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AUTHOR(S):

Srisawat, Pisanee; Higuchi-Takeuchi, Mieko;  
Numata, Keiji

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# Microbial autotrophic biorefineries: Perspectives for biopolymer production

Pisane Srisawat<sup>1</sup> · Mieko Higuchi-Takeuchi<sup>1</sup> · Keiji Numata<sup>1,2</sup> 

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## Abstract

The use of autotrophic microorganisms to fabricate biochemical products has attracted much attention in both academia and industry. Unlike heterotrophic microorganisms that require carbohydrates and amino acids for growth, autotrophic microorganisms have evolved to utilize either light (photoautotrophs) or chemical compounds (chemolithotrophs) to fix carbon dioxide (CO<sub>2</sub>) and drive metabolic processes. Several biotechnological approaches, including synthetic biology and metabolic engineering, have been proposed to harness autotrophic microorganisms as a sustainable/green production platform for commercially essential products such as biofuels, commodity chemicals, and biopolymers. Here, we review the recent advances in natural autotrophic microorganisms (photoautotrophic and chemoautotrophic), focusing on the biopolymer production. We present current state-of-the-art technologies to engineer autotrophic microbial cell factories for efficient biopolymer production.

## Introduction

Within this past decade, petroleum fuel inadequacy, together with global warming due to greenhouse gas emissions (e.g., CO<sub>2</sub>), has motivated scientists to search for a new and renewable resource for commodity chemical production. CO<sub>2</sub> gas has high potential as a renewable carbon source when utilized through an autotrophic system. The ability of autotrophic microorganisms to fix CO<sub>2</sub> and convert it into biomass and potentially valuable products is of particular interest in the field of green production within academia and industry. Autotrophic microorganisms require an energy source, either light (photoautotrophy) or an inorganic electron donor (chemolithoautotrophy), to incorporate CO<sub>2</sub> into biomass [1] (Fig. 1A). Autotrophic biorefineries, which are considered a more sustainable use of carbon and an energy-efficient alternative to heterotrophic cultivation, are used to convert organic carbon materials into similar products.

In recent decades, the bioproduction of commodity chemicals in autotrophic microorganisms has been well studied. For example, photoautotrophic cyanobacteria have been well optimized to enable the sustainable bioproduction of small molecules such as butanol, propanol, ethylene, and fatty acids. Such studies have been well documented in review articles elsewhere [2–5]. Recently, autotrophic microorganisms have also attracted great interest as promising platforms for biopolymer production. Biopolymers biosynthesized from microbial hosts are obtained through a green process for industrial-scale production. They are synthesized through an enzymatic process inside the cytoplasm or other cell organelles by harnessing biotechnological approaches. There have been several attempts to produce biopolymers from either model heterotrophs or autotrophs. However, research conducted on biopolymer production under autotrophic conditions is still limited due to production efficiency. In this review, we focus on the recent biotechnological aspects of autotrophic microbial cell factory development and provide insight into microbial-based biopolymer production.

