Title: Demonstration of Bioplastic Production from CO_2 and Formate using the Reductive Glycine Pathway in *E. coli*

Authors: Daria Fedorova[1], Roee Ben-Nissan[1], Eliya Milshtein[1], Ghil Jona[2], Nili Dezorella[3], Gil Feiguelman[1], Rostislav Fedorov[4], Aya Gomaa[5], Ariel B. Lindner[5], Elad Noor[1], Ron Milo[1]*

Affiliations:

[1]Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot, Israel

[2] Department of Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel

[3] Electron Microscopy Unit, Weizmann Institute of Science, Rehovot, Israel

[4] Heidelberg Institute for Theoretical Studies, 69118 Heidelberg, Germany

[5]Université Paris Cité, INSERM U1284, Center for Research and Interdisciplinarity (CRI), Paris, France

**Corresponding author. Email: ron.milo@weizmann.ac.il*

Abstract: There is a strong need to develop technologies that reduce greenhouse gas emissions. Recently, progress was made in engineering the model organism *E. coli* to grow using CO_2 and formate as its only carbon and energy sources using the reductive glycine pathway (rGlyP). Here, we use this engineered strain of *E. coli* as a host system for the production of polyhydroxybutyrate (PHB), a biologically derived and biodegradable plastic. We confirmed the production of PHB in this strain using Nile red fluorescent microscopy, transmission electron microscopy and GC measurements. Since formate can be efficiently generated from CO_2 via electricity, this study serves as a proof of concept for the emerging field of electro-bioproduction and opens a new avenue for the production of carbon-neutral chemicals.

Introduction

Currently, a large fraction of essential chemicals are produced from natural gas and petroleum. These petrochemicals (such as ethylene and acetylene) are used as a feedstock for the production of plastics, fuel, textiles, drugs, cosmetics and fertilizers. Using petrochemicals as a feedstock raises several important issues. Firstly, fossil fuels are a finite resource. Secondly, according to the IEA, chemicals are responsible for 18% of industrial CO_2 emissions or 2% of global greenhouse gas emissions.

Alternatives to petroleum based on sustainable feedstocks such as maize, sugar cane, or other plant-based sources have been proposed and have been gaining momentum in recent years. However, these methods have a large land footprint and compete with land used for producing food. This could have consequences for food security, especially in the context of a growing population and projected yield reductions from climate change (Hultgren et al. 2022). Indeed, according to the Agricultural outlook 2015-2024 released by OECD-FAO, more than 10% of cropland is being used for bio-production rather than food production. There is therefore a need to find another sustainable replacement for petrochemical production which is scalable, efficient to, and decoupled from human food production.

Poly-β-hydroxybutyrate (PHB) production in microorganisms

Poly-β-hydroxybutyrate (PHB) is a naturally biodegradable polyester, synthesized natively in various microorganisms as an energy and carbon storage under nutrient-depleted conditions. PHB is mainly used for food packaging, as well as biocompatible and biodegradable implants, medical scaffolds, and encapsulation of medicines (dos Santos et al. 2017; Nagarajan et al. 2021). PHB can be produced in *E. coli* heterologously by expression of a PHB operon. As shown in Figure 1, PHB production in *E. coli* requires expressing three non-native enzymes: PhaA (3-ketothiolase), PhaB (acetoacetyl-CoA reductase) and PhaC (PHB synthase). PhaA condenses two molecules of acetyl-CoA to form acetoacetyl-CoA, which is then reduced by PhaB to create (R)-3-hydroxybutyl-CoA (3HB). PhaC then uses 3HB as the monomer for polymerising poly-3-hydroxybutyrate (PHB). On an industrial scale, PHB is produced heterotrophically in fermenters by bacteria such as *Cupriavidus necator* or recombinant *Escherichia coli*, using glucose as a feedstock (G.-Q. Chen 2009).

