-producing bacteria (Lopar et al., 2013). The PHB synthesis pathway (Fig. 1) has been investigated extensively in multiple organisms (Chen, 2009; Chen and Jiang, 2017; Leaf and Srienc, 1998; Ranaivoarisoa et al., 2019; Tyo et al., 2010; Van Wegen et al., 2001; Kocharin et al., 2012; Sekar and Tyo, 2015; Uchino et al., 2007) but different studies primarily point to three different bottlenecks that constrict the rate through the pathway (Chen and Jiang, 2017; Leaf and Srienc, 1998; Ranaivoarisoa et al., 2019; Tyo et al., 2010; Van Wegen et al., 2001; Kocharin et al., 2012; Sekar and Tyo, 2015; Uchino et al., 2007). First, a number of studies have indicated that the rate of PHB synthesis is dependent on the acetyl-CoA pool size or the acetyl-CoA/CoA ratio (Van Wegen et al., 2001; Kocharin et al., 2012). Since acetyl-CoA is the first substrate in the pathway, it is intuitive to postulate that increasing its intracellular concentration will lead to an increase in the rate of PHB synthesis. Indeed, this has been shown in *Saccharomyces cerevisiae* (Kocharin et al., 2012). Furthermore, the first reaction in the PHB pathway is catalyzed by the thiolase enzyme (*phaA*), and is thermodynamically unfavorable in the forward direction ($\Delta G^\circ = 26 \text{ kJ/mol}$). This means that the ratio of reactants to products should be high in order for this reaction to proceed. This hypothesis is related to the pathway's role as a carbon sink, as any increase in the production of the central metabolite (i.e., acetate) will be funneled towards PHB for storage (McKinlay et al., 2014a). Second, other studies have reported that the rate of PHB synthesis is correlated to the redox state of NADPH ([NADPH]/[NADP]) (Centeno-Leija et al., 2014; Sacomboio et al., 2017; Chohan and Copeland, 1998; Lee et al., 1996). This observation can be connected to the pathway's role as an electron sink (McKinlay et al., 2014a). Furthermore, an increase in the NADPH to NADP ratio increases the thermodynamic driving force of the second reaction in the pathway (*phaB*). Third, a study investigating the effect of overexpressing different enzymes in the pathway found that the expression of *phaB* was the limiting factor in PHB synthesis (Tyo et al., 2010). This result is consistent with a number of previous kinetic analyses which revealed that the catalytic efficiency of the reductase reaction (*phaB*) was substantially lower than that of other reactions in the pathway (Wang and Lee, 1997; Sim et al., 1997). Interestingly, this study also shows that an increase in the expression of the thiolase (*phaA*) actually leads to a slight decrease in PHB production, which further points to the thermodynamic driving force favoring the reverse direction of the reaction (Tyo et al., 2010).

Based on these findings, it is apparent that the overall rate through the pathway is highly dependent on metabolic factors, such as the relative abundance of the participating metabolites and cofactors.

Furthermore, due to the inherently high connectivity of the participating metabolites, their condition-specific availability is reliant on their overall generation and consumption rates from a number of different pathways. It is therefore vital to make use of a systems-wide framework such as genome-scale metabolic models (GSMMs) that can account for changes that occur in all parts of the metabolic network. These models translate the repertoire of all known metabolic functionalities performed by an organism into a mathematical representation which can then be used to predict the activity throughout the metabolic network (Islam et al., 2018; Fondi et al., 2015; Saha et al., 2014). With the aid of methods such as Flux Balance Analysis (FBA) (Orth et al., 2010), GSMMs have been successful in accurately predicting how metabolism changes under different genetic and environmental perturbations, as well as generating testable hypotheses on what drives such changes. Thus far, GSMMs of several PHB producing bacteria have been reconstructed (Tyo et al., 2010; Park et al., 2011; Bordel et al., 2019a, 2019b; Tajparast and Frigon, 2018). These models were mainly used to explain observed behavior (e.g., storage metabolism under feast-famine cycles) in the specific organism of interest, and accordingly only considered a subset of the possible metabolic states. Therefore, a holistic understanding of how the PHB pathway interacts with the rest of the metabolic network is crucial for obtaining a set of generalized design strategies to increase PHB productivity.

The metabolically diverse *Rhodopseudomonas palustris* CGA009 (hereafter *R. palustris*) is an ideal candidate for deciphering key engineering design strategies for PHB production using a GSMM. *R. palustris* can utilize a wide variety of carbon sources in both aerobic and anaerobic conditions, including over two dozen aromatic compounds derived from lignin (Austin et al., 2015; Harwood and Gibson, 1988), an underutilized abundant raw material that would be toxic to many microbes used in industrial bioprocessing (Lee et al., 2019). Of note, *R. palustris* is renowned for its metabolism of the lignin breakdown product (LBP) *p*-coumarate, as well as its ability to perform anoxic photosynthesis and fix CO₂ (Larimer et al., 2004). During anaerobic growth, *R. palustris* utilizes the Calvin Benson Bassham (CBB) cycle as an electron sink (McKinlay and Harwood, 2010). This allows a direct comparison between the redox state of the cell and the rate of CO₂ fixation (McKinlay and Harwood, 2010, 2011a). This feature of the bacterium's metabolism is especially pronounced under conditions where other electron consuming routes (e.g., N₂ fixation and biomass production) are halted, as is the case during nitrogen starvation (McKinlay et al., 2014b). The ability of *R. palustris* to utilize substrates of varying properties (Larimer et al., 2004; McKinlay and Harwood, 2011b), the limited metabolic

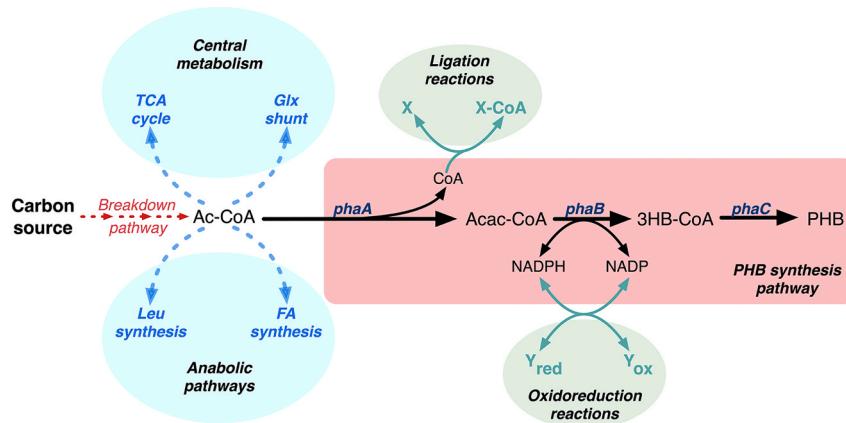


Fig. 1. PHB synthesis in the context of whole-cell metabolism. The pathway is connected to central metabolism through acetyl-CoA, the cofactor CoA, and the cofactor pair NADP/NADPH. The highly connected nature of these metabolites causes their concentration to be dependent on the overall activity of the pathways they are active in.

routes it can operate to maintain redox balance (McKinlay et al., 2014b), and its constant expression of *pha* genes across different conditions (Ranaivoarisoa et al., 2019; McKinlay et al., 2014b) make it an ideal candidate for probing the metabolic elements that control PHB production.