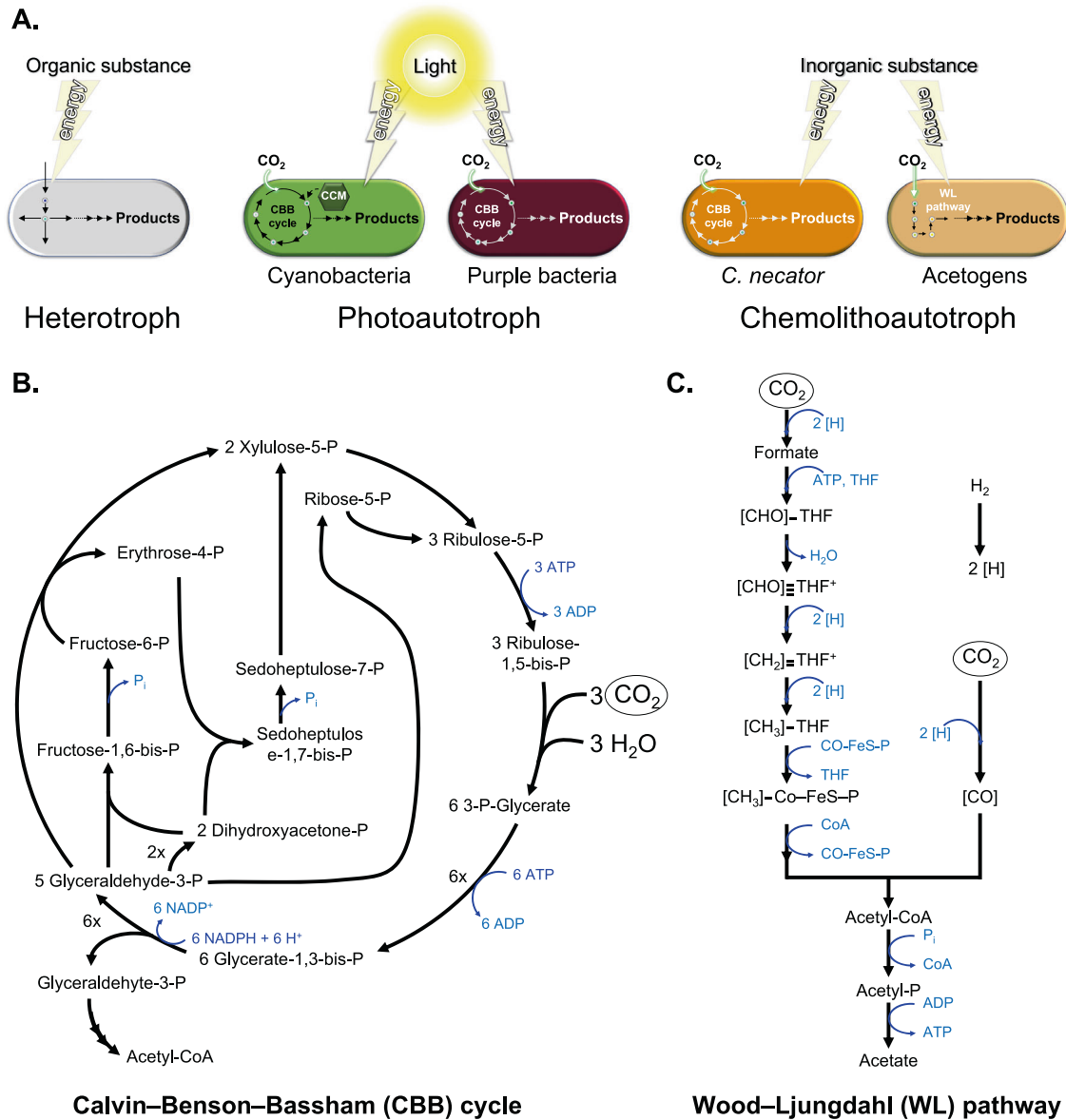
✉ Keiji Numata  
[keiji.numata@riken.jp](mailto:keiji.numata@riken.jp)

<sup>1</sup> Biomacromolecules Research Team, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

<sup>2</sup> Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Kyoto-Daigaku-Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

## Natural photoautotrophic hosts

Photoautotrophic microorganisms, which refer to both oxygenic and anoxygenic photoautotrophs, use light as an energy source to generate phosphate bond energy (ATP). Like plants and algae, oxygenic photoautotrophic



**Fig. 1** Summary of the different types of microbial trophic growth modes. **A** Heterotrophic microorganisms utilize organic carbon as an energy source and convert it into biomass and bioproducts. In contrast, autotrophic microorganisms harness energy from nature (e.g., light or inorganic electron donors) to incorporate an inorganic carbon source

microorganisms, such as cyanobacteria, can generate a reducing power and proton gradient to be used for ATP regeneration in the metabolic pathway by the water-splitting oxygen-generating photosystem I and II complexes. Light-induced electron transport generates energy and reducing power, which drives  $\text{CO}_2$  fixation via the Calvin–Benson–Bassham (CBB) cycle, which produces cellular materials and other valuable metabolites autotrophically (Fig. 1A, B). Moreover, cyanobacteria exhibit highly efficient solar energy capture and a  $\text{CO}_2$  concentrating mechanism to overcome negative effects from photorespiration [5]. Owing to their ability to perform photosynthesis and