As of today, the economic value of PHB production is questionable, mainly due to high feedstock prices which reach up to 50% of the total production costs (Halami 2008). Much effort has been invested in finding cheap raw materials for cost-effective PHB production (Koller et al. 2017) (Zhang et al. 2022). One solution is using waste materials such as wastewater, starch food waste, and lignocellulosic materials. Although this solution seems to be sustainable and bacteria can reach a high PHB content, there are limitations related to the pre-treatment procedures of the feedstock as these materials cannot be directly consumed by microorganisms. Additionally, this solution is not scalable as it relies on the availability, upstream processing, and the conversion efficiency of raw materials into feedstock.

It has been shown that cyanobacteria can reach a high PHB content per dry biomass ratio either when growing heterotrophically or autotrophically. In *Synechocystis* sp PCC 6803, 38% (w/w) was achieved (Panda and Mallick 2007) in the presence of fructose and acetate;. in *Spirulina* sp. LEB 18 44% (w/w) was achieved (Martins et al. 2014) while growing autotrophically. *Synechococcus MA19* reached a PHB content of 55% under phosphate-limited culturing conditions (Nishioka et al. 2001). Despite the high yield and inexpensive carbon source, this solution is not scalable due to techno-economic limitations of light-transparent photobioreactors necessary for the growth of the photosynthetic organisms described above. The problems of sunlight distribution and long-term fermentation still cause this direction to be uncompetitive.

C. necator is also capable of producing PHB natively from CO_2 as the sole carbon source (using aerobic respiration of H_2 or formate as the energy source) and reaching high PHB production levels - up to 80% of its dry cell weight. However, using *C. necator* as a platform for bioproduction of chemicals that are not native to their metabolism from CO_2 can be challenging due to complex metabolism, uncharacterized genome and limited availability of genetic toolkits as compared to *E. coli*. Therefore, we aimed to explore the alternative possibility of utilizing a synthetically engineered C1-feeding *E. coli* as a platform for PHB bioproduction. Naturally, this can serve as a proof-of-concept for bioproduction of other biochemicals, assuming their pathways can be readily introduced into this well-characterized model organism.

Engineering E. coli to feed on C1 compounds

Recently, progress has been made on several fronts in engineering *E. coli* to feed on C1 compounds (Kim et al. 2020; Gleizer et al. 2019; F. Y.-H. Chen et al. 2020; Keller et al. 2022).

Kim and co-workers (Kim et al. 2020) introduced the reductive glycine pathway (rGlyP), an efficient route for direct formate and CO_2 assimilation into central metabolism (Fig. 1, left). By combining rGlyP with laboratory evolution approaches, they successfully achieved formatotrophic growth of synthetically engineered *E. coli*. This strain had a doubling time of less than 8 hours and a biomass yield of 2.3 gCDW per mol of formate. However, strain growth is strongly coupled to formate as alternative electron donors would not be effective with rGlyP. Additionally, this strain lacks a CO_2 concentrating mechanism, requiring artificially high concentrations of CO_2 for biomass accumulation.

Gleizer and co-workers established complete synthetic autotrophy in *E. coli* by combining rational design and adaptive laboratory evolution (Gleizer et al. 2019). The evolved strain utilizes the reductive pentose phosphate cycle (rPP) cycle for all its biomass production, using CO_2 as the only carbon source and oxidizing formate for energy. This strain displays a doubling time of 18 ± 4 hours and has a formate-to-biomass conversion yield of 2.8 ± 0.8 gCDW/mol formate. Further engineering of such a strain may enable bacteria to grow at ambient CO_2 levels to become industrially relevant. Furthermore, since the autotrophic design separated the carbon fixation from the energy supply, the system is modular and can be adapted for the use of other electron donors (*e.g.,* methanol, hydrogen.)

In another study Keller and co-workers (Keller et al. 2022) transformed *E. coli* into a synthetic methylotroph that assimilates methanol via the energy-efficient ribulose monophosphate (RuMP) cycle. Methylotrophy wasachieved after evolving a methanol-dependent *E. coli* strain over 250 generations in continuous chemostat culture, using methanol as the sole source of carbon and energy. This strain has a strong coupling to methanol and cannot use any other energy source.