This study delivers an integrated experimental and computational modeling approach to decipher metabolic factors controlling PHB production and offers engineering design strategies to boost production. In the metabolically robust *R. palustris*, PHB production significantly increased when grown on the carbon- and electron-rich lignin breakdown product (LBP) *p*-coumarate ($C_9H_8O_3$) compared to virtually no PHB titer from acetate ($C_2H_4O_2$). The maximum PHB titer did not improve when grown on the LBP coniferyl alcohol ($C_{10}H_{12}O_3$), but comparison of the PHB profiles showed that coniferyl alcohol's higher carbon content resulted in a higher rate of PHB production. Combined experimental results revealed that cytoplasmic space may be a limiting

factor for maximum PHB titer. This study also reveals that *R. palustris* can create a copolymer called poly-3-hydroxybutyrate-co-hydroxyvalerate (PHBV) (Supplementary Fig. S1), which boosts the thermo-mechanical properties compared to PHB alone. It was unclear why the LBPs yielded more PHB production compared to virtually no titer from acetate. Furthermore, measurements prior to nitrogen starvation revealed the presence of PHB, which indicated that PHB production was not strictly a stress response. H_2 production measurements were also conducted to qualitatively assess electron secretion. These observations along with previously described data on PHB production (Brown et al., 2020) lead to the hypothesis that the rate of PHB production was mainly controlled by metabolic factors, especially since expression of *pha* genes has been reported to remain constant under different conditions (Ranaivoarisoa et al., 2019; McKinlay et al., 2014b). To examine this hypothesis, a thermo-kinetic analysis of the PHB pathway was conducted to determine the specific metabolic factors controlling pathway

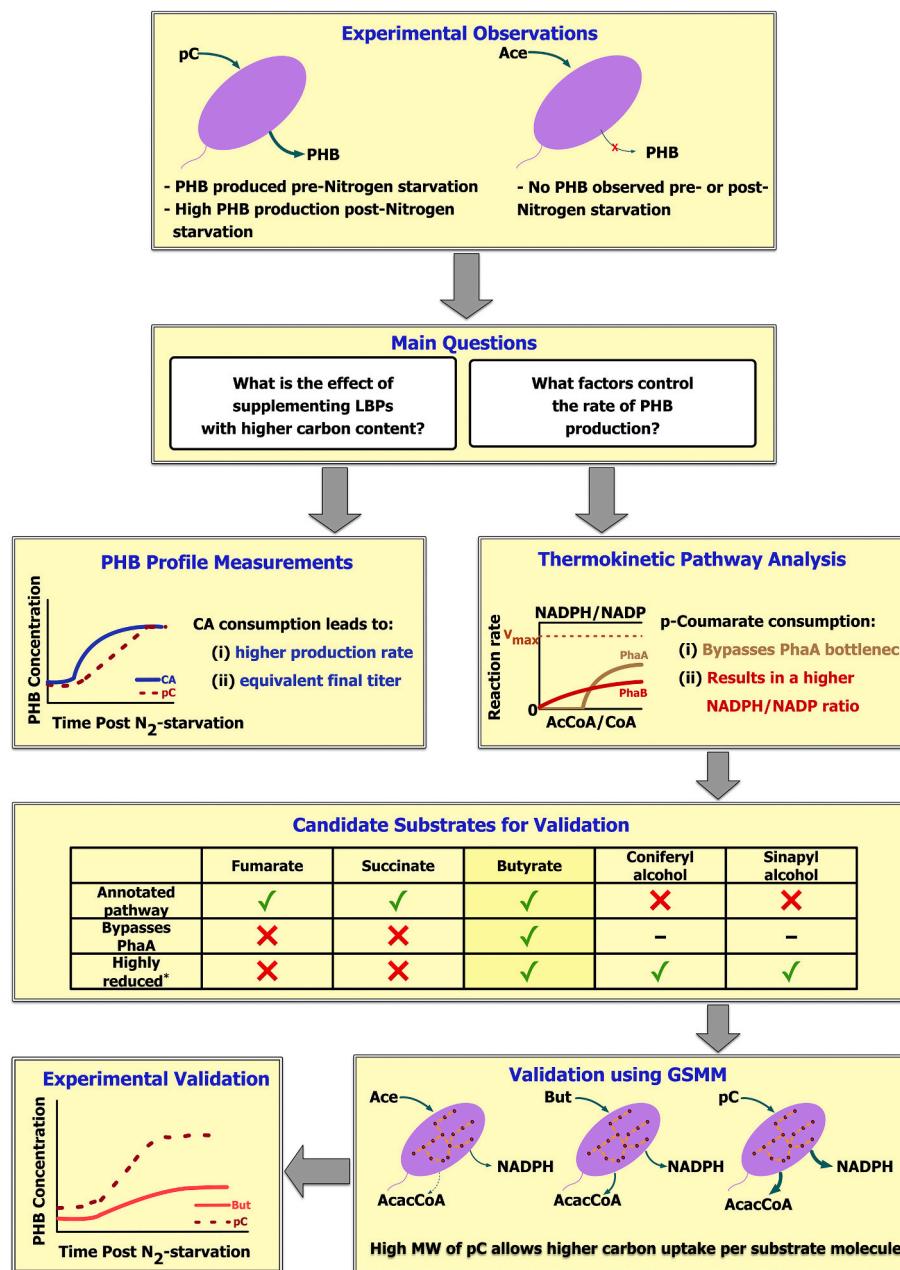


Fig. 2. Workflow followed to identify the metabolic factors controlling PHB production under different conditions.

activity. This analysis revealed a significant thermodynamic bottleneck associated with the thiolase reaction (*phaA*) and a high dependence of the reductase reaction (*phaB*) on the NADP(H) redox state. Based on this analysis, butyrate ($C_4H_8O_2$) was identified as a potential substrate for PHB production due to its ability to bypass the *phaA* reaction and its high electron content.

To combine the study's findings within the context of whole-cell metabolism, a recently constructed GSMM of *R. palustris*, *iRpa940* (Alsiyabi et al., 2019), was used to compare growth simulations on substrates with annotated breakdown pathways. Fig. 2 shows the general workflow followed in this study. *iRpa940* was chosen due to its ability to simulate growth under different conditions, including an array of organic acids (i.e., acetate, butyrate, fumarate, and succinate) and LBPs and very high correlation of model-predicted fluxes with experimental flux measurements. The model was also used to capture the observed complex trade-offs between different metabolic modules required for *R. palustris* to maintain optimum redox balance. Hence, the GSMM was applied to uncover possible causes for these experimental findings and to offer engineering design strategies that optimize PHB production. Four major findings from the GSMM include (i) a very high AcCoA/CoA ratio is required to drive the thiolase reaction (*phaA*), (ii) substrates with high carbon uptake rates accumulate higher amounts of acyl-CoA, (iii) the rate through reductase (*phaB*) is linearly dependent on the NADPH/NADP ratio, and (iv) *phaB* will likely be rate-limiting even under optimal metabolic conditions due to the very low catalytic efficiency. These findings suggest novel engineering design strategies to optimize PHB production including choosing substrates that bypass the thiolase reaction (e.g. *p*-coumarate), utilizing high molecular weight substrates for high carbon uptake rates, and using highly reduced substrates. Furthermore, this study shows that renewable and abundant LBPs are ideal candidates for PHB production. Ultimately, this study showcases how an integrated approach utilizing GSMMs can help identify key factors associated with a bioconversion that would have otherwise not been recognizable. The engineering design strategies derived from this integrated approach could be applied to other bacteria with similar metabolic features, advancing the replacement of petroleum-derived plastics with sustainable biopolymers.

2. Materials and methods

2.1. Growth curves and PHB/V production

R. palustris seed culture preparation, growth curves, and PHB production were performed as described (Brown et al., 2020). Briefly, seed cultures were diluted to an optical density (660 nm) of 0.2 and grown anaerobically at 30 °C with 100 µE white light in the same media as the seed cultures but with different carbon sources. Instead of the 20 mM sodium acetate used for seed cultures, either 10 mM sodium acetate, 5 mM sodium butyrate, 1 mM *p*-coumarate, or 1 mM coniferyl alcohol were supplied. All substrates were supplemented with 10 mM sodium bicarbonate unless specified otherwise, and this carbon content was factored into the analyses. Growth data were fitted to a modified logarithmic growth model as described previously (Chen and Jiang, 2017; Lee et al., 2019).

Once growth parameters were determined from the growth models (Fig. 3A), cultures were grown to mid-exponential in the respective conditions (except when specified otherwise) and subsequently nitrogen starved as described previously (Brown et al., 2020). Briefly, for nitrogen starvation the anaerobic vials were centrifuged to generate a pellet, the supernatant was discarded, and the pellet was resuspended in media

with the desired carbon sources without ammonium sulfate as a nitrogen source. For phosphorus starvation regarding the starvation analysis (Fig. 3B), the same methods were applied but instead of removing nitrogen from the media phosphorous was omitted.

2.2. PHB/V quantification via gas-chromatography mass spectrometry (GC-MS)

PHB/V extraction and quantification was conducted as described previously (Brown et al., 2020). A triplicate of anaerobic culture vials was harvested from each condition for analysis at the desired growth phase (i.e., mid-exponential or stationary phase) and after each day of nitrogen starvation. Each vial was subjected to acidic methanolysis, and PHB/V was quantified with conventional GC-MS as described (Brown et al., 2020). External standards were created from serial dilutions of sodium 3-hydroxybutyrate or (–)-Methyl (R)-3-hydroxyvalerate (Sigma-Aldrich™).