( $\text{CO}_2$ ) into their metabolism. **B** The Calvin–Benson–Bassham (CBB) cycle is found in most photoautotrophic microorganisms, including some chemoautotrophs, such as *C. necator*. **C** The Wood–Ljungdahl (WL) pathway is a noncyclic carbonic fixation pathway that forms acetyl-CoA from  $\text{CO}_2$  and is found in acetogenic microorganisms

fix  $\text{CO}_2$ , cyanobacteria have ideal biosynthetic machinery for the sustainable production of various commodity chemicals and biofuels. Metabolic engineering and synthetic biology approaches have been employed to enable cyanobacteria to autotrophically produce commodity chemicals such as ethanol, 1-butanol, L-lactic acid, 2,3-butanediol, and polyhydroxyalkanoate biopolyester [6, 7]. Among the cyanobacteria strains, the freshwater species *Synechococcus elongatus* PCC7942, *Synechocystis* PCC6803, marine *Synechococcus* PCC7002, and *Anabaena* sp. PCC7120 are considered model strains due to their extensive study and the availability of genetic toolkits [8, 9].

Unlike oxygenic photoautotrophs, anoxygenic photolithoautotrophs have only one photosystem, which is not powerful enough to split water. Anoxygenic photolithoautotrophs can use both organic and inorganic electron donors (e.g.,  $H_2$  and sulfur) to generate reducing power to drive light-dependent  $CO_2$  fixation via the CBB cycle (Fig. 1A, B). Their versatile metabolism that enables aerobic, anaerobic, autotrophic, and heterotrophic growth, ability to adapt to extreme conditions, low maintenance cost, and high biomass yield make anoxygenic photolithoautotrophs ideal for the production of high-value chemicals [10, 11]. Anoxygenic purple nonsulfur photosynthetic bacteria such as the freshwater *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Rhodospirillum rubrum*, *Rhodospirillum rubrum*, *Rhodospirillum rubrum*, *Rhodospirillum rubrum*, and marine *Rhodovulum sulfidophilum* have been demonstrated to be bioproduction hosts for several value-added chemicals, including biopolymers such as poly- $\beta$ -hydroxyalkanoates and recombinant spider silk (Fig. 3A, B) [12–17].

This section provides the current developments in bioengineering photoautotrophic microorganisms, focusing on cyanobacteria and purple photosynthetic bacteria, as an autotrophic bioproduction platform related to biopolymer production. In addition, the current insights into the metabolic engineering of autotrophic metabolism in photoautotrophs for further biotechnological purposes will be reviewed.

### Recent developments of cyanobacteria as an autotrophic cell factory and their use for biopolymer production

Cyanobacteria have been proposed as an ideal photoautotrophic host for sustainable bioproduction. Recently, metabolic engineering techniques in cyanobacteria have rapidly developed, which has expanded the diversity of the bioproducts derived from the intracellular metabolites of cyanobacteria into a range of alcohols, organic acids, fatty acids, biofuels, and bioplastic precursors [6, 7]. Under photoautotrophic conditions, bioproducts are synthesized from the central intracellular metabolism of cyanobacteria, and the CBB cycle is the main contributor to this process. Thus, the use of metabolic engineering has been considered to improve  $CO_2$  fixation efficiency, endogenous carbon flux distribution, redox balance, and product conversion efficiency, which facilitates the development of cyanobacterial cell factories.