In light of the studies described above, we opted to incorporate a PHB production pathway from *C. necator* into the *E. coli* strain that was previously equipped with rGlyP (Kim et al. 2020). This choice was motivated by several factors, including the compatibility of this strain with both formate and CO_2, which aligns with the concept of formate economy (as elaborated in the discussion section). Additionally, this strain exhibits a faster growth rate and is amenable to straightforward genetic engineering, as compared to a synthetic autotroph.

Results

We used genes from the bacterium *C. necator* H16 which naturally produces polyhydroxybutyrate (PHB) as a carbon storage polymer that accumulates as granules in the cytoplasm. The three enzymes producing PHB from acetyl-CoA are located within a single operon. Our focus was on introducing the PHB production pathway into formate-utilizing strains of *E. coli* that rely solely on formate and CO_2 as their sources of carbon and energy using the reductive glycine pathway (rGlyP). It is a synthetic pathway that offers an efficient route for the direct assimilation of formate and CO_2 into the rest of metabolism (Fig. 1B). Notably, a variant of this pathway was identified in the anaerobic bacterium *Desulfovibrio desulfuricans* (Sánchez-Andrea et al. 2020). Building upon previous work by (Kim et al. 2020), who successfully achieved formatotrophic growth in the engineered *E. coli*, we sought to adapt this strain for production of PHB. This strain proved amenable to genetic manipulations, as it maintained fast growth on rich media. Furthermore, its doubling time on M9 minimal media, supplemented with formate and CO_2 as the only reducing and carbon sources, was less than 8 hours, sufficiently fast for our experimental setup. To produce PHB in *E. coli*, we introduced a pHB-4 plasmid (Liu et al. 2020) containing the PHB operon under the control of the auto-inducible promoter PthrC3 (Liu et al. 2020). This promoter was selected due to its advantages over traditional inducible promoters, such as pT7 (Anilionyte et al. 2018). It is an endogenous, short *E. coli* promoter that can auto-induce during early exponential growth, thereby enhancing efficiency and cost-effectiveness by eliminating the need for inducer chemicals in the media, which could potentially be used as a carbon source (e.g., in Arabinose-inducible systems).