2.3. Hydrogen quantification via gas chromatography-thermal conductivity detector (GC-TCD)

Samples were nitrogen-starved as described above. Precisely 3 mL of the nitrogen-starved culture was placed into a 10 mL air-tight glass vial with 18 mm headspace (Thermo Scientific®). The glass vials were immediately closed with air-tight magnetic screw caps that contained self-sealing septums (Thermo Scientific®). The vials were flushed with argon for 30 min by piercing the septums with a needle attached to the argon tank and another needle for outlet gas. Once the samples were purged with argon, they were placed into the lighted growth chamber until testing, using the same light intensity, temperature, and shaking as described above. Hydrogen quantification was conducted by drawing 500 µL of gas from the headspace into an airtight, fixed needle syringe (Thermo Scientific®) and manually injected into the thermal conductivity detector (TCD) (Thermo Scientific Trace 1300) of the gas chromatograph. The characterization methods were the same as described, including the carrier gas, column, and GC parameters (Tiryaki and Irmak, 2020). A gas mixture of known composition was used as a standard, and had the same composition as described (Tiryaki and Irmak, 2020).

2.4. Transmission electron microscopy (TEM)

To visualize the PHB/V granule(s) inside the cell, samples from 10 mM acetate, 1 mM *p*-coumarate, and 1 mM coniferyl alcohol after five days of nitrogen starvation were subjected to TEM. The bacteria were fixed in 2% glutaraldehyde and 1.5% paraformaldehyde in 100 mM sodium cacodylate buffer for >1 h at room temperature and then 4 °C overnight per established procedures (Graham and Orenstein, 2007). The samples were washed in sodium cacodylate buffer three times (10 min each) and post-fixed in 1% osmium tetroxide in deionized water at room temperature for 1 h. After washing in water twice, the samples were dehydrated through an ethanol series and embedded in Spurr medium using a conventional TEM processing protocol. Ultrathin sections were cut using a Leica UC7 ultramicrotome and stained with 1% uranyl acid and 1% lead citrate. Images were collected using a Hitachi H7500 TEM at the Microscopy Core Research Facility of the Center for Biotechnology, University of Nebraska-Lincoln.

2.5. Pulse amplitude modulation (PAM) fluorometry

PAM fluorometry was conducted using the LI-6800 Portable Photosynthesis System, Software Version 1.4. LI-COR, Inc. To determine the photosynthetic yield (Φ_{PSII}) following previously established procedures (Phongjarus et al., 2018; Ritchie, 2008, 2013; Ritchie and Mekjinda, 2015; Ritchie and Larkum, 2012). Briefly, anaerobically growing cells were centrifuged during the mid-exponential phase and re-suspended in 400 μ L of PM media (with no carbon source) (Kim and Harwood, 1991). Precisely 250 μ L of cell suspension was then uniformly pipetted onto a Whatman® glass fiber disk and air-dried for 20 min at room temperature. Next, the fiber disks were dark-adapted in an opaque container for 10–30 min and subsequently placed in the chamber of the LI-6800's multiphase flash fluorometer. Both the measuring and actinic light sources were set to ~ 465 nm (blue light) (Ritchie, 2013). After setting the irradiance, the cells require approximately 15 min to reach a new steady-state (characterized by constant fluorescence), at which time a saturating light pulse is introduced to measure Φ_{PSII} (Maxwell and Johnson, 2000). Since the electron transport rate (ETR) is proportional to Φ_{PSII} (Phongjarus et al., 2018; Maxwell and Johnson, 2000) the relative photosynthetic rates during growth on any two conditions can be inferred from the relative photosynthetic yields (see Eqn. (3)).

2.6. Thermo-kinetic analysis

The rate law of a bi-molecular reaction can be re-written into the separable form below, as derived (Noor et al., 2013a):

$$v = E k_{cat}^+ \left(\frac{s/K_s}{1 + s/K_s + p/K_p} \right) (1 - e^{\Delta_r G' / RT}) \quad (1)$$

where the first factor refers to the enzyme's catalytic capacity (V^{max}) and is a product of the enzyme concentration (E) and the maximal catalytic efficiency of the reaction in the forward direction (k_{cat}^+). The second term refers to the fractional substrate saturation (κ) and is a function of the substrate (s) and product (p) concentrations and their associated Michaelis constants K_s and K_p . The third factor denotes the thermodynamic driving force (γ) and is mainly a function of the Gibbs free energy of a reaction ($\Delta_r G'$), which can be calculated from the standard Gibbs free energy ($\Delta_r G^0$) and the metabolite concentrations (or product to substrate ratios) (Noor et al., 2013a):

$$\Delta_r G' = \Delta_r G^0 + RT \ln \left(\frac{p}{s} \right) \quad (2)$$

The $\Delta_r G^0$ values of the thiolase (*phaA*) and reductase (*phaB*) reactions were calculated using the component contribution method (Noor et al., 2013b). For thiolase, the acetoacetyl-concentration was set to the minimum physiological concentration of 1 μ M (Noor et al., 2013b) and the acetyl-CoA:CoA ratio was sampled to determine its effect on γ . For reductase, simulations were conducted using 3-hydroxybutyryl-CoA concentrations of 30 μ M and 300 μ M to capture the range of reported concentrations (Leaf and Sreenc, 1998). The NADPH:NADP ratio was sampled to determine its effect on γ .

Michaelis constants (K_s and K_p) used during simulation were reported for the soil bacterium *Cupriavidus necator* (Leaf and Sreenc, 1998) as no values have been reported for *R. palustris*. Due to the high homology of both *phaA* and *phaB* (E value $< 10^{-80}$) between the two organisms, the substrate saturation effects were assumed to be similar. Supplementary Tables S1–S2 contain the parameters used in this analysis and the references they were obtained from.

2.7. GSMM simulations

Growth simulations under different environmental conditions were performed through parsimonious Flux Balance Analysis as in (Lewis et al., 2010). The rate through the photosynthetic electron transport reaction PSII (light-induced reduction of quinol) was fixed based on previous analysis for growth on acetate and butyrate (Alsiyabi et al., 2019). The rate of this reaction during growth simulations on *p*-coumarate was calculated based on the relative photosynthetic yield (Φ_{PSII}) between *p*-coumarate and acetate:

$$v_{PSII}^{pC} = v_{PSII}^{ace} \frac{\Phi_{PSII}^{pC}}{\Phi_{PSII}^{ace}} \quad (3)$$

where “pC” and “ace” refer to *p*-coumarate and acetate, respectively.

Finally, the generation rate of a metabolite k (e.g., acetyl-CoA) was calculated using Eq. (4) below:

$$genRate_k = \sum_{j \in J} v_j \forall j \in J \text{ s.t. } S_{kj} > 0 \quad (4)$$

2.8. Statistical methods

Independent, two-tailed t tests were conducted to decipher statistically significant differences in maximum optical densities between maximum optical densities from the substrates. Similarly, t tests were conducted to compare maximum PHB titers between *p*-coumarate, coniferyl alcohol, and butyrate at 5 days of nitrogen starvation (acetate was not included since it yielded no PHB titer). Paired, one-tailed t tests were conducted to decipher the time of maximum PHB production from *p*-coumarate, coniferyl alcohol, and butyrate. For example, a paired t test was conducted between days 4, 5, and 6 of nitrogen starvation on *p*-coumarate to decipher the time of maximum PHB production. All t tests were conducted with a 95% confidence level ($\alpha = 0.05$).