#### Increased $CO_2$ fixation in cyanobacterial cell factories

$CO_2$  is the primary carbon source in cyanobacteria. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the central enzyme that mediates  $CO_2$  fixation by catalyzing ribulose-1,5-bisphosphate (RuBP) carboxylation to generate

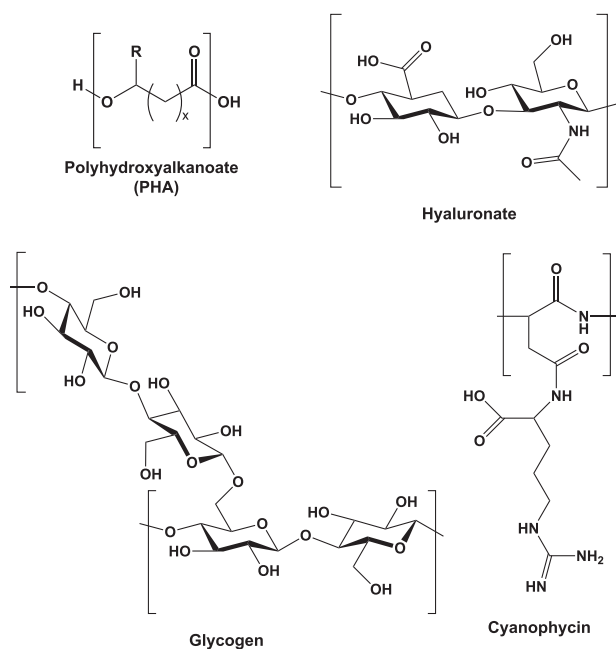
two molecules of 3-phosphoglycerate (3-PGA), which are further used in the CBB cycle. However, Rubisco is one of the most inefficient enzymes found in nature because it cannot differentiate  $O_2$  from  $CO_2$ . During photorespiration (using  $O_2$  as a substrate), Rubisco catalyzes the oxygenation of RuBP and releases one molecule of 2-phosphoglycolate (2-PG), which is toxic to cyanobacterial cells. To convert 2-PG into a nontoxic substance, cyanobacteria have applied rescue strategies to recycle two molecules of 2-PG to generate one molecule of 3-PGA while releasing  $CO_2$  and consuming ATP (Fig. 1B). These processes decrease the carbon fixation efficiency and energy conversion of the CBB cycle [18, 19].

Several strategies have been applied to engineer Rubisco, either to enhance its carboxylation activity or to suppress its oxygenation activity. However, limited success has been achieved due to the complexity of Rubisco. A direct evolution strategy has been employed to enhance Rubisco activity. Libraries of Rubisco mutants were generated and screened for their activities in an *E. coli* reporter strain that was engineered to grow dependently on the carboxylation function [20]. Using this *E. coli* screening system, a single mutation in the large subunit of *Synechocystis* sp. PCC 6803 Rubisco (RbcLF140I) significantly increased the carboxylation efficiency by 2.9-fold compared to the wild-type [21]. Further reintroduction of this Rubisco point mutation into *Synechocystis* sp. PCC 6803 improved the photosynthesis rate by ~55% [21]. Apart from improving Rubisco activity, the overexpression of CBB-related enzymes also increased the total  $CO_2$  fixation rate and heterologous ethanol production in an engineered *Synechocystis* sp. PCC 6803 [22].

The cyanobacterial  $CO_2$  concentrating mechanism (CCM) is one strategy to avoid competing oxygenase activity since the carboxylation reaction of Rubisco is restrained in the carboxysome structure. Manipulating the cyanobacterial CCM is also an approach to enhance  $CO_2$  fixation. Increasing the activity of the bicarbonate transporter helps improve the Rubisco  $CO_2$  fixation rate. It has been demonstrated that introducing an additional bicarbonate transporter resulted in a 2-fold increase in the growth and biomass of *Synechocystis* sp. PCC6803 [23]. Furthermore, the overexpression of two bicarbonate transporters (SbtA and BicA) in marine *Synechococcus* sp. PCC7002 enhanced biomass and intracellular glycogen accumulation by 50% and increased extracellular carbohydrates up to 3-fold [24].