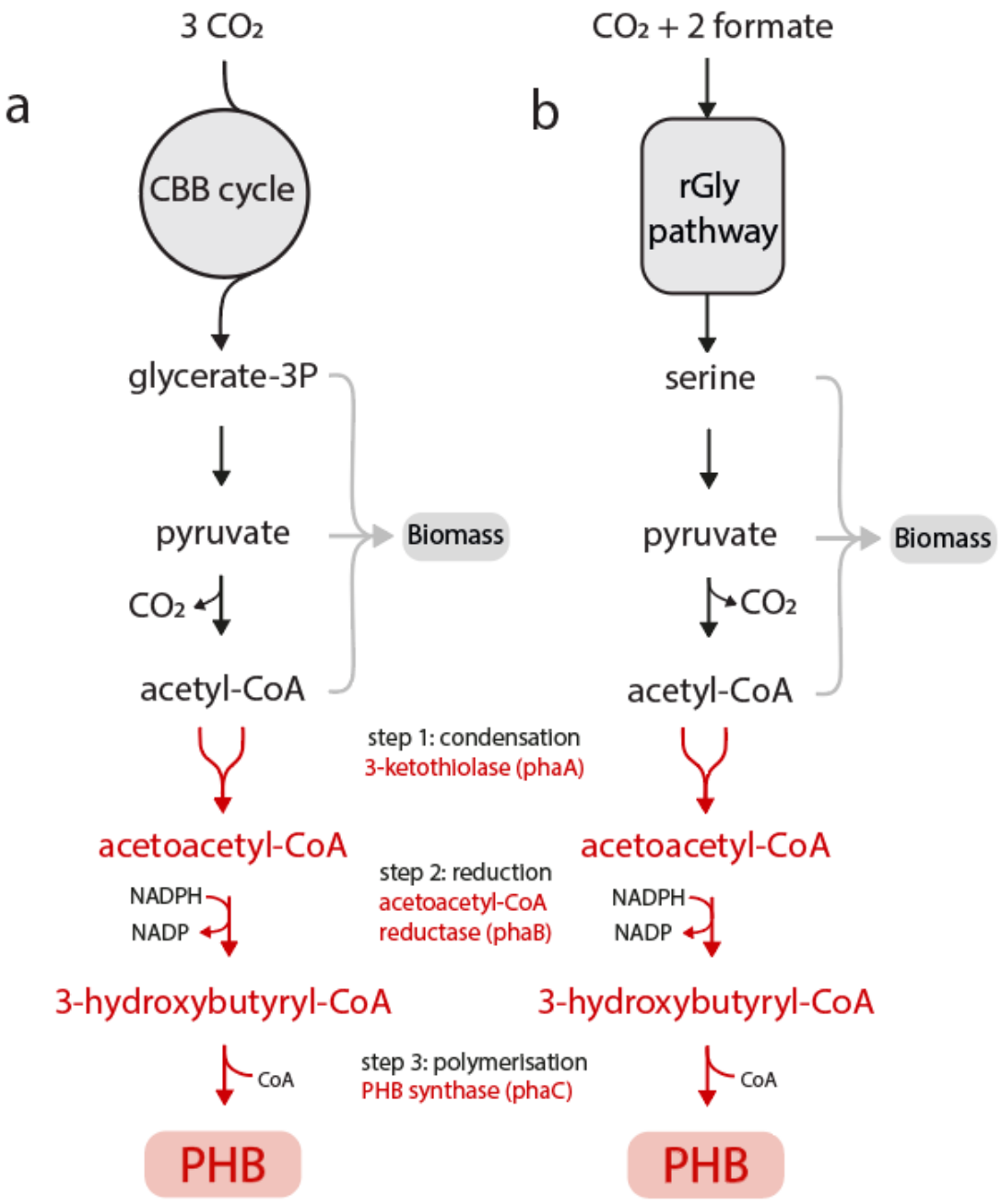

Figure 1: Metabolic pathway of PHB production in *C. necator (A)* and *rGlyP E. coli (B).*

(A) Metabolic pathway of PHB production in C. necator using the Calvin-Benson-Bassham (CBB) cycle for carbon assimilation. The PHB pathway is highlighted in red

(B) Metabolic design of rGlyP E. coli producing PHB. Formate and CO_2 are assimilated into carbon metabolism using the reductive Glycine pathway. PHB is produced from Acetyl-CoA by heterologous expression of the PHB operon (in red).

The formatotrophic strain carrying the PHB genes was cultivated in a fermenter using minimal media supplemented with 60 mM formate (as previously optimized (Kim et al. 2020)) and an overlay of 10% CO_2 in the headspace as the sole carbon source. The PHB-producing strain cultivated in the fermenter exhibited a prolonged lag phase when compared to the

non-producing strain, suggesting that the synthesis of PHB slows down bacterial growth (Fig. 2C). This was expected, as it is well-established that heterologous production of chemicals or proteins tends to slow down microbial growth (Glick 1995).

To initially confirm PHB production, we employed a widely-used technique: Nile red staining (Juengert, Bresan, and Jendrossek 2018) (Kavitha et al. 2016) (Jendrossek, Selchow, and Hoppert 2007). Nile red is a lipophilic stain that becomes fluorescent within lipid-rich environments, enabling detection through fluorescent confocal microscopy. Our findings revealed the formation of granules (in red) in a transformed strain of *E. coli* (Fig. 2D), in contrast to a strain lacking the PHB-producing plasmid. As Nile Red also fluoresces when binding to bacterial membranes, we complemented these results with transmission electron microscopy, which additionally reveals the morphology of the granules (Fig. 2E). To validate the chemical composition of PHB, we conducted Gas Chromatography with Flame Ionization Detection (GC-FID) analysis. Gas Chromatography is often used for the detection and quantification of PHB (Juengert, Bresan, and Jendrossek 2018) (Oehmen et al. 2005). The verification of PHB's chemical structure was substantiated by performing an analysis using conventional PHB standards (SupFig. 1). We could not quantify the yield of PHB using GC-FID due to the low levels of accumulation achieved (See Methods: Quantification of PHB by GC-FID).