3. Results and discussion

3.1. Disparities in PHB/V titers from cells grown on various carbon sources

In our previous study, *R. palustris* was shown to grow on *p*-coumarate and produce PHB to a much higher titer than on acetate (Brown et al., 2020). It was unclear as to why more PHB production was observed from the LBP *p*-coumarate compared to virtually no PHB production from acetate. In this study, PHB production was produced from *R. palustris* on the LBP coniferyl alcohol ($C_{10}H_{12}O_3$) for the first time, which has higher carbon content than *p*-coumarate. *R. palustris* was indeed able to grow on the LBP coniferyl alcohol (Fig. 3A) and reached a significantly higher maximum optical density (3.22) compared to *p*-coumarate (2.17) or acetate (0.87). A higher maximum OD suggests more CO_2 is being utilized during the degradation of the LBPs compared to the other carbon sources (McKinlay and Harwood, 2010, 2011a). Furthermore, the increased growth rates of the LBPs signify faster carbon uptake rates. Before proceeding with PHB analysis between the carbon sources, a starvation analysis was conducted to decipher if nitrogen or phosphorous limitation yields more PHB since this has been shown previously to boost production (Wen et al., 2010). Starvation analysis was conducted on *p*-coumarate and employed either nitrogen, phosphorus, or both nitrogen and phosphorous starvation conditions. Nitrogen starvation yielded a significantly higher PHB titer, and thus it was used to compare PHB production across the substrates (Fig. 3B). A subsequent analysis

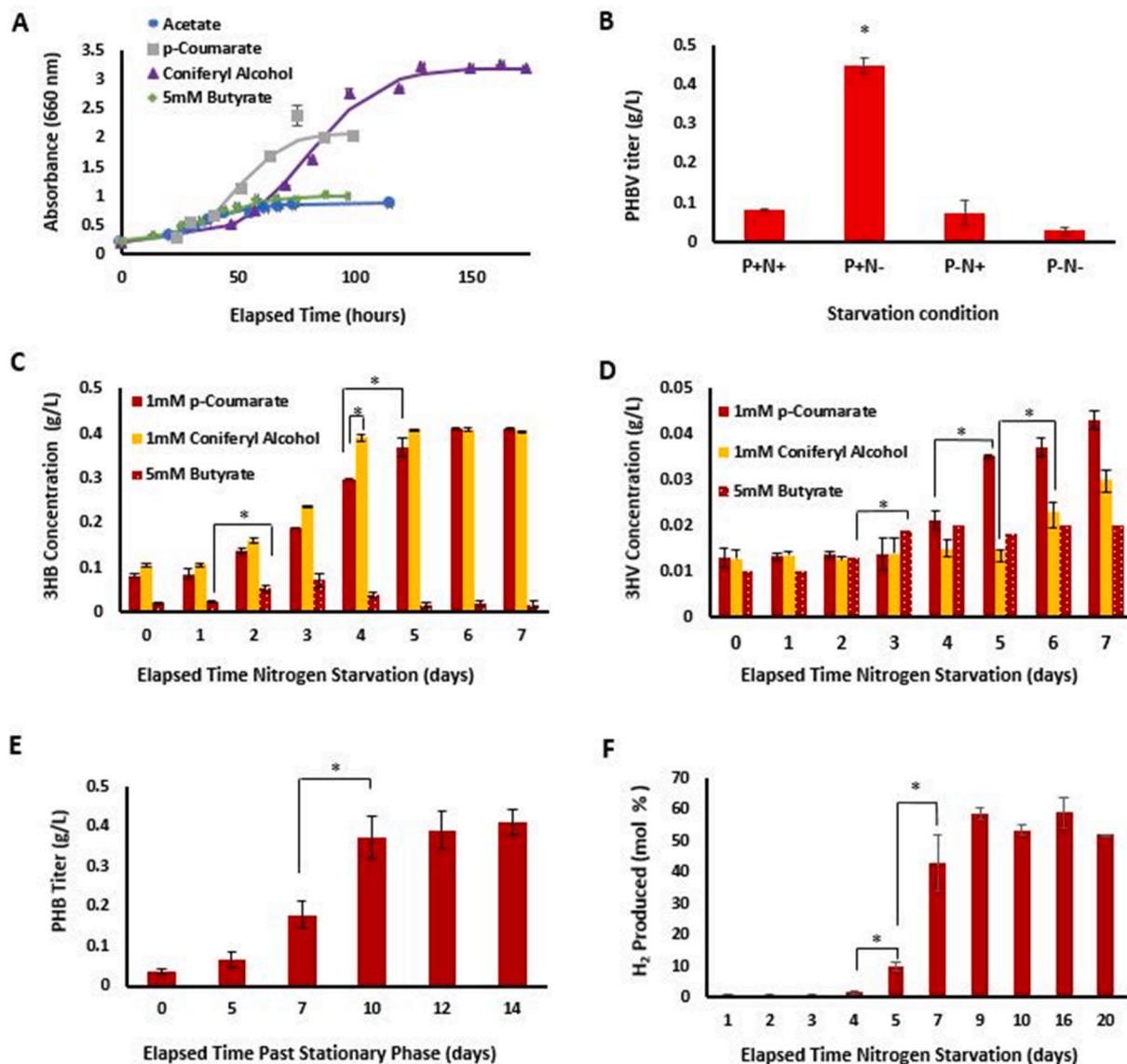


Fig. 3. Unique findings from experimental data that motivated application of the GSMM. (A) Anaerobic growth analyses. All substrates were supplemented with 10 mM sodium bicarbonate. (B) Starvation condition analysis comparing nitrogen starvation, phosphorous starvation, and a combination of both nitrogen and phosphorus starvation. (C) Comparison of 3HB titers on LBPs vs. butyrate. (D) Comparison of 3HV titers on LBPs vs. butyrate. (E) PHB production from 1 mM *p*-coumarate from stationary phase (i.e. without resuspending in nitrogen-starved media). (F) H₂ production from 1 mM *p*-coumarate after nitrogen starvation. All error bars represent the standard error for the population and are derived from the mean of biological triplicates for each data point. The (*) represents statistical significance at a 95% confidence level ($\alpha = 0.05$).

Table 1

Carbon conversion efficiency to PHB from each substrate.

Substrate	Formula	Concentration Used (mM)	Carbon Invested (moles) ^a	Carbon Conversion to PHB (%) ^b
Sodium acetate	C ₂ H ₃ NaO ₂	10	0.02	N/A
Sodium butyrate	C ₄ H ₇ NaO ₂	5	0.02	10.83
<i>p</i> -Coumarate	C ₉ H ₇ O ₃ ⁻	1	0.009	68.4
Coniferyl alcohol	C ₁₀ H ₁₂ O ₃	1	0.01	65.0

^a This does not include 10 mM sodium bicarbonate (NaHCO₃).

^b The percent conversion to PHB includes 10 mM sodium bicarbonate to account for total carbon invested.

was conducted to decipher PHB production on *p*-coumarate without nitrogen starving the cells (Fig. 3E). PHB production peaked at approximately 10 days past stationary phase with a similar maximum titer as that of the nitrogen-starved cells (0.41 g/L).

Despite the higher growth rate on coniferyl alcohol, the maximum PHB titer (0.41 g/L) did not improve. The carbon conversion efficiency

for the maximum PHB titer on each substrate is summarized in Table 1. Comparison of the PHB profiles showed that coniferyl alcohol's higher carbon content resulted in a higher rate of PHB production without changing the final titer.

Combined experimental results from growth rates (Fig. 3A), PHB production (Fig. 3C), H₂ production (Fig. 3F), and transmission electron

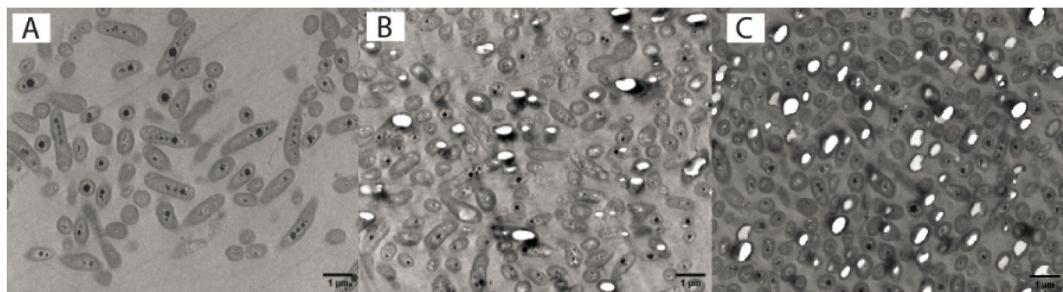


Fig. 4. Transmission Electron Microscopy (TEM). TEM images of *R. palustris* CGA009 cells grown anaerobically in photosynthetic media supplemented with (A) 10 mM acetate and 10 mM sodium bicarbonate, (B) 1 mM *p*-coumarate and 10 mM sodium bicarbonate, and (C) 1 mM coniferyl alcohol and 10 mM sodium bicarbonate. All samples were grown to mid-exponential phase, and subsequently nitrogen starved for five days. White inclusions inside the cytoplasm denote PHB granules.

microscopy (Fig. 4) revealed that cytoplasmic space may be a limiting factor for maximum PHB titer. Transmission electron microscopy was conducted to image granules inside of the cells on the LBPs vs. acetate, and revealed stark discrepancies between the LBPs and acetate as expected based on the measured titers (Fig. 4). Virtually no granules were present inside cells grown on acetate, whereas there was an abundance of cells that contained one large granule when grown on the LBPs. Of note, one large granule is produced in the cells utilizing the LBPs, which indicated cytoplasmic space might be a major limiting factor for the maximum PHB titer as shown in *Halomonas bluephagenesis* (Shen et al., 2019).