#### Biopolymer production in cyanobacteria

Glycogen is a branched  $\alpha$ -polyglucan (Fig. 2), one of the carbon biopolymers that naturally accumulates in cyanobacteria. Glycogen metabolism is the most important natural



**Fig. 2** Chemical structures of the biopolymers discussed in this review

carbon sink mechanism and cellular carbon reservoir, storing a large portion of carbon and energy from the CBB cycle. Thus, this process is a promising target to optimize the efficiency of cyanobacterial cell factories [25, 26]. Many studies have tried to manipulate glycogen metabolism to rewire the carbon flow to increase the production of other metabolites. However, disturbing glycogen metabolism results in weakened cellular physiology and diminishes metabolic robustness because glycogen metabolism is vital for cellular homeostasis [27]. Flexible and dynamic regulation tools seem to be essential for the engineering of glycogen metabolism. The theophylline-responsive riboswitch has been applied to control the gene expression of *glgC* (this gene encodes ADP-glucose pyrophosphorylase, which is the rate-limiting step of glycogen biosynthesis) in glycogen metabolism, resulting in a 300% increase in glycogen accumulation (approximately 70% of the cell dry weight, Table 1) and a slightly increase in the growth in the presence of theophylline in *S. elongatus* PCC7942 [28]. In the absence of theophylline, glycogen accumulation was significantly decreased, and cellular tolerance toward environmental stresses was weakened, confirming the importance of glycogen accumulation in cellular homeostasis [28]. In addition, the fast-growing cyanobacterium *Synechococcus* sp. PCC 7002 successfully demonstrated CO<sub>2</sub> bioconversion into hyaluronic acid, a natural biopolymer composed of N-acetylglucosamine and glucuronic acid units (Fig. 2). Heterologous overexpression of hyaluronic acid synthases from gram-negative *Pasteurella multocida* (encoded by *pmHAS* genes) together with metabolic alterations to impair the competitive route resulted in

the photoautotrophic production of hyaluronic acid in a quantity reaching 112 mg/L [29] (Table 1).

The other important carbon biopolymers are the polyhydroxyalkanoates (PHAs), a class of polyester consisting of (R)-hydroxyalkanoic acid units (Fig. 2). The most common PHA polymer produced in cyanobacteria is poly[(R)-3-hydroxybutyrate] (PHB). Unlike glycogen metabolism, which is conserved in all cyanobacteria, only a few cyanobacteria genera, such as *Nostoc*, *Arthrospira*, *Synechocystis*, and *Synechococcus*, naturally synthesize PHB as additional cellular carbon storage [30–32]. Unlike *Capriavidus necator*, the main PHA producer that naturally accumulates PHB at a high level (~70% of its biomass weight) [33], cyanobacteria accumulate PHB in a lower amount (~4.1% of its biomass weight) [34]. One of the differences between them is the form of PHA synthase (PhaC), the key enzyme in PHA biosynthesis. In cyanobacteria, PHB is synthesized by class III PHA synthase (PhaC), consisting of two subunits, PhaC and PhaE [35]. The *in vitro* specific activity of the class III PHA synthase (PhaCE) from *Synechocystis* sp. PCC 6803 has been demonstrated using cell-free synthesis, and it was revealed that the specific activity of PhaCE is comparable to that of PhaC (a class I PHA synthase, single subunit) from *C. necator* H16 [36]. These results suggest that the low PHA productivity of cyanobacteria is not due to PHA synthase activity but might be caused by other pathways related to PHA synthesis [36]. Several genetic manipulation strategies have been applied to enhance PHB production in cyanobacteria. The overexpression of the RNA polymerase sigma factor *sigE* in *Synechocystis* sp. PCC 6803 resulted in the alteration of the metabolic pathway starting from glycogen to enhance PHB production under nitrogen starvation conditions. PHB production reached 14 mg/L (1.4% of its biomass weight, Table 1), showing a 2.3-fold increase compared to the control strain [37]. In another study, overexpression of the endogenous *pha* genes for PHB biosynthesis using a high copy number vector in *Synechocystis* sp. PCC 6803 successfully enhanced PHB production up to 10.59 mg/L (7% of its biomass weight, Table 1), which showed a 12-fold increase in productivity compared to the control strain [38]. Furthermore, random mutagenesis using UV radiation has been employed to generate the high PHB-producing strain *Synechocystis* sp. PCC 6714. The resulting strain could produce PHB up to 37% of its biomass weight, which is a more than 2.5-fold increase in PHB production compared to the wild-type under photoautotrophic growth [39] (Table 1). The introduction of heterologous *phaCAB* genes from *C. necator* into the fast-growing cyanobacteria *S. elongatus* UTEX 2973, which does not have a natural PHB production pathway, yielded a strain that produced PHB up to 420 mg/L (16.7% of its biomass weight, Table 1) under photoautotrophic growth [40].