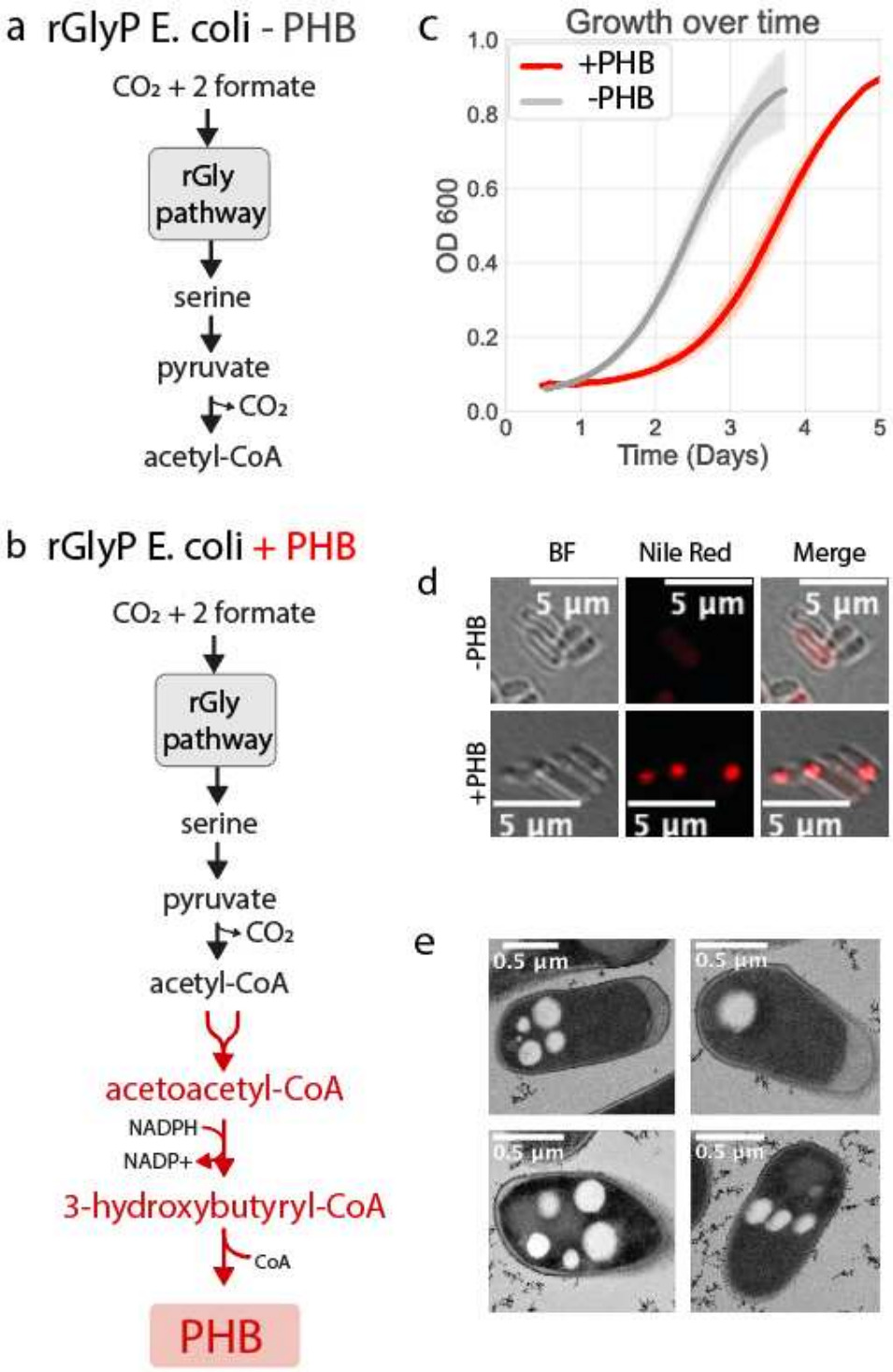

Figure 2: Validation of bioplastic production in *rGlyP E. coli*.