This study also reveals that *R. palustris* can create a copolymer called poly-3-hydroxybutyrate-co-hydroxyvalerate (PHBV) (Supplementary Fig. S1), which boosts the thermomechanical properties compared to PHB alone. The LBPs and butyrate all fostered a 3-hydroxyvalerate (3HV) component that creates the copolymer PHBV (Fig. 3D), with *p*-coumarate yielded the highest 3HV titer of approximately 0.04 g/L.

3.2. Tradeoffs between PHB and H₂ production

H₂ production measurements were also conducted to qualitatively assess electron secretion (Fig. 3F). H₂ production significantly increases at approximately five days of nitrogen starvation, which is when PHB production peaks.

These observations along with previously described data on PHB production (Brown et al., 2020) lead to the hypothesis that the rate of PHB production was mainly controlled by metabolic factors, especially since expression of *pha* genes has been reported to remain constant under different conditions (Ranaivoarisoa et al., 2019; McKinlay et al., 2014b).

3.3. The need for metabolic modeling

It was still unclear why the LBPs yielded more PHB production compared to virtually no titer from acetate. Furthermore, measurements prior to nitrogen starvation revealed the presence of PHB, which indicated that PHB production was not strictly a stress response (Fig. 3C, E). The GSMM was next employed to investigate the potential mechanisms behind these findings and identify specific design strategies that can be implemented to optimize PHB production. Due to the lack of kinetic parameters and concentration ranges for PHBV-producing reactions and their corresponding metabolites, the model was strictly used to investigate PHB production. However, since both monomer products (3-hydroxybutyrate and 3-hydroxyvalerate) are produced by the same enzymes, it is likely that the identified design strategies are applicable to both products. Furthermore, coniferyl alcohol is not included in the GSMM analysis because its degradation pathway is not yet known, which we aim to illuminate through an ongoing transcriptomics analysis.

3.4. Computational analysis of PHB production

Computational methods were employed to investigate the observed experimental findings. The main question that the GSMM intended to address was what underlying factors cause the observed disparity in PHB production between the tested carbon sources. More importantly, can a set of generalized design strategies for PHB overproduction be deduced by incorporating the observed experimental findings into a whole-cell metabolic modeling framework? To guide the analysis of the GSMM, a detailed thermokinetic study of the pathway was conducted to determine how the overall production rate of PHB is affected by the cell's metabolic state. The goal of this analysis was to identify the different metabolic factors (e.g., NADPH redox state) governing the activity of the PHB pathway. Growth simulations facilitated by the GSMM can then be used to determine how consumption of each substrate affects the identified factors and therefore the rate of PHB synthesis.

3.4.1. Thermo-kinetic analysis of the PHB pathway

The rate of an unregulated enzymatic reaction that follows Michaelis-Menten kinetics can be broken down into a product of three main constituents: (i) the enzyme's catalytic capacity (V^{\max}), (ii) the reaction's thermodynamic driving force (γ), and (iii) enzyme substrate saturation (κ) (Noor et al., 2013a).

This can be expressed mathematically as shown in Eq. (5):

$$v = V^{\max} * \gamma * \kappa \quad (5)$$

where, the maximal rate of the reaction is determined by its catalytic efficiency and enzyme concentration ($V^{\max} = [E]k_{cat}$). The thermodynamic driving force can take on values of 0–1 when the reaction is in the forward direction and –1 to 0 when the reaction is going in the reverse direction. Moreover, the enzyme saturation factor has a range of 0–1. Since the rate law, written in this form, is separable, the effect of each factor on the overall rate can be analyzed individually (Noor et al., 2013a). Starting with the first reaction in the pathway (*phaA*), it was found that this reaction is significantly constrained thermodynamically (Fig. 5A). As described previously, the rate of the reaction was highly dependent on the acetyl-CoA/CoA ratio (Van Wegen et al., 2001; Kocharin et al., 2012). Surprisingly, the reaction was found to be infeasible under a ratio 12, which is relatively high compared to ratios observed in most bacteria (<5) (Bennett et al., 2009; Chohnan et al., 1998; Gameiro et al., 2016; Park et al., 2016; Thauer et al., 1977). This means that a significant accumulation of acetyl-CoA is required for thiolase (*phaA*) to become thermodynamically viable and start forming acetoacetyl-CoA. This limitation is likely a major contributor to why no PHB was observed during growth on acetate, even after nitrogen starvation. On the other hand, the breakdown pathways of the other carbon substrates produced acetoacetyl-CoA as an intermediary metabolite (Fig. 6), therefore bypassing this thermodynamic obstacle. Moreover, changes to this ratio displayed a minimal effect on κ (Fig. 5A), meaning

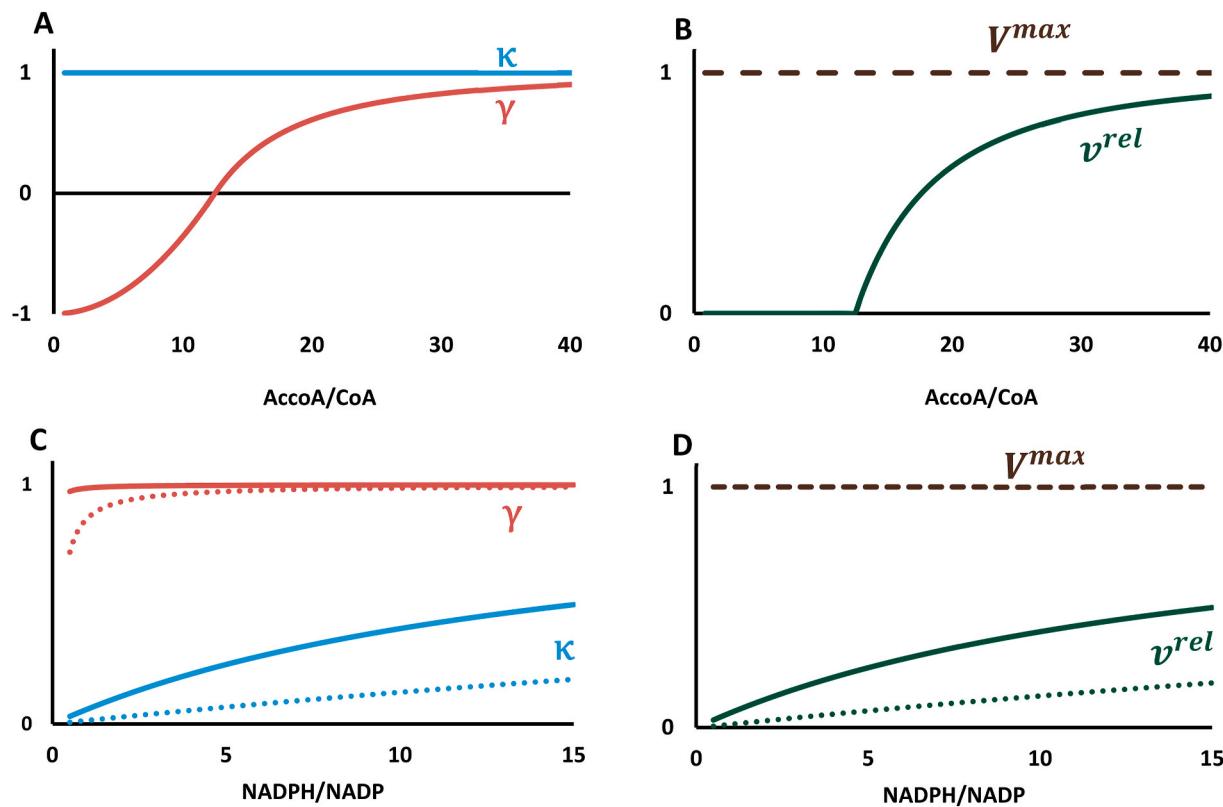


Fig. 5. Thermo-kinetic analysis of thiolase (*phaA*) and reductase (*phaB*) activity. Effect of the acetyl-CoA/CoA ratio on the *phaA*'s (A) thermodynamic driving force (γ) and substrate saturation (κ) and (B) Overall rate relative to V^{\max} . Effect of the NADPH/NADP ratio on the *phaB*'s (C) thermodynamic driving force (γ) and substrate saturation (κ) and (D) Overall rate relative to V^{\max} . Solid and dotted lines in (C) and (D) correspond to a 3-hydroxybutyryl-CoA concentration of 30 μM and 300 μM , respectively. The y-axis is unitless in all four panels.

that during any given condition, the rate of the reaction, relative to V^{\max} , can be estimated solely based on γ (Fig. 5B). Due to the high thermodynamic burden of the *phaA* reaction, substrates that breakdown into acetoacetyl-CoA as an intermediary metabolite could help maximize PHB production.

