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Rhodopseudomonas palustris CGA009 polyhydroxybutyrate production from a lignin aromatic and quantification via flow cytometry

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Abstract

Polyhydroxyalkanoates are biopolymers with the potential to replace petroleum-based plastics but are limited by higher productions costs. For the first time, (i) polyhydroxybutyrate (PHB) was produced by the metabolically versatile *Rhodopseudomonas palustris* CGA009 from the lignin breakdown product *p*-coumarate as a renewable carbon source, and (ii) quantification of PHB via flow cytometry was conducted with this highly pigmented bacterium. PHB titer ranged from 0.08 to 0.41 g/L and yielded a 68.4% carbon conversion efficiency. An optimized protocol using either Nile Red or BODIPY 493/503 lipophilic stains yielded high linear fitness for fluorescence intensity with both PHB titer (R^2 = 0.9384 and R^2 = 0.9747 respectively) and cell count (R^2 = 0.9383 and R^2 = 0.9955 respectively). This quick and precise method for high-throughput quantification of both PHB and cell count coupled with the highest PHB titer from a lignin breakdown product to date from *R. palustris* make this study novel.

1. Introduction

Polyhydroxyalkonoates (PHAs) are biopolymers produced by microorganisms under certain stress conditions and offer a sustainable alternative to petroleum-based plastics. PHA metabolism has been shown to not only act as a carbon sink for bacteria, but also to provide a pool for reducing redox potential (Liebergesell et al., 1991; Higuchi-Takeuchi et al., 2016). These biopolymers have thermomechanical properties similar to conventional plastics, are biodegradable, and can be produced from a variety of substrates. Polyhydroxybutyrate (PHB) is a short-chain-length PHA that is commonly studied and can be modified to generate various

copolymers depending on the metabolism of the bacteria and carbon sources provided for fermentation (Li et al., 2016). Due to its thermomechanical properties, biodegradation, and biocompatibility, PHB has been used in applications ranging from packaging materials to drug delivery systems (Poltronieri and Kumar, 2017). However, widespread use of PHAs is inhibited by high production costs due primarily to the carbon source and extraction methods (Aramvash et al., 2018).

The carbon source for bacterial PHB production accounts for about half of the total production costs (Mozumder et al., 2015). Lignocellulosic biomass is considered to be the most economic carbon source in the world since it is a common agricultural waste and highly renewable (Qian, 2013; Ponnusamy et al., 2018). The production of biopolymers from lignin, such as PHAs, is arguably the most promising route for boosting the valorization of lignin (Banu et al., 2019). However, there is still a significant research gap for microbes that are capable of producing high-value byproducts from lignocellulosic biomass. In fact, many bacteria are not capable of catabolizing the complex phenolic structure of lignin due to its recalcitrance. However, *Rhodopseudomonas palustris* CGA009 (hereafter *R. palustris*) is a purple non-sulfur, gram-negative bacterium that has been shown to grow on lignin breakdown products and lignocellulosic biomass (Austin et al., 2015; Salmon et al., 2013). A particular lignin aromatic, *p*-coumarate, is a major lignin breakdown product that has been highly characterized for *R. palustris* catabolism (Pan et al., 2007; Hirakawa et al., 2012). *R. palustris* is one of the most metabolically versatile organisms on the planet (Larimer et al., 2004), and its ability to exploit a wide array of carbon sources in diverse environments could reduce PHB production costs. A variety of *R. palustris* strains have been shown to produce PHB. However, many studies of *R.*

palustris involving PHB production have focused on hydrogen gas production (McKinlay et al., 2014; Wu et al., 2012). Furthermore, many studies use nitrogen fixation for PHB production by *R. palustris*, which is time consuming and adds to production costs (McKinlay et al., 2014; Ranaivoarisoa et al., 2019; Wu et al., 2012). Although *R. palustris* is known to grow on lignin aromatics and lignocellulosic biomass, PHB production on phenolic lignin breakdown products has yet to be assessed. As mentioned previously, PHA metabolism serves as a redox sink for the bacterium, and since *p*-coumarate has more reducing potential than acetate it was hypothesized in this study that *R. palustris* PHB production would increase using *p*-coumarate as a substrate.

The extraction process also accounts for a large portion of PHB production costs (Aramvash et al., 2018). Extraction and quantification of PHB generally entails depolymerization so that the monomers can subsequently be analyzed with gas chromatography or UV spectrophotometry. This typically involves solvent extraction via acidic methanolysis with chlorinated hydrocarbons, which is time consuming and includes toxic solvents that are nonrecyclable (Anis et al., 2013; Kunasundari and Sudesh, 2011).

For an alternative approach, there have been several studies that show staining bacteria with lipophilic stains followed by flow cytometry analysis yields precise quantification of PHB titers (Kacmar et al., 2006; Karmann et al., 2016; Lagares Jr. and Valverde, 2017; Li and Wilkins, 2020). Recently, Li and Wilkins (2020) developed a flow cytometry protocol for quantifying *Cupriavidus necator* PHB concentrations and cell counts for cultures that were grown on alkaline pretreated liquor from corn stover and stained with Nile Red (9-diethylamino-5-benzo[a]phenoxazinone). While Nile Red fluorescence had a good linear fitness for PHB

concentrations (R^2=0.9939), the protocol showed less linear fitness for cell counts (R^2=0.8614). Nile Red is a lipophilic fluorescent dye that has a high affinity for lipids, an excitation wavelength of over 528 nm, emission wavelengths between 570-630 nm, has been used in a variety of organisms from yeasts to animal cells, and can be used with cells expressing green fluorescent proteins (Wang et al., 2018). A possible explanation for the lower fitness for cell count correlation could be that Nile Red has a higher sensitivity to polarity, large spectrums for adsorption and emission, low relative fluorescent stability, and limited permeability capacity compared to other fluorescent dyes (Wang et al., 2018). Although studies have shown Nile Red to be ideal for evaluating some lipids, its efficiency varies across species and experimental parameters. BODIPY (a boron dipyrromethene dye) is a relatively newer lipophilic stain that also has a high specificity for lipids, but does not have some of the drawbacks of Nile Red. Unlike Nile Red, BODIPY is not sensitive to changes in polarity or pH, has better photochemical stability, lower background signal, and a narrower emission spectrum (Wang et al., 2018). Compared to Nile Red that emits red fluorescence, BODIPY emits green fluorescence in the 510-540 nm range and can thus be used with cells expressing red fluorescent proteins. Several studies have shown BODIPY to be a better fluorescent marker for lipids than Nile Red (Wang et al., 2018). Ultimately, factors such as spectral properties, incubation periods, and recalcitrance of cells can be an issue with lipophilic stains and flow cytometry analysis. For bacteria with many pigments such as *R. palustris*, the various pigments can absorb light at different wavelengths (Muzziotti et al., 2017). It is therefore essential to develop high-throughput quantification methods across different PHB-producing strains on various carbons sources. To the best of the author's knowledge, a flow cytometry method for PHB quantification from *R.*

palustris has never been reported, and this will also be the first general application of BODIPY with *R. palustris*.

Thus, this study aims to i) produce PHB from *R. palustris* on the lignin aromatic *p*-coumarate for the first time. ii) apply flow cytometry for quantification of PHB in this highly pigmented bacterium for the first time, and iii) optimize a flow cytometry method for accurate correlations of *both* PHB titers and cell counts using Nile Red and BODIPY 493/503 stains. A schematic of the overall experimental workflow is depicted in the graphical abstract. This approach addresses the two leading causes of increased PHB production costs (carbon and quantification), is the first reported analysis for *R. palustris* PHB production on the lignin aromatic *p*-coumarate, is the first reported *R. palustris* PHB quantification with flow cytometry, and delivers a multi-pronged optimization protocol for flow cytometry analysis.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

R. palustris was obtained from the American Type Culture Collection (ATCC) and cells were stored at -80°C in 20% glycerol until ready for further processing. Cells were taken from -80°C and streaked onto agar plates. For seed culture preparation, cells were inoculated into 50 mL of prepared media in 250 mL flasks. Seed culture media was photosynthetic mineral medium (PM) containing 10mM sodium bicarbonate, 15.2mM ammonium sulfate, 0.1457mM thiosulfate, and 50mM of both monopotassium phosphate and sodium phosphate dibasic

(Gibson et al., 1990). Sodium acetate (20mM) was also added for seed cultures. Cultures were grown aerobically in the dark in an AlgaeTron AG 230 (Photon Systems Instruments™) at 30°C with continuous shaking at 275 rpm with an Innova® 2000 (New Brunswick™). Cell growth was monitored by optical density at 660nm with a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific™).

2.2 Growth curves and PHB production

Cultures were diluted to an optical density of 0.2 for all experiments. *R. palustris* was grown anaerobically inside 14 mL Falcon™ Round-Bottom Polystyrene Tubes, which were completely filled and sealed to the second stop. Samples were grown at 30°C with 100 µE white light using an AlgaeTron AG 230 (Photon Systems Instruments™). The media used was the same PM used for seed cultures but with either 10mM sodium acetate or 1mM *p*-coumarate substituted for 20mM sodium acetate. Cell growth was monitored by optical density at 660nm with a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific™). To determine growth parameters, growth data were fitted to the modified logarithmic growth equation in Eq. 1 (Zwietering et al., 1990).

$$y = \frac{A}{\{1+\exp\left[\frac{4\mu}{A}(\lambda - t)+2\right]\}} \quad (1)$$

Where µ is the maximum growth rate, A is the maximum optical density, and λ is the lag time.

Cultures were nitrogen starved at mid-exponential growth (Figure 1) on either 10mM sodium acetate or 1mM *p*-coumarate in the anaerobic conditions described previously. Each culture tube was centrifuged at 3800 rpm for 30 minutes to generate a pellet, the supernatant was discarded, and the pellet was resuspended in PM that did not contain ammonium sulfate as a nitrogen source. Either 10mM sodium acetate or 1mM *p*-coumarate was added. Culture tubes were completely filled and sealed at the second stop.

2.3 PHB quantification via gas-chromatography mass spectrometry (GC-MS)

PHB extraction and quantification was conducted by an adapted protocol used previously (Juengert et al., 2018; Li and Wilkins, 2020). Each 14 mL culture tube was centrifuged to obtain a pellet, washed twice with 0.89% NaCl, and frozen at -80°C until ready for processing. External standards were created from serial dilutions of sodium 3-hydroxybutyrate (Sigma-Aldrich™). Cell pellets and standards were vortexed with 1 mL acidic methanol (15% H_2SO_4) and 1 mL chloroform, and methanolized at 105°C for 2.5 hours. Samples were allowed to come to room temperature, and 1 mL deionized water (ELGA PURELAB® flex 1 and 2 Water Systems, 18.2 MΩ*cm) was added and then vortexed. Once phase separation was complete, the methyl-ester derivatives were extracted from the lower organic phase with a Pasteur pipette. At least 200 µL was extracted into GC-MS vial inserts. GC-MS operating and processing parameters were the same as outlined in (Li and Wilkins, 2020), including software analysis. *2.4 Permeability and stain volume assay*

In order to decipher the ideal combination of permeabilizer and stain volume for flow cytometry analysis, a 96-well plate assay was developed. It has been shown that ethylenediaminetetraacetic acid (EDTA) and polyethylenimine (PEI) are efficient permeabilizers for other gram-negative bacteria, and thus they were chosen for the assay on *R. palustris* (Alakomi et al., 2005). To compare staining efficiency, solutions of either 10mM PBS, 40% ethanol + PBS, 50% ethanol + PBS, 1mM EDTA + PBS, 2mM EDTA + PBS, 1mM PEI + PBS, and 2mM PEI + PBS were assessed. PBS concentration was 10mM for all conditions. To decipher the optimal volume of stain, each solution was stained with either 3 µL, 6 µL, or 9 µL Nile Red or BODIPY 493/503. All conditions were run using live cells from the maximum PHB production on *p*-coumarate (approx. 0.41 g/L) to ensure the parameters would be sufficient for staining the largest titer. All conditions were conducted with biological duplicates. Both stains were created with equal concentrations of 1 mg stain per mL dimethyl sulfoxide (DMSO).

Samples were loaded into a 96-well plate with a E1-ClipTip™ Electronic Multichannel Pipette for refined accuracy (Thermo Scientific™). Precisely 100 µL of live cell cultures that had been grown on 1mM *p*-coumarate and nitrogen starved for seven days were placed into each well. The plate was centrifuged at 4200 rpm for 30 minutes to generate pellets, and the pellets were washed with 1mM PBS before proceeding with the permeability assay. After cells were washed, the pellets were resuspended with the multichannel pipette in the corresponding permeabilizers described previously. Samples were incubated with the permeabilizer for 15 minutes, and the corresponding amount of the required stain was placed into the sample. The samples were incubated in the dark for another 15 minutes with the stain and then centrifuged to generate pellets in each well. Samples were washed twice with 10mM PBS and resuspended

in precisely 100 µL of 10mM PBS. The 100 µL of each sample was diluted in 1 mL 10mM PBS inside 5 mL BD Falcon™ Round-Bottom Tubes for analysis via flow cytometry. All samples were run in a FACS Aria II flow cytometer (BD Biosciences, San Jose, CA) with a 488 nm wavelength laser. BODIPY signal was detected using a 530/30 bandpass filter, while Nile Red was detected with a 610/20 bandpass filter. Samples were analyzed with BD FACSDiva 8.0.1 software.

2.5 Analysis of live and frozen samples

Once the ideal permeability condition of 50% ethanol/PBS and 3 µL of either stain was established from the permeability assay, this combination of parameters was used to compare the stain efficiency of live and frozen samples. The remaining amount of the same live cell cultures that were used for the permeability assay that had not been processed were saved as pellets in -80°C. Samples were allowed to thaw for 10 minutes before processing and were run with at least two biological duplicates for each condition. Precisely 100 µL of thawed cell culture in 10mM PBS was centrifuged in an Eppendorf tube at 17,000 rpm for 4 minutes and resuspended in 50% ethanol/PBS solution for 15 minutes. Either 3 µL of Nile Red or BODIPY stains was mixed into the sample by pipetting and incubated for another 15 minutes in the dark. Samples were washed twice with 10mM PBS, resuspended in exactly 100 µL 10mM PBS, and transferred to 5 mL BD Falcon™ Round-Bottom Tubes that contained 1 mL 10mM PBS. The samples were immediately analyzed with the same flow cytometry parameters described in section 2.4.

2.6. PHB and cell count analysis

Previous analyses revealed that 50% ethanol/PBS with either 3 µL of Nile Red or BODIPY stains was ideal for staining efficiency, and that frozen cells still yield high stain efficiency similar to that of live cells. Thus, flow cytometry was conducted with frozen PHB samples across a range of concentrations (approximately 0.001 – 0.41 g/L) on cultures grown from 10mM sodium acetate and 1mM *p*-coumarate. All conditions were run with a minimum of biological duplicates, with the same processing procedures outlined in section 2.5. To compare cell counts and decipher if this staining method is efficient for cell number calculations, 25 µL of 5 µm AccuCount particles (ACFP-50–5, Spherotech Inc, IL, USA) of known number was injected into each sample before flow cytometry analysis. Particles were excited with a 561 nm laser and detected with a 780/60 bandpass filter during flow cytometry. Two unstained samples in PBS and two unstained samples in PBS + AccuCount particles were used as controls. The signals of these controls were removed to determine the regions of bacterial cells and stained cells (also known as gates). Cell number was calculated with Eq. 2 below:

$$Stained\ cell\ \# = (\#\ stained\ events/\#\ events\ of\ particles) * (\#\ particles\ added\ by\ volume/initial\ volume\ of\ culture\ sample) \quad (2)$$

The initial volume of culture sample was 100 µL, and the number of particles added by volume is supplied by the manufacturer and specific for each solution of AccuCount particles.

2.7 Fluorescence microscopy

After the permeability assay, fluorescence microscopy was conducted to image PHB-producing cells stained with either Nile Red or BODIPY. The optimal combination of permeabilizer and stain volume was 50% ethanol and 3 µL of stain for both Nile Red and BODIPY. Thus, 100 µL of live cells that were grown anaerobically on 1mM *p*-coumarate and nitrogen starved were subjected to these conditions. Cell cultures were centrifuged at 17,000 rpm in microcentrifuge tubes for 4 minutes and washed twice with 10mM PBS. The cell pellet was then resuspended in 100 µL of 50% ethanol/PBS solution, and incubated for 15 minutes. Either 3 µL of Nile Red or BODIPY was placed into the sample, mixed by pipetting, followed by another 15 minutes of incubation in the dark. Cell cultures were then washed twice with 10mM PBS. The washed cells were resuspended in 100 µL of 10mM PBS for imaging by a Nikon A1R confocal system (Thermo Scientific™). Nile red stained cells were subjected to a red fluorescent protein channel, while BODIPY stained cells were subjected to a green fluorescent protein channel. Each sample was subjected to a 100x lens with an additional 1.5x optical magnification set on the scope.

2.8 Statistical Analyses

Growth curves and GC-MS data represent the averages of biological triplicate data points (Figure 2). All error bars represent the calculated standard deviation from raw data based on the entire population. All flow cytometry experiments were run with biological duplicates (Figures 3-5). One-way Analyses of Variance (ANOVAs) were conducted for both Nile

Red and BODIPY data to compare differences in 40% and 50% ethanol solution, 3 µL and 9 µL stain volumes in the 50% ethanol/PBS solution data, and live vs. frozen stain efficiency ($p < 0.05$).

3. Results and discussion

3.1 Growth curves and PHB production results

It was hypothesized that nitrogen starvation would encourage PHB production since this has been shown with *R. palustris* previously (McKinlay et al., 2014). Furthermore, *R. palustris* was intentionally not subjected to nitrogen fixation to decipher PHB yields without having to incur that processing burden or increase in production cost. The anaerobic growth curve and growth constants for both 10mM sodium acetate and 1mM *p*-coumarate are shown in Figure 1. Cells grown on 10mM sodium acetate produced a PHB titer from 0.001-0.06 g/L over a five-day period (data not shown). The PHB production from 1mM *p*-coumarate with CO_2 fixation from 10mM sodium bicarbonate reached a maximum titer of approximately 0.41 g/L (or 3.25mM sodium 3-hydroxybutyrate) at six days of nitrogen starvation (Figure 1). Using Eq. 3 below for the conversion of 1mM *p*-coumarate and 10mM sodium bicarbonate to 3.25mM 3-hydroxybutyrate, this equates to approximately 68.4% carbon conversion efficiency.

$$\frac{0.013\ moles\ carbon\ produced\ in\ PHB}{0.019\ moles\ carbon\ invested}\ x\ 100 = 68.4\%\ carbon\ conversion \qquad (3)$$

Cells produced PHB without nitrogen fixation, which simplifies processing and could reduce production costs. Maximum production capacity could be due to a number of factors such as the amount of carbon source, the type of nutrient deprivation (i.e. nitrogen starvation), and fermentation conditions. Future efforts could focus on further increasing the carbon to nitrogen ratio, manipulating the size or quantity of the granules inside the cytoplasm via a synthetic biology approach, and engineering an optimized fermentation strategy to increase titer.

3.2 Permeability and stain volume assay results

It was hypothesized that incorporating a permeabilizer into the experimental workflow would increase staining efficiencies, thus optimizing PHB titer and cell count predictions using flow cytometry. Furthermore, it is likely that an efficient permeabilizer would reduce the amount of stain required, resulting in less washing and a reduction in false positives. Developing a method using a permeabilizer could also reduce the amount of time necessary for staining, which has been previously performed with a 30-minute incubation period (Li and Wilkins, 2020). Results of the permeability assay and stain volume analysis are depicted in Figure 2. Ethanol and PEI were the most efficient permeabilizers for staining *R. palustris* for both Nile Red and BODIPY. Since ethanol is far more common than PEI generally, it was chosen as the permeabilizer for further testing. A one-way ANOVA was conducted to decipher if the values for 40% and 50% ethanol solutions were statistically different from one another for Nile Red and BODIPY ($p < 0.05$). No significant difference was observed between 40% and 50% data.

Since data for the 40% solution showed larger standard deviations, the 50% ethanol solution was chosen as the permeabilizer for further PHB analysis. Furthermore, a one-way ANOVA was conducted to evaluate differences in staining efficiencies for 3 µL and 9 µL in 50% ethanol solution. No significant difference was observed between the amounts of stain for either dye, so 3 µL was used for further testing. Overall, the 50% ethanol solution with 3 µL of either stain was chosen as the ideal parameters for future analysis. Ultimately, the permeability method developed here helps increase staining efficiency compared to samples with no permeabilizer, optimizes efficiency with only a 15-minute stain incubation, and helps reduce the amount of washes and false positives by limiting the amount of stain required.

3.3 Analysis of live and frozen samples results

The results of live and frozen stain efficiencies are depicted in Figure 3. Frozen cells yielded similar staining efficiencies as live cells, with no confirmed statistical significance based on a one-way ANOVA ($p > 0.05$). However, frozen cells produced higher standard deviations. Some recommended remedies for this are to freeze the pellets in new vials after washing them to ensure there is no media present and to vortex the samples after adding the stains to ensure even staining. Since frozen samples still provided high staining efficiencies, subsequent cultures were nitrogen starved, pelleted after each day of nitrogen starvation, and stored in -80°C until ready for processing with flow cytometry. Nitrogen starved samples were saved over a seven-day period from both 10mM sodium acetate and 1mM *p*-coumarate to assess a range of PHB concentrations (0.001 – 0.41 g/L).

3.4 PHB and cell count analysis results

The linear regressions for PHB concentration and cell count predictions are shown in Figure 4. Linear models for PHB concentration and fluorescence intensity for both Nile Red and BODIPY yielded strong fitness (R^2 = 0.9495 and R^2 = 0.9928 respectively). Both stains yielded good fitness for cell count predictions as well (R^2 = 0.9383 and R^2 = 0.9967 respectively). It is notable that BODIPY was a better fit than Nile Red for both PHB concentration and cell count. This is in agreement with other studies that show BODIPY has a very high affinity for PHA granules (Rumin et al., 2015). Nile Red also has spectral properties that are very sensitive to polarity in the surrounding environment, which could contribute to the decrease in fitness as compared to BODIPY (Rumin et al., 2015). Adding ethanol as the permeabilizer would decrease the polarity, which could increase the overall absorption efficiency of Nile Red compared to other studies. *R. palustris* has a high pigment concentration, which could be yet another factor attributing to the variation in Nile Red staining. *R. palustris* can typically be found as a clump of rod-shaped bacteria, and it is hypothesized that the permeabilizer helped break up these clumps and make the cells more ideal for flow cytometry. This could be a factor for why Nile Red cell count predictions are a good fit for this optimized method compared to a previous study in which it was not (Li and Wilkins, 2020). Permeation of BODIPY is also faster than that of Nile Red typically, and thus it might be beneficial to use a slightly longer incubation time for Nile Red (Wang et al., 2018).

Unlike previously reported methods, this protocol is accurate for cell count and enables estimations of PHB production efficiency on a per cell basis. Moreover, this study shows that frozen cells can be used with this protocol, enabling more flexible analyses over time. Compared to the conventional PHB quantification method with methanolysis and GC-MS analysis that takes hours to perform and requires toxic chemicals, the method delivered here can be conducted in 30 minutes without organic solvents. Additionally, PHB is not depolymerized in the process, providing more flexibility for analysis or extraction. Since Nile Red and BODIPY can be used concurrently with cells expressing green and red fluorescent proteins respectively, this method also provides flexibility for future synthetic biology experiments. If cells expressing red or green fluorescent proteins is not an issue, it is recommended that BODIPY be used since it showed higher linear fitness than Nile Red for both PHB concentration and cell count linear regressions. Ultimately, producing PHB from such a metabolically versatile organism, using a renewable and cheap carbon source, and developing a quick and reliable quantification method helps promote the valorization of PHB. Our future efforts with *R. palustris* will focus on optimizing PHB production from *p*-coumarate and lignocellulosic biomass, analyzing PHB granules with transmission electron microscopy, engineering the overproduction of PHB, and upscaling fermentation.

3.5 Fluorescence microscopy results

Fluorescent microscopy was used to image *R. palustris* Nile Red and BODIPY stained cells that had been subjected to nitrogen starvation (Figure 5). As expected, cells showed rod shapes

with both stains. PHB granules are visible with a transmitted light image overlaid onto the fluorescent image, and the majority of cells show polarity to the granules. Variations in brightness are thought to be due to either different sizes of a single PHB granule inside each cell or several PHB granules within the cytoplasm, such that these would have picked up more stain and appear brighter. Variations would cause Nile Red to appear brighter red while BODIPY would be brighter green. Other imaging techniques, such as transmission electron microscopy, will be employed in the future to analyze the shape, polarity, number, and other factors of the PHB granules when *R. palustris* is grown on lignocellulosic biomass.

4. Conclusions

This study demonstrates for the first time that *R. palustris* produces PHB on the lignin aromatic *p*-coumarate and delivers an optimized process for quantifying both PHB concentration and cell count via flow cytometry. *R. palustris* produced approximately 0.41 g/L on 1mM *p*-coumarate after six days of nitrogen starvation, resulting in a 68.4% carbon conversion efficiency. The protocol created here is a multi-pronged optimization that allows for a quick and accurate assessment of both *R. palustris* PHB titer and cell concentrations using either Nile Red or BODIPY coupled with flow cytometry analysis.

Author Statement

Brandi Brown: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Writing - Original Draft, Resources, Data Curation, Visualization. **Cheryl Immethun:** Resources, Supervision, Writing - Review & Editing. **Mark Wilkins:** Resources,

Funding acquisition, Supervision, Writing - Review & Editing, Resources. **Rajib Saha:** Resources, Funding acquisition, Supervision, Writing - Review & Editing.

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Figure 1. (A) *R. palustris* anaerobic growth on 10mM sodium acetate and 1mM *p*-coumarate. Data have been fitted to a logarithmic growth model as described in section 2.2. (B) *R. palustris* PHB production on 1mM *p*-coumarate from nitrogen starved cells at mid-exponential growth. All data points represent averages of biological triplicates and error bars are the calculated standard deviation of the population.

Figure 2. Permeability stain assay results for Nile Red and BODIPY with *R. palustris*. Values are averages of the percent of staining from flow cytometry data using biological duplicates. Error bars are the calculated standard deviations based on the population.

Figure 3. Staining efficiency comparison of *R. palustris* live and frozen cells. Data represent averages of biological duplicates using the same cultures for both live and frozen cells. Error bars are the calculated standard deviation based on the entire population.

Figure 4. Fluorescence intensity of (A) Nile Red and (B) BODIPY stained cells compared to PHB production. (C) Nile Red and (D) BODIPY stained cell counts compared to the calculated cell counts per µL of sample.

Figure 5. Nile Red (A) and BODIPY (B) stained *R. palustris* cells under fluorescence microscopy. Cells were grown on *p*-coumarate, nitrogen starved, and contain PHB granules. For interpretation of the references to colors in this figure, the reader is referred to the web version of this article.

Figure 1

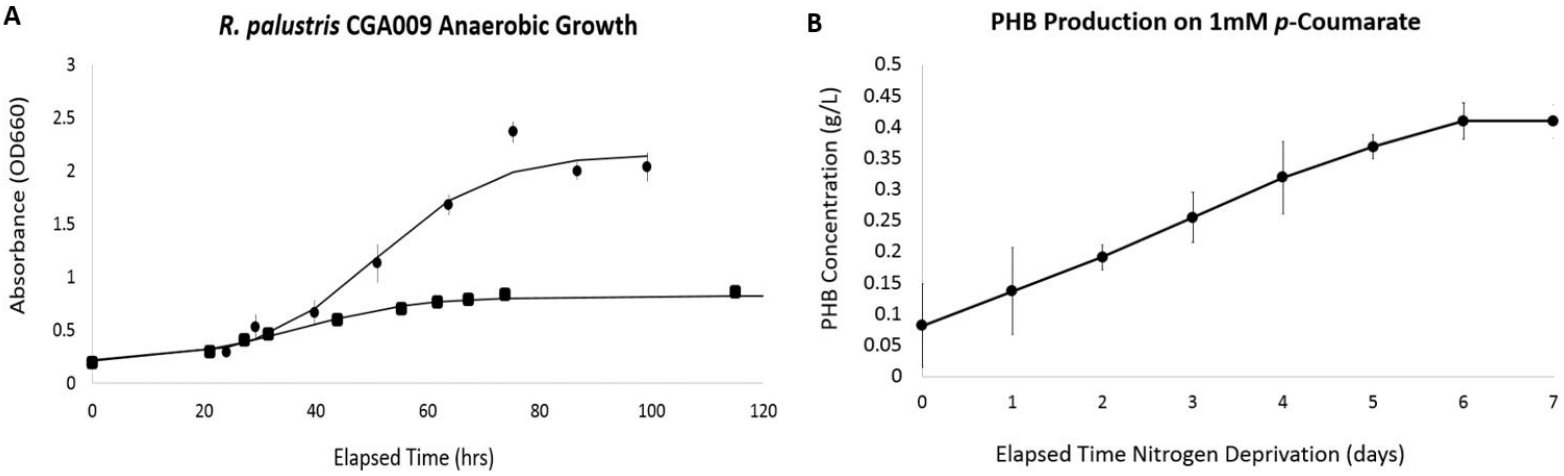


Figure 2

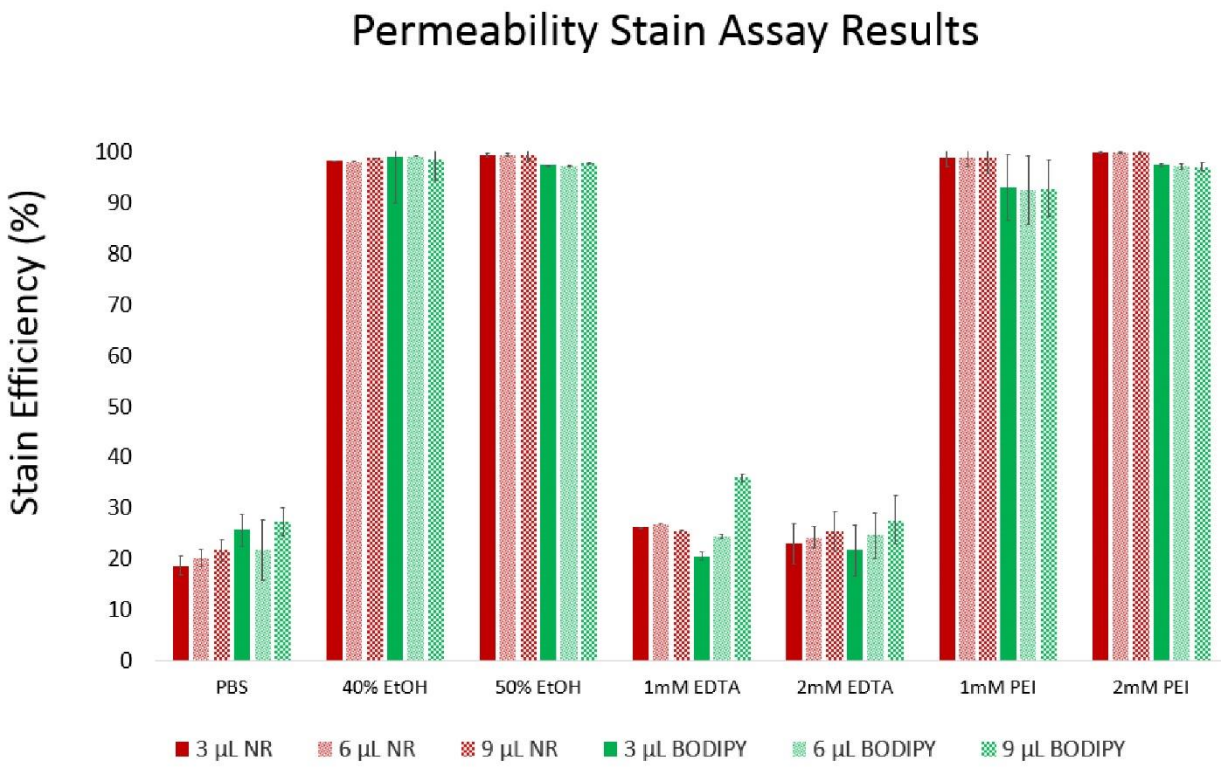


Figure 3

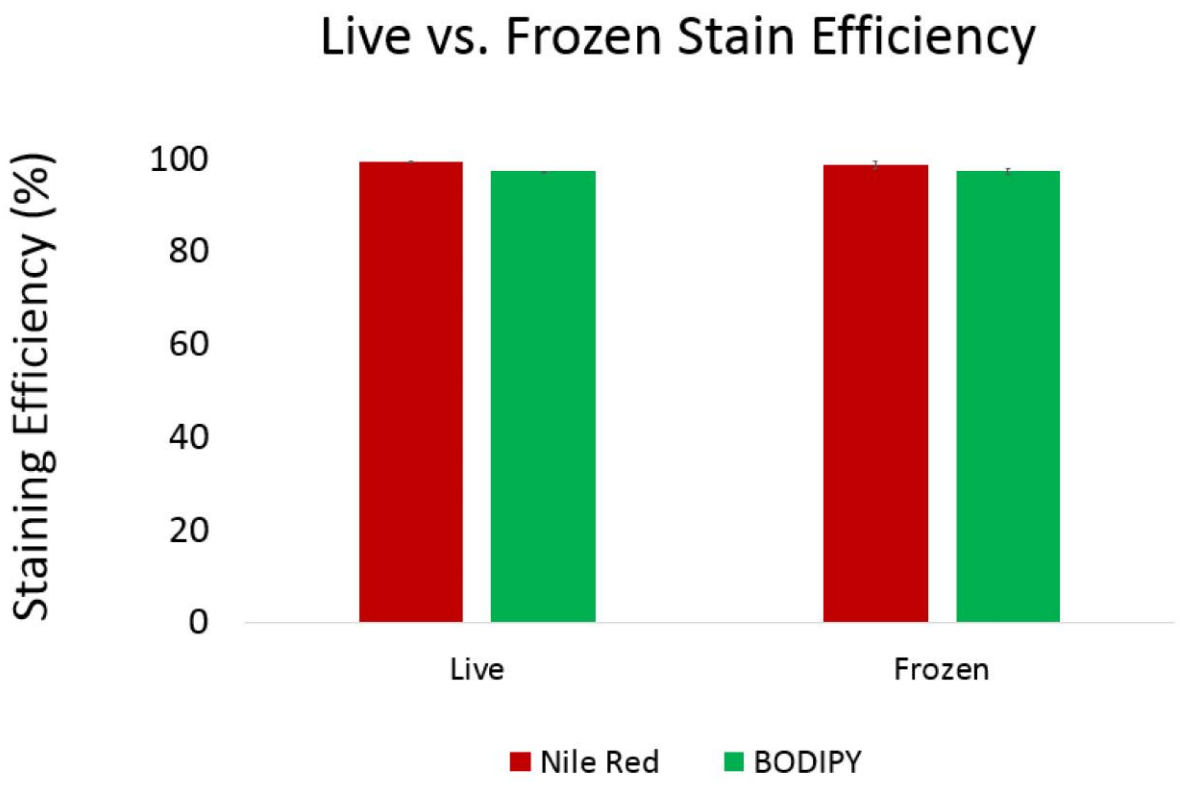


Figure 4

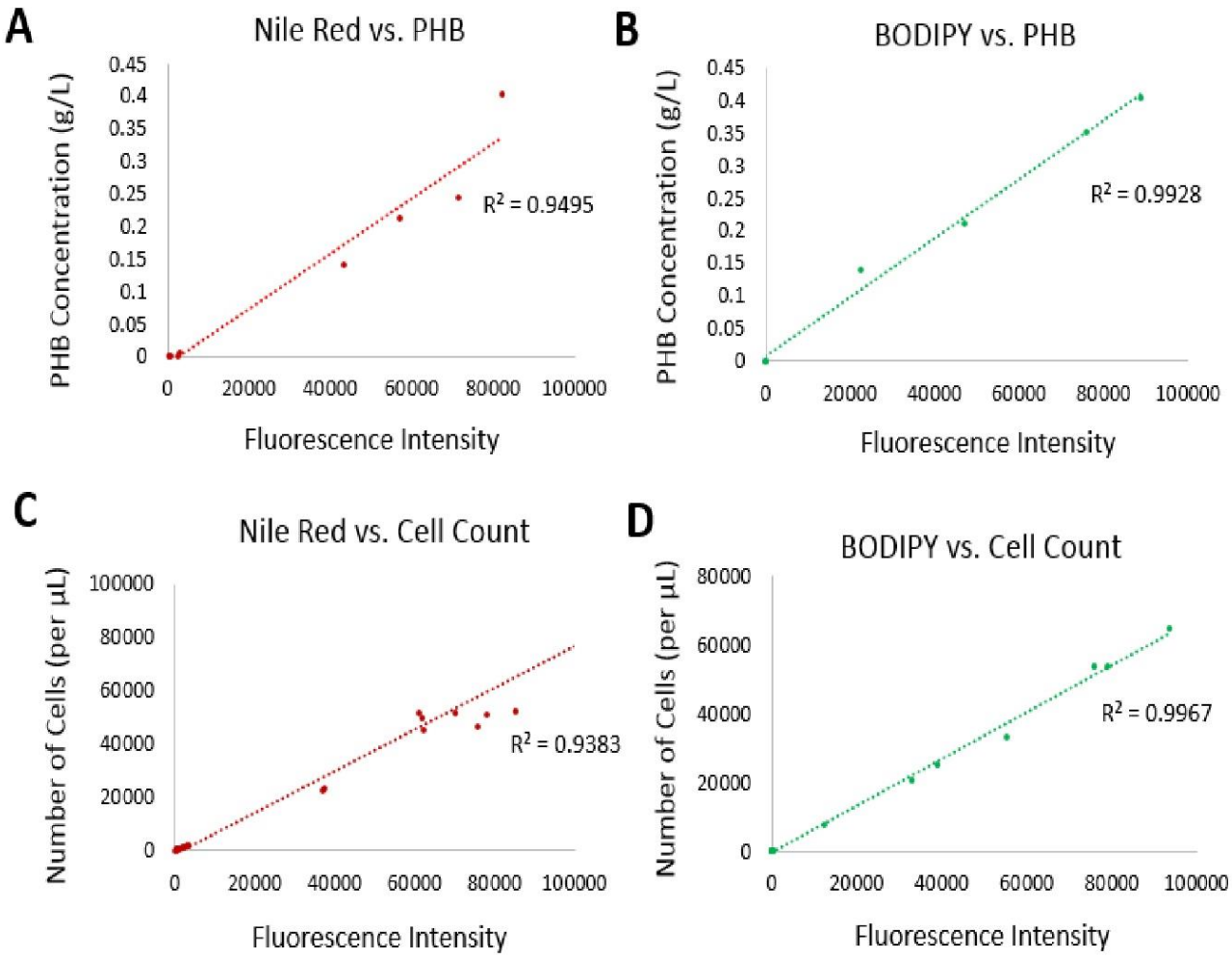
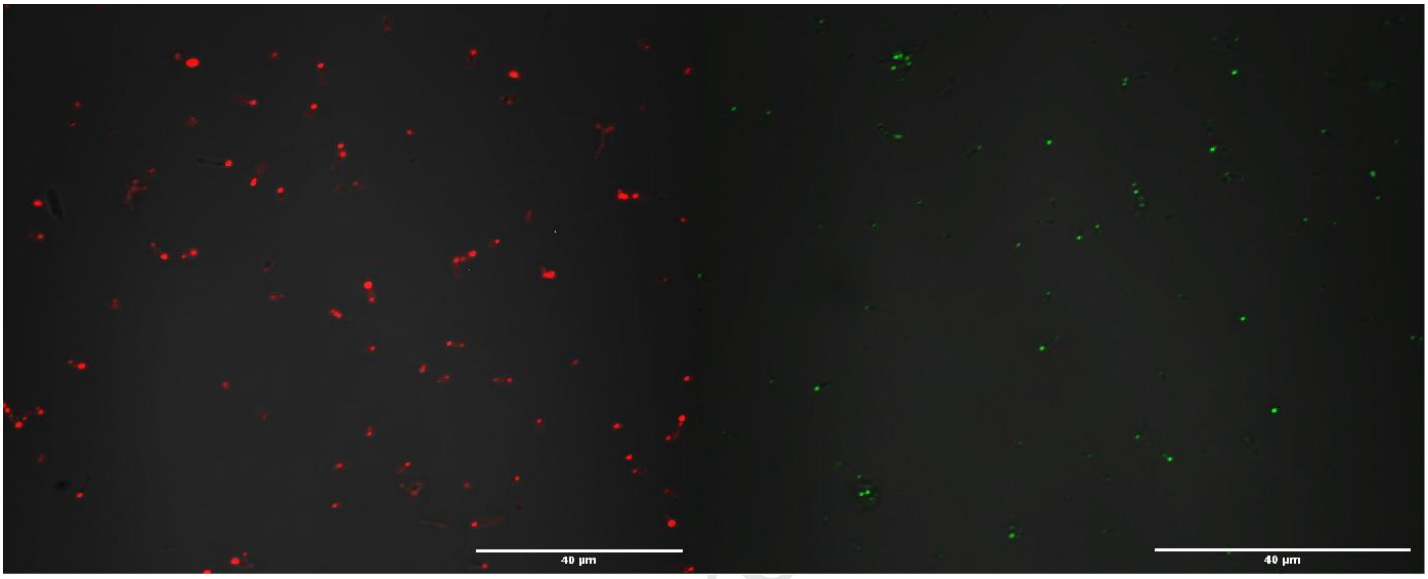

A
Nile Red vs. PHB
PHB Concentration (g/L)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
R² = 0.9495
0
20000
40000
60000
80000
100000
Fluorescence Intensity
B
BODIPY vs. PHB
PHB Concentration (g/L)
R² = 0.9928
Fluorescence Intensity
C
Nile Red vs. Cell Count
Number of Cells (per µL)
100000
80000
60000
40000
20000
0
R² = 0.9383
Fluorescence Intensity
D
BODIPY vs. Cell Count
Number of Cells (per µL)
80000
60000
40000
20000
0
R² = 0.9967
Fluorescence Intensity

Figure 5



Credit Author Statement

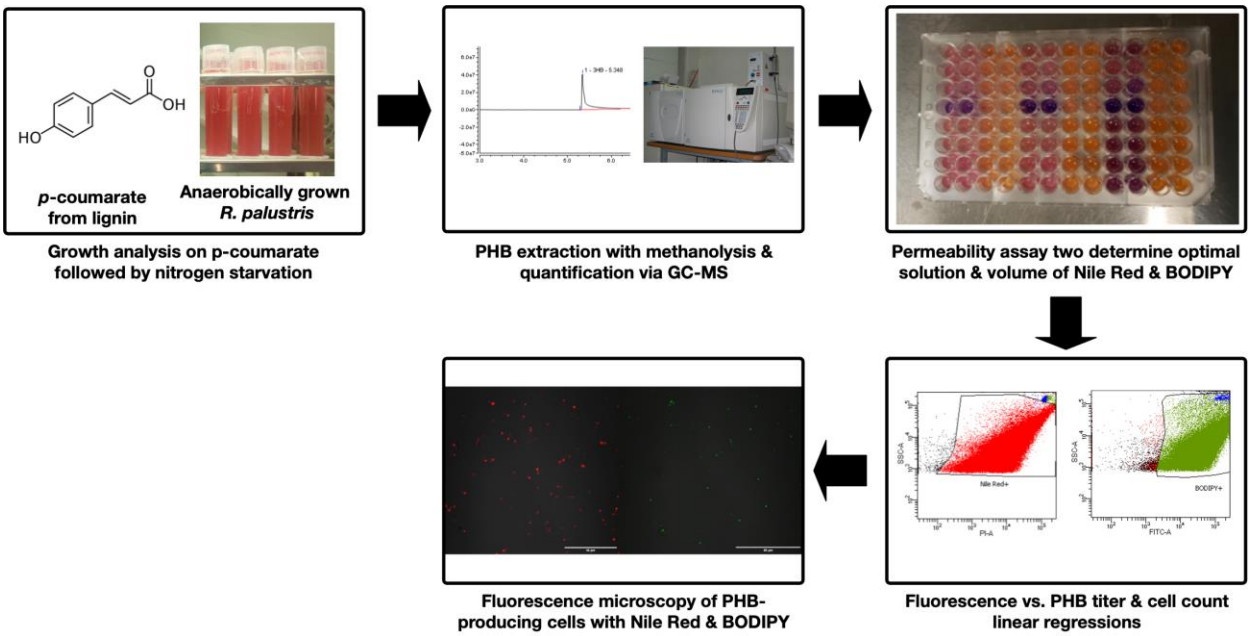
Brandi Brown: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Writing - Original Draft, Resources, Data Curation, Visualization. **Cheryl Immethun:** Resources, Supervision, Writing - Review & Editing. **Mark Wilkins:** Resources, Funding acquisition, Supervision, Writing -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

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

Rhodopseudomonas palustris CGA009 produced bioplastic from a lignin aromatic.

Polyhydroxybutyrate titer ranged from 0.08 to 0.41 g/L with 68.4% carbon conversion.

An optimized flow cytometry protocol using Nile Red and BODIPY stains was developed.

Polyhydroxybutyrate and cell count yielded strong linear fitness with fluorescence.

This study promotes the valorization of both lignin and polyhydroxybutyrate.