**Table 1** Summary of biopolymers and related chemicals produced by autotrophic microorganisms discussed in this review

Host	Modification	Product	Product content	Product titer	Ref
<i>S. elongatus</i> PCC7942	Theophylline-responsive riboswitch to control gene expression during glycogen biosynthesis	Glycogen	–	39.4 mg L <sup>-1</sup>	[28]
<i>Synechococcus</i> sp. PCC 7002	Heterologous overexpression of hyaluronic acid synthases from <i>Pasteurella multocida</i> and pathway manipulation	Hyaluronic acid	–	112 mg L <sup>-1</sup>	[29]
<i>Synechocystis</i> sp. PCC 6803	Overexpression of RNA polymerase sigma factor <i>sigE</i>	PHB	1.4% CDW	14 mg L <sup>-1</sup>	[37]
<i>Synechocystis</i> sp. PCC 6803	Overexpression of the endogenous <i>pha</i> genes using high copy number vector	PHB	7% CDW	10.59 mg L <sup>-1</sup>	[38]
<i>Synechocystis</i> sp. PCC6714	Random mutagenesis using UV radiation	PHB	37% CDW	735 mg L <sup>-1</sup>	[39]
<i>S. elongatus</i> UTEX2973	Introduction of heterologous <i>phaCAB</i> genes from <i>C. necator</i>	PHB	16.7% CDW	420 mg L <sup>-1</sup>	[40]
<i>Synechocystis</i> sp. PCC6803 ( <i>ApirC</i> )	Introduction of heterologous <i>phaCAB</i> genes from <i>C. necator</i>	PHB	61% CDW	–	[41]
<i>Synechocystis</i> sp. PCC6803	Replacing the nitrogen assimilation regulatory gene with its mutated variant	Cyanophycin	57.3% CDW	–	[47]
<i>Synechocystis</i> sp. PCC6803	Replacing the nitrogen assimilation regulatory gene with its mutated variant and culture optimization	Cyanophycin	45% CDW	~1 g L <sup>-1</sup>	[48]
<i>R. rubrum</i>	Syngas fermentation	PHA	20% CDW	–	[53]
<i>R. rubrum</i>	Introduction of heterologous genes encoding the transhydrogenase <i>PntAB</i> and <i>phaB1</i>	P(HB-HV))	5.1% CDW	–	[55]
<i>R. rubrum</i>	Introduction of genes encoding enzymes responsible for producing mel-PHA from <i>Pseudomonas putida</i>	P(3HD-co-3HO)	7.1% CDW	–	[56]
<i>R. rubrum</i>	Fermentation using syngas obtained from microwave-induced pyrolysis	PHA	16% CDW	–	[54]
<i>R. sulfidophilum</i>	Culture optimization	PHA	32.4% CDW	–	[59]
<i>R. sulfidophilum</i>	Culture optimization	PHB	33% CDW	–	[60]
<i>R. sulfidophilum</i>	Genome-wide mutagenesis	PHA	35% CDW	16 mg L <sup>-1</sup> h <sup>-1</sup>	[61]
<i>R. sulfidophilum</i>	Implemented the use of engineered nanogel particles into photoautotrophic cultivation	PHA	–	21.3 mg L <sup>-1</sup>	[62]
<i>R. sphaeroides</i>	Insertion of <i>phaA3</i> , <i>PhaB2</i> , and <i>phaC1</i> together with the deletion of <i>phaZ</i>	PHB	79% CDW	1.9 g L <sup>-1</sup>	[16]
<i>R. sulfidophilum</i>	Introduction of the MaSp1 gene from <i>Nephila clavipes</i>	Spider silk	–	52.3 mg L <sup>-1</sup>	[13]
<i>C. necator</i>	Engineering the CBB cycle and hydrogen utilization pathway	PHB	34% CDW	–	[77]
<i>C. necator</i>	Heterologously expressing the cyanophycin synthetase gene	Cyanophycin	5.5% CDW	–	[81]
<i>C. necator</i>	Heterologous construction of the synthetic pathway in PHB-abolished <i>C. necator</i>	1,3-Butanediol	–	2.9 g L <sup>-1</sup>	[66]
<i>C. coskatii</i>	Heterologous expression of the synthetic PHB pathway	PHA	1.2% CDW	–	[91]
<i>C. ljungdahlii</i>	Heterologous expression and fine-tuning the acetic acid reassimilation pathway	3-HB	–	3 g L <sup>-1</sup>	[92]