Interestingly, an opposite conclusion was reached during analysis of the reaction through acetoacetyl-CoA reductase (*phaB*). Although both factors (γ and κ) contributed to the overall rate, substrate saturation was significantly more limiting (Fig. 5C). An increase in the NADPH/NADP ratio reduces the bottleneck through this reaction by increasing both the thermodynamic driving force and substrate saturation. Furthermore, the concentration of the main reaction product (3-hydroxybutyryl-CoA) has a pronounced effect on substrate saturation. The solid and dotted lines in Fig. 5C correspond to the minimum and maximum 3-hydroxybutyryl-CoA concentrations reported in literature (Leaf and Srienc, 1998; Van Wegen et al., 2001). The combined effect of both factors on the overall flux through the reaction is shown in Fig. 5D. Although the reaction is viable under different biologically relevant NADPH:NADP ratios, flux through *phaB* is significantly hindered during high NADP(H) oxidation states (i.e., low NADPH:NADP ratios) due to the enzyme being sub-saturated ($\kappa \ll 1$). Finally, reports from previous kinetic studies had identified that *phaB*'s kinetic capacity (V^{\max}) was notably lower than that of *phaA* and *phaC* (Wang and Lee, 1997; Sim et al., 1997), meaning that for steady-state PHB accumulation to occur, a relatively higher expression rate of this enzyme is required for the fluxes to be balanced. The implications of this difference in catalytic efficiency are discussed in the Supplementary Methods. Therefore, even under optimal metabolic conditions (high NADPH:NADP ratio), the catalytic inefficiency of the reductase enzyme (*phaB*) may limit the overall rate of the PHB pathway.

3.4.2. Genome-scale prediction of metabolic activity through fluorometry and *iRpa940*

To investigate the cause of the disparities between PHB production during growth on *p*-coumarate compared to that on butyrate, *iRpa940* model was employed to explore differences in the generation of both carbon and electron sources. It had been previously observed that the activity of *R. palustris*' metabolic network during anaerobic growth conditions can be predicted accurately if the rate of photosynthesis, or the electron transport rate (ETR), was known *a priori* (Alsiyabi et al., 2019). When ^{13}C metabolic flux analysis (MFA) measurements are available, the ETR can be determined by fitting pFBA-generated flux predictions to those measured through MFA (Alsiyabi et al., 2019). The ETR values during growth on acetate and butyrate were available *a priori* (Alsiyabi et al., 2019). However, due to the absence of MFA measurements during growth on *p*-coumarate, pulse amplitude modulation (PAM) fluorometry was conducted to determine the relative ETR through cells growing on *p*-coumarate compared to those growing on acetate (see Supplementary Fig. S2). First, ETR values predicted through MFA measurements (Alsiyabi et al., 2019) during growth on acetate and butyrate were validated. Based on the MFA measurements, the model had predicted that the ETR, and therefore the photosynthetic yield, was similar during growth on these two substrates (Alsiyabi et al., 2019). Fluorometry measurements show that the photosynthetic yields are indeed similar (Fig. 7). These results gave confidence in the validity of subsequent model predictions. Next, PAM fluorometry was used to measure the photosynthetic yield during growth on *p*-coumarate. Interestingly, the ETR was found to be significantly higher during growth on *p*-coumarate compared to growth on acetate and butyrate (Fig. 7). These measurements provided the parameters required for the GSMM to simulate anaerobic growth on each of the carbon sources. Supplementary Figs. S3–5 shows the metabolic activity through the

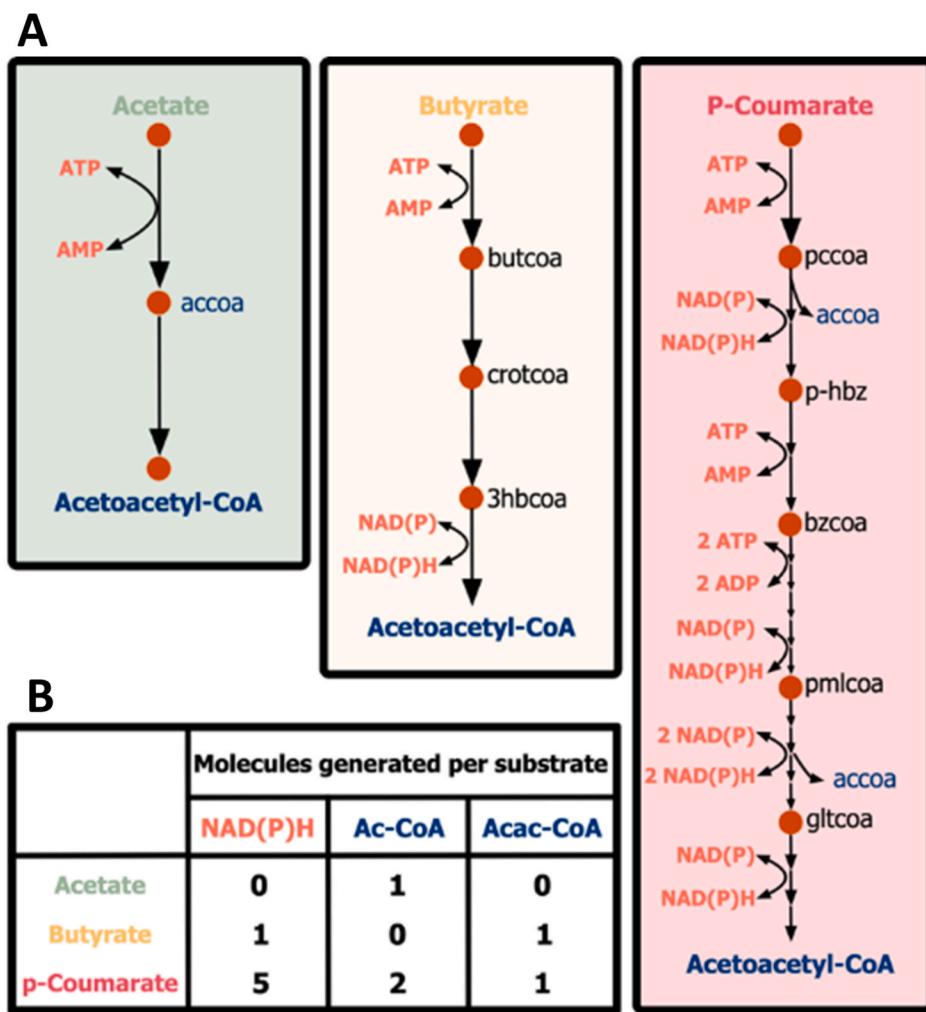


Fig. 6. Substrate consumption of the three modeled carbon sources: acetate, butyrate, and *p*-coumarate depicting the (A) breakdown pathways and the (B) relevant cofactors and PHB precursors produced from each carbon source.

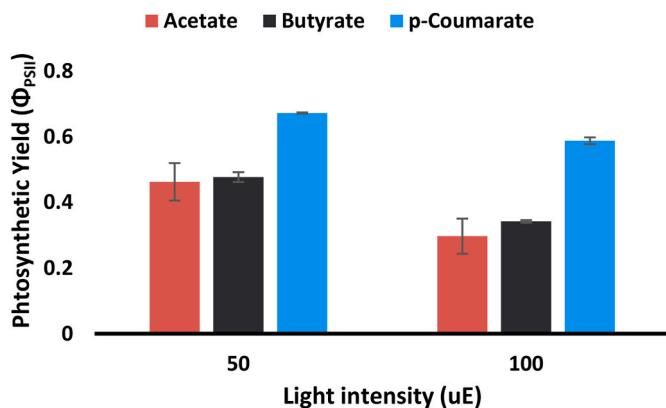


Fig. 7. Photosynthetic yields of *R. palustris* cells grown on different carbon sources as measured at 50 μ E and 100 μ E using PAM fluorometry.

central metabolism during growth on the three substrates. Next, these predictions were used to compare how the generation rates of different metabolites participating in PHB synthesis varied between carbon sources.