(A) *Metabolic design of rGlyP E. coli*

(B) *PHB pathway integrated in rGlyP E. coli*

(C) *Growth of isolated PHB producing strain in liquid M9 minimal media with 60 mM sodium formate and overlayed with a gas mixture of 10% CO_2, 90% air (in duplicates). Growth was carried out in DASGIP fermenters (150 mL working volume).*

(D) *Production of PHB in Formatotrophic E. coli as seen by confocal Nile red microscopy (red granules stained with Nile Red). Images were taken using a Nikon A1R HD25 confocal microscope, 60x. Images were cropped to show relevant fields of view and no post-processing was performed.*

(E) *Transmission electron micrographs showing PHB granules inside the PHB producing rGlyP E. coli strain.*

Discussion

It was previously demonstrated that *E. coli* can be engineered to feed on C1 compounds such as CO_2, formate or methanol (Kim et al. 2020; Gleizer et al. 2019) (Keller et al. 2022). In this study, we use one of these modified strains as a platform for the production of PHB. Through heterologous expression of the PHB producing pathway from *C. necator*, we successfully produced bio-plastic and confirmed its presence using analytical techniques.

The gold standard in the field is to perform 13C labeling experiments to ensure that all the carbon is derived from CO_2 and formate (Gleizer et al. 2019; Kim et al. 2020). Since this method had already been applied to demonstrate that the formatotrophic strain of *E. coli* synthesizes all of its biomass exclusively from CO_2 and formate in minimal media conditions, we did not sought to repeat the 13C labeling in the context of PHB production, as in our experiments we used the identical strain described in the study of Kim and colleagues, at identical growth conditions (in both the growth and in the production phases).

Decoupling and other strategies for improving the production capacities

A high yield of PHB is critical for its competitiveness against traditional synthesis of plastics as increased yield directly influences the cost-effectiveness of production, making PHB more economically viable and accessible. We will delve into various strategies aimed at maximizing PHB yields, thereby advancing its potential for commercial viability. We also note that a pipeline for rapid quantification of PHB will be instrumental for progress in these efforts.

As expected, the production of PHB is an additional burden on cell growth. It is known that production of value-added chemicals also leads to the accumulation of toxins and mutations (Borkowski et al. 2016). Expressing every gene continuously would demand a substantial energy expenditure for an organism, thereby making it more energy-efficient to tune gene expression only when they are needed. To overcome these problems in bioproduction and reduce metabolic stress, it is common to decouple biomass accumulation from chemical production. Current decoupling strategies include using inducible promoters triggered by physical (e.g. temperature) or chemical (e.g. pH, nutrient depletion, IPTG and galactose) signals. Recently, complex circuit designs for dynamic regulation, such as optogenetics (Lalwani

et al. 2021) and biosensing (Lo et al. 2016), were shown to successfully increase the product yield in several bioproduction strains. An additional decoupling strategy for synthetic or natively autotrophic organisms is to use a two-stage cultivation system (Koller 2018; Baumschabl et al. 2022). This consists of a heterotrophic growth phase to reach a high OD and an autotrophic growth phase for production. For formatotrophs, different strategies can be used to decouple growth from production including using formate inducible promoters (Hanko et al. 2020). A two-stage cultivation system is a more robust and reliable approach for sustainable bioproduction compared to constitutive production of a molecule of interest. Further investigation is warranted to explore the potential of a two-stage cultivation system using synthetic C-1 feeding organisms.

Another approach to enhance PHB accumulation is by increasing the growth rate of initial strains. This can be accomplished through laboratory evolution or genetic modifications. Another viable strategy is metabolic engineering, involving the modification of bacterial metabolic pathways to boost PHB production. Specific genetic manipulations include modulating phasin expression, which is a surface-binding protein of polyhydroxyalkanoate (PHA) granules that is encoded by the phaP gene. *E. coli* strain harboring novel phasins from a PHB high-producer strain, *Halomonas* sp. YLGW01 had enhanced PHB production by 2.9-fold. (Lee et al. 2023).

Additionally, the deletion of *ldhA, pta*, and *adhE* genes, which encode enzymes in competing metabolic pathways, can be employed to redirect resources toward PHB production. The composition of the growth medium can exert a significant impact on PHB accumulation. By regulating factors like nitrogen and phosphorus concentrations, it is possible to create stress conditions that prompt bacteria to store excess carbon as PHB. Restricting these essential nutrients compels the cells to prioritize PHB production (Wang et al. 2009).