A recent strategy to maximize PHB production was developed using the *Synechocystis* sp. PCC 6803 *pirC* mutant strain  $\Delta pirC$  [41]. The PirC protein was found to play a role in glycolytic carbon flux in a P<sub>II</sub>-dependent manner to regulate cyanobacterial carbon flow [42]. By introducing heterologous *phaCAB* genes into *Synechocystis* sp. PCC 6803 ( $\Delta pirC$ ), the resulting strain produced PHB up to 61% of its biomass weight (Table 1), which represents an approximately 6.1-fold increase in PHB production compared to the wild-type under photoautotrophic growth [41].

In addition to carbon biopolymers, cyanobacteria also accumulate amino acid polymer granules intracellularly for nitrogen storage, known as cyanophycin (multi-L-arginylpoly-L-aspartic acid, Fig. 2). Cyanophycin is of particular interest in biotechnology as a source of polyaspartic acid, a biocompatible and biodegradable polymer used in several industrial, agricultural, and medical applications [43, 44]. In cyanobacteria, the cyanophycin accumulation pattern is correlated with the nitrogen fixation phases, in which nitrogenase enzymes are inactivated in aerobic environments [45, 46]. Therefore, cyanophycin is synthesized during low-light periods, during which aerobic photosynthesis does not occur, and is consumed during high-light periods [46]. Maximizing the accumulation of cyanophycin in cyanobacteria was demonstrated in an engineered *Synechocystis* sp. PCC 6803. By replacing the PII protein (which regulates nitrogen assimilation) with its mutated variant (PII(I86N)), arginine content is enhanced more than tenfold compared to its wild-type and maximizes the accumulated cyanophycin to reach up to 57.3% of its biomass [47] (Table 1). Furthermore, the cyanophycin production yield in this strain can be enhanced by applying the high-density cultivation technique, thus reaching up to 1 g/L [48] (Table 1).

### Recent developments in purple photosynthetic bacteria as an autotrophic cell factory and their use for biopolymer production

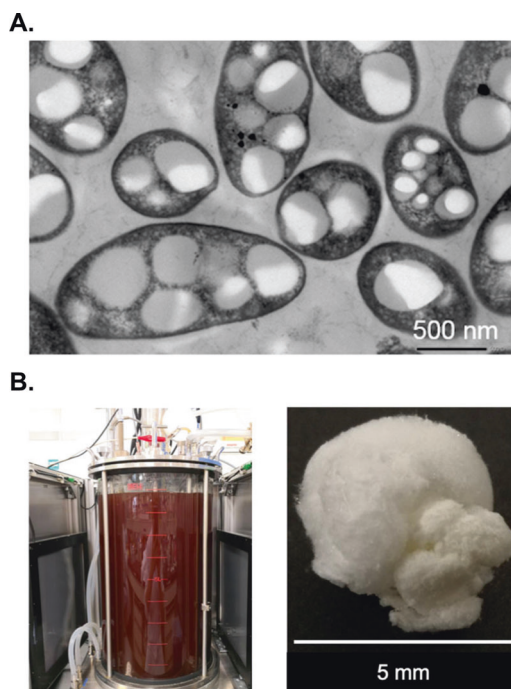
The metabolic versatility and ability of anoxygenic purple photosynthetic bacteria (PPB) to assimilate CO<sub>2</sub> are highly promising properties to develop them as autotrophic cell factories for the production of a wide-range of bioproducts. They have been