3.4.3. PHB synthesis within the context of metabolism

First, the overall generation rate of NAD(P)H was determined during

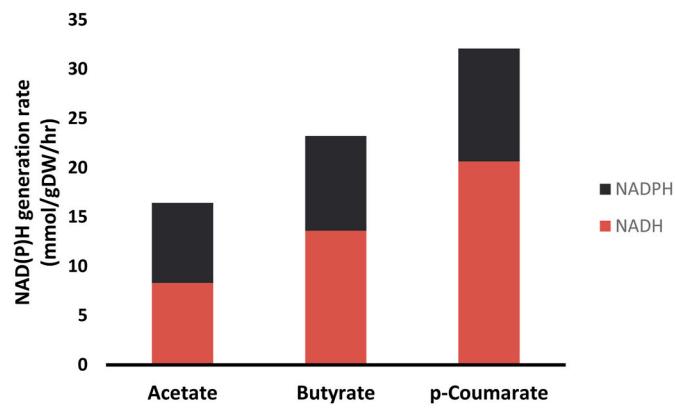


Fig. 8. Predicted reduction rates of the cofactors NAD and NADP during growth on acetate, butyrate, and *p*-coumarate.

growth on each carbon source based on the model-generated growth predictions (see Methods). The rationale behind this analysis was that a higher generation rate of reducing power results in a more reduced state and a higher driving force through the reductase (*phaB*). It has also been reported that *R. palustris*' metabolism is constrained by its inability to oxidize cofactors through the electron transport chain due to the lack of a terminal electron acceptor (McKinlay and Harwood, 2010, 2011a),

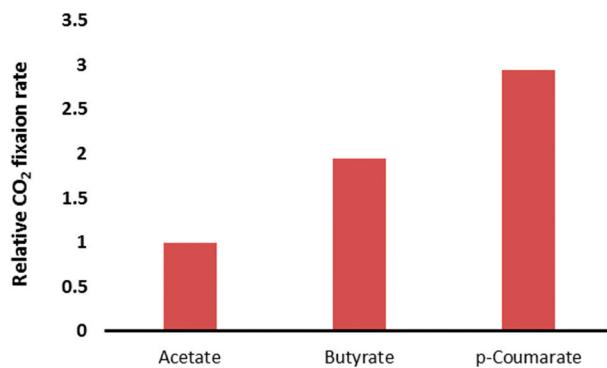


Fig. 9. Relative predicted rate of CO₂ fixation during growth on acetate, butyrate, and *p*-coumarate.

which forces it to utilize other routes such as carbon fixation, nitrogen fixation or PHB synthesis to accomplish this (McKinlay and Harwood, 2011a; McKinlay, 2014). During steady-state growth, the rate at which cofactors are oxidized needs to be equivalent to the rate of reduction to avoid the accumulation of electrons. As can be seen from Fig. 8, growth on butyrate and *p*-coumarate generate approximately 40% and 95% more reducing equivalents compared to acetate, respectively. Since reduced cofactors (NAD(P)H) are generated during substrate breakdown, the differences in generation rates (Fig. 8) correspond to differences in substrate oxidation states, uptake rates, and breakdown pathways (Fig. 6). Furthermore, since both of these substrates (butyrate and *p*-coumarate), generate acetoacetyl-CoA during their breakdown, the redox state of their respective NADP(H) pool can be considered the rate-limiting factor for PHB production. It is noted that since both nitrogen fixation and biomass production are major electron sinks, this effect becomes more pronounced during nitrogen-starvation when both of these processes are halted. Results from this analysis indicate that the higher rate of NAD(P)H generation likely leads to a significantly more reduced intracellular state. In the closely related strain *R. palustris* TIE-1, the measured NADPH:NADP ratio during anaerobic growth on butyrate was approximately 2 (Guzman et al., 2019). Therefore, as indicated by the thermo-kinetic analysis, a higher ratio during growth on *p*-coumarate will lead to a higher rate through the reductase reaction (*phbB*) in the PHB pathway.

During anaerobic growth conditions in which a reduced nitrogen source (e.g. ammonia) is present and hence nitrogen fixation is not required, *R. palustris* utilizes CO₂ fixation through the Calvin Cycle as its primary electron sink (McKinlay and Harwood, 2010, 2011a). Therefore, the rate of carbon fixation was expected to show a similar trend to that of NAD(P)H generation (McKinlay and Harwood, 2010). Indeed, as

shown on Fig. 9, the relative rate of carbon fixation is proportional to the rate of reducing cofactors. Relative rates were calculated by normalizing the predicted CO₂ fixation rates by the rate predicted during growth on acetate. The predicted rates during growth on each carbon source are shown on the metabolic maps in Supplementary Figs. S3–5. Due to the high NAD(P)H generation rate during growth on *p*-coumarate, the model predicts a 3-fold increase in carbon fixation compared to growth on acetate (Fig. 9). However, due to known inefficiency of the carbon fixing enzyme Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) (Cummins et al., 2018), it is plausible to postulate that kinetics of the RuBisCO enzyme do not permit such a high rate. Therefore, the cell would be required to store the remaining excess electrons in PHB, which is the only alternative electron sink under such conditions. It is also noted that model predictions indicate that both NAD(P)H generation and carbon fixation rates increase with increasing ETR during growth on any of the three substrates.

Finally, the model was used to investigate how utilization of each of the carbon substrates affected the rate of acetyl-CoA and acetoacetyl-CoA generation (Fig. 10). As previously described, although acetyl-CoA is considered to be the first metabolite in the PHB synthesis pathway, its conversion to acetoacetyl-CoA is thermodynamically very unfavorable. It was therefore hypothesized that exploring the rate of acetoacetyl-CoA generation may give a better indication of PHB production. Growth simulations on acetate showed that all of the consumed substrate was converted into acetyl-CoA before entering the central metabolic pathways through the TCA cycle. Since acetate assimilation into central metabolism does not produce acetoacetyl-CoA, model predictions indicate that only a small fraction of the starting carbon gets converted to this metabolite, while the majority is used to synthesize other biomass precursors and constituents. Conversely, consumption of butyrate mandates that all of the starting substrate goes through acetoacetyl-CoA before entering central metabolism. In fact, butyrate's breakdown pathway also goes through 3-hydroxybutyrate CoA (Fig. 6), which is the monomer form of PHB. Furthermore, due to the constitutive nature of the genes involved in PHB synthesis, generation of acetoacetyl-CoA during assimilation of the carbon source into central metabolism is expected to result in PHB production even under conditions when carbon or electron storage is not required. As shown in Fig. 3C, PHB production is observed in cells growing even prior to nitrogen starvation (day 0). Finally, growth on *p*-coumarate results in relatively high generation rates of both acetyl- and acetoacetyl-CoA. Model analysis reveals that based on the measured growth rates, the rate of carbon uptake is 4.5-fold higher during growth on *p*-coumarate compared to acetate. Therefore, this analysis reveals that the basis behind the highly observed PHB production rate on *p*-coumarate is likely due to both an excess of carbon and electrons. It is likely that due to the large size of *p*-coumarate

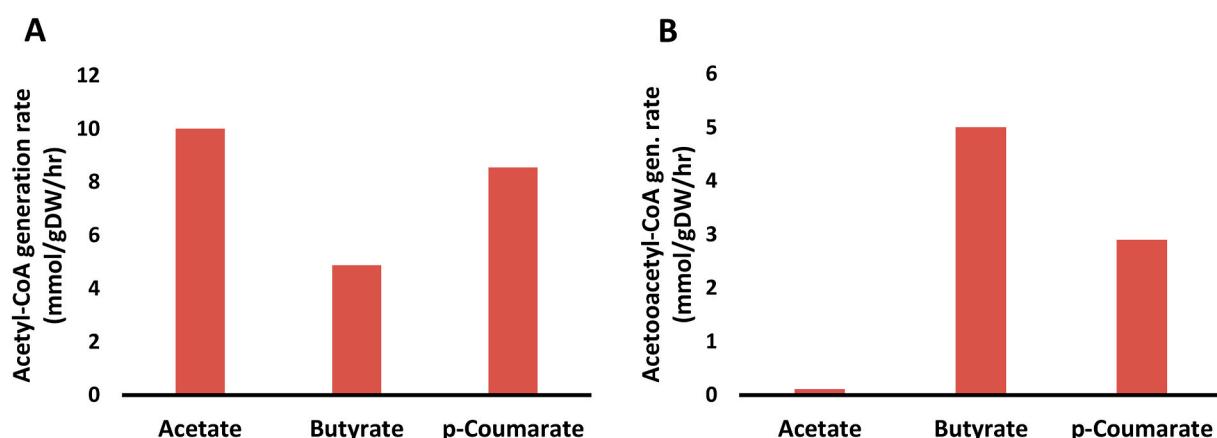


Fig. 10. Predicted generation rates of the first two substrates in the PHB pathway (acetyl-CoA (A) and acetoacetyl-CoA (B)) during growth on acetate, butyrate, and *p*-coumarate.