Modifying the physical characteristics of bacterial cells can also positively influence PHB accumulation. Strategies may encompass altering cell size and shape, division pattern or structure of the cell wall (Wu et al. 2016) . For instance, by overexpressing *sulA*, a cell division inhibitor, in E. coli harboring PHB synthesis operon, engineered cells became long filaments and accumulated more PHB compared with the wild-type (Wu, Chen, and Chen 2016).

In vitro polymerization of PHB represents an innovative approach. Traditional PHB production occurs within bacterial cells, however, developing techniques for in vitro (outside the cell) polymerization of PHB has the potential to streamline the production process. This approach may offer greater control over the polymer's characteristics and reduce energy and resource consumption.

Electro-fermentation

A cost-effective bioproduction method could utilize microorganisms that grow using molecules containing only a single carbon atom (C1), such as carbon dioxide, formate or methane. Recently, (Leger et al. 2021) analyzed the efficiency of using solar energy for converting CO_2

derived from direct air capture into formate and other electron donors and using it as a feedstock for microbial production of protein for human consumption. The study found that photovoltaic-driven production of protein outperforms agricultural cultivation of staple crops in terms of caloric and protein yields per land area and could thus be used in the future to save limited land resources.

Electron carriers, such as formate and hydrogen, are essential in photovoltaic-driven production to shuttle electrons generated through water electrolysis to microorganisms that grow on C1 feedstocks. Formate can be produced by the electrochemical reduction of CO_2 with a current Faradaic efficiency of greater than 90% (Pletcher 2015; Taheri and Berben 2016). Compared to hydrogen, the electrochemical production of formate is less efficient, but it has the advantage of being soluble and non-flammable, and thus more convenient for large-scale usage. Compared to other C1 and C2 electron carriers (e.g. methane, methanol, or acetate), formate can donate electrons to cellular redox carriers more efficiently as it has a lower reduction potential. Exploring the bioproduction capabilities of organisms that use formate and CO_2 as an energy and carbon source might therefore be a solution for achieving sustainable production of goods such as plastics.

Engineering an *E. coli* strain with rGlyP to heterologously express the PHB operon, as described in this study, holds significant implications for the field of electro-fermentation as it serves as a pioneering proof of concept for synthetically engineering *E. coli* strains that can utilize formate and CO_2 as a feedstock for the production of plastics. More generally our study supports the potential of photovoltaic-driven bioproduction as a future sustainable production method. To the best of our knowledge, bioproduction in synthetically feeding C1 *E. coli* has not been reported before.

Materials and methods

Chemicals and reagents

Primers were synthesized by Sigma-Aldrich. PCR reactions were performed using KAPA HiFi HotStart ReadyMix or Taq Ready Mix. Glycine, Sodium formate, D-xylose, D-glucose, Nile-Red, PHB and 3HB standards were purchased from Sigma-Aldrich.

Bacterial strains

The synthetic formatotrophic rGlyP *E.coli* strain was described previously by (Kim et al. 2020) and used as host organism for bioproduction of plastic by transformation of this strain with recombinant plasmid/s.

Recombinant plasmids

pHB-4 was a gift from Kang Zhou (Addgene plasmid # 140957 ; http://n2t.net/addgene:140957 ; RRID:Addgene_140957)

Fermentation of E. coli strains

The growth of the various strains was done in a DASBox multi-parallel fermentation system (Eppendorf). Briefly, 10 ml starters from each strain were used to inoculate each bioreactor that contained 140 ml minimal M9 media supplemented with vitamin B1, trace elements, and 60mM filter sterilized sodium formate, to an OD 600 of ~0.05. Cultures were grown at 37 C with constant agitation and were supplemented with gas composed of 10% CO_2 and 90% air. The growth of the culture was constantly monitored along the when the cultured reached saturation the cultures were diluted to the initial OD by aspirating the appropriate volume of the culture and replacing it with fresh M9 media as above. Samples from each strain were routinely collected.