(and LBPs in general), the rate at which central metabolites are generated is too high to enter central metabolism, which is why PHB production can be observed even during exponential growth. Similar behavior has been observed in *E. coli* under high carbon uptake rates and has been referred to as Janusian growth (O'Brien et al., 2013).

3.5. General design strategies for PHB production

These findings can be generalized into a set of design strategies for optimal PHB production. Due to the constitutive expression of the metabolic *pha* genes in *R. palustris*, changes in the intracellular metabolic state in the form of increased carbon and electron accumulation have a direct effect on the pathway's activity. Therefore, carbon sources used for PHB production should ideally have a high molecular weight and be more reduced than the organism's biomass. This will lead to the generation of an excess in both reducing agents (NAD(P)H) and carbon that can subsequently be shuttled towards PHB production. Moreover, the substrates should preferably produce acetoacetyl-CoA as part of their breakdown. This is necessary to bypass the extremely unfavorable first reaction of the PHB pathway. Based on these characteristics, LBPs such as *p*-coumarate and coniferyl alcohol appear to be ideal candidates for PHB production. Finally, under optimal metabolic conditions, it is expected that the catalytic efficiency of *phaB* may play a limiting role in the overall rate (Supplementary Methods). Therefore, overexpression of *phaB* may be necessary to increase PHB production rates under such conditions. Most importantly, the generality of these design rules allows them to be applied to any PHB-producing microbe with similar metabolic features.

3.6. Selection of sodium butyrate as an ideal substrate for PHB production

To better understand how the choice of substrate impacts PHB production, butyrate ($C_4H_8O_2$) was tested since an annotated pathway was available, it bypasses the *PhaA* pathway, and is more reduced than acetate. Butyrate was assessed via metabolic modeling (Section 3.4), and PHB production was subsequently experimentally validated. After assessment with the GSMM to substantiate the hypotheses, we experimentally validated the growth and PHB production analyses on butyrate (Fig. 3). Butyrate was compared to acetate on a carbon-to-carbon basis to test the impact on PHB titer from a more reduced substrate that bypasses the energy intensive keto-thiolase reaction (*phaA*). Significantly more PHB was produced from butyrate compared to virtually none from acetate, despite having the same carbon content (Fig. 3C). The percent carbon conversion from butyrate (10.83%) was lower than the LBPs (Table 1), which can be attributed to the higher molecular weights of the LBPs that likely leads to higher rates of NAD(P)H and acyl-CoA generation.

The approach taken to arrive at these findings highlights how experimental observations can be used to generate a broad hypothesis that can subsequently be narrowed down through the application of metabolic modeling frameworks. Furthermore, it illustrates the synergistic effect of combining a detailed modeling analysis of the pathway of interest with a coarse-grained genome scale analysis to test the generated hypothesis and to suggest subsequent validation experiments. As illustrated in this work, the advantage of utilizing a detailed thermokinetic analysis is the ability of identifying specific metabolic factors that exert control over the production pathway of interest. Once those factors are identified, genome-scale metabolic modeling can be employed to predict how different growth conditions influence the state of the identified factors. We believe such an approach can be generalized to other engineering applications where the production pathway of interest is highly connected to the rest of the metabolic network.

4. Conclusions

Novel design strategies for improved PHB production have been identified through an integrated experimental and computational approach. *R. palustris* was an ideal candidate for probing the metabolic elements that control PHB production due to its ability to utilize substrates of varying properties (Larimer et al., 2004; McKinlay and Harwood, 2011a), the limited metabolic routes it can operate to maintain redox balance (McKinlay et al., 2014b), and its constant expression of *pha* genes across different conditions (Ranaivoarisoa et al., 2019; McKinlay et al., 2014b). Unique experimental findings from the metabolically versatile *R. palustris* motivated the application of metabolic modeling. These findings included 1) significantly higher PHB production rates from LBPs, 2) PHB production without stress induction, 3) growth and PHB production on coniferyl alcohol, 4) increased hydrogen production as PHB production peaks, 5) production of the copolymer PHBV, and 6) cytosolic space as a major limiting factor for PHB production. These findings led to the hypothesis that PHB production was driven by the metabolic state of the cell. Therefore, a metabolic modeling approach was taken to gain a deeper understanding of the underlying factors causing the discrepancies in observed PHB production. Three main design strategies were identified for optimizing PHB production in hosts with similar metabolic features.

First, PHB production efforts should utilize carbon sources that can bypass the thiolase reaction. Modeling results indicated that under normal acetyl-CoA:CoA ratios, the first reaction in the PHB synthesis pathway was infeasible. This result explains, at least partly, why no PHB production was observed during growth on acetate. On the other hand, growth on butyrate and *p*-coumarate does not require this reaction for PHB production, as both substrates produce acetoacetyl-CoA as part of their consumption pathway. Although the assimilation route of coniferyl alcohol is not known, it is likely that breakdown of this substrate produces acetoacetyl-CoA as part of its consumption pathway, similar to *p*-coumarate. Second, carbon substrates used for PHB production should be more reduced than the cell's biomass. Model analysis indicates that activity of the acetoacetyl-CoA reductase (*phaB*) is highly dependent on the redox state of NADPH, especially under physiologically relevant NADPH:NADP ratios. The importance of this result is magnified due to the inherently low catalytic efficiency of this enzyme. Therefore, use of a highly reduced substrate or interruption of processes that require significant amounts of electrons (e.g. nitrogen fixation) is predicted to lead to a higher NADPH:NADP ratio and therefore enhance the rate through this reaction. Third, the utilized substrates should have high molecular weights, or a relatively large number of carbons. Growth measurements and model predictions indicate that the relatively large size of *p*-coumarate and coniferyl alcohol leads to a higher carbon uptake rate. At the observed uptake rates, catalytically inefficient enzymes within central metabolism, such as RuBisCO, are likely to operate close to their maximum capacity, leading to an increased production of storage metabolites like PHB. The increased uptake rate is also what leads to the predicted generation rates of NAD(P)H and acyl-CoAs being significantly higher during growth on *p*-coumarate.

Utilizing *R. palustris*' ability to consume a wide array of substrates, a number of carbon sources were screened to identify a carbon substrate that satisfies the identified design requirements. Due to the lack of annotation of consumption pathways associated with many substrates, butyrate was identified as the ideal candidate for model validation. Consistent with model predictions, butyrate consumption led to PHB production both before and after nitrogen starvation. However, the lower molecular weight of butyrate compared to *p*-coumarate likely lead to lower rates of NAD(P)H and acyl-CoA generation, which resulted in lower PHB titers despite butyrate being more reduced. As evident from the characteristics of *p*-coumarate and coniferyl alcohol, and from the measured PHB profiles associated with them, it appears that LBPs are ideal substrates for PHB production and can lead to an economically viable process for PHB production. Overall, this study showcases how an

integrated experimental-modeling approach can identify a set of novel engineering design strategies associated with sustainable biopolymer production, thus advancing the replacement of petroleum-derived plastics. Most importantly, the generality of these design rules allows them to be applied to any PHB-producing microbe with similar metabolic features.

Author statement

Adil Alsiyabi: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Writing - Original Draft, Resources, Data Curation, Visualization. Brandi Brown: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Writing - Original Draft, Resources, Data Curation, Visualization. Dianna Long: Methodology, Formal Analysis, Investigation. Cheryl Immethun: Methodology, Formal Analysis, Investigation, Resources, Supervision, Writing - Review & Editing. Mark Wilkins: Resources, Funding acquisition, Supervision, Writing - Review & Editing. Rajib Saha: Resources, Funding acquisition, Supervision, Writing - Review & Editing.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymben.2021.08.008>.

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