Growth tests of rGlyP E. coli strains

Method was adapted from (Kim et al. 2020)

Sample preparation for Nile Red confocal microscopy

Nile red is a fluorescent dye used to visualize lipid-like inclusions. This dye binds to PHB granules and can be detected by fluorescence microscopy. Nile red staining was performed according to the protocol detailed in (Juengert, Bresan, and Jendrossek 2018). Cells were harvested by centrifugation (6,000x). 4 µl of cells were taken and pipet mixed with 1 µl of Nile red (10 µg/ml in DMSO). Then, 1 µl of the stained cell suspension was put on a microscopic slide and covered with an agarose pad. The cells were imaged using a Nikon A1R HD25 confocal microscope. Nile Red fluorescence was detected with DU4 detector, excitation of 561.5 nm laser, and emission filter 593/46 nm. Fluorescence intensity was adjusted to the negative control and imaged with the SR Plan Apo IR AC 60x water immersion objective (numerical aperture 1.27). Images were cropped to show relevant fields of view.

PHB extraction, depolymerization and derivatization to methyl 3-hydroxybutanoate

Cell pellets were dried by lyophilization overnight, weighed, and transferred to an air-tight glass tube. 2 mL of 3% H_2SO_4 in methanol containing 190 µg/mL of benzoic acid as internal standard and 2 mL of chloroform were added to the pellet. PHB standards were prepared in chloroform and mixed 1:1 (v/v) with 3% H_2SO_4 in methanol containing benzoic acid. For PHB extraction, depolymerization by methanolysis, and derivatization, samples were incubated for 2.5 h in a boiling water bath. To initiate phase separation, 1 mL of ultra-pure water (MilliQ) was added to the solution and the samples subsequently incubated for 10 min in a sonication bath. The aqueous phase (upper) was discarded and the organic phase (lower) used for GC analysis.

Quantification of PHB by GC-FID

By acidic methanolysis processed PHB (3-hydroxybutyrate methyl ester) was analyzed by gas chromatography (GC 6850, Agilent Technologies, Basel, Switzerland) equipped with a 7683B Series injector coupled to a flame ionization detector (FID). A DB-WAX column (15 m x 0.32 mm x 0.50 µm; Agilent Technologies, Basel, Switzerland) was used for metabolite separation with helium as the carrier gas at a flow of 30 mL/min. The following temperature gradient was applied: 3.5 min from 90 °C to 230 °C, 3 min at 230 °C, 1.25 min to 90 °C, 2.5 min at 90 °C. 1 uL of sample was injected. The split ratio was 2.0 and the detector temperature set to 270 °C. Peaks were confirmed by standards. Methyl benzoate was used as an internal standard to correct for methodological variation.

Electron microscopy

Samples for electron microscopy were routinely collected along the experiments and n cells were harvested. ells were then placed in an aluminum disc with a depression of 100 µm and outer diameter of 3 mm (Engineering Office M. Wohlwend GmbH), then covered with a matching flat disc. The sandwiched sample was high-pressure frozen using an EM ICE high pressure-freezing device (Leica Microsystems, GmbH, Germany). Frozen samples were dehydrated in a temperature-controlled AFS2 Freeze substitution device (Leica Microsystems). Substitution was performed in dry acetone containing 1% glutaraldehyde, 1% osmium tetroxide and 0.1% Uranyl Acetate at − 90 °C for 64 h. The temperature was gradually increased to −20 °C (2.9 °C/h) and then raised to 4° C (12 °C/h). The samples were washed five times with acetone and infiltrated for 4 days at room temperature in a series of increasing concentrations of Epon in acetone. After polymerization at 60 °C for 48 h, ultrathin sections (90 nm) were obtained using an EMUC7 ultramicrotome (Leica microsystems) and were mounted on formvar coated 200 mesh nickel grids. Sections were stained with Reynolds lead citrate and examined using a Thermo Fisher Scientific Tecnai T12 transmission electron microscope operating at 120 kV. Digital electron micrographs were acquired using a bottom mounted TVIPS TemCam-XF416 4k x 4k CMOS camera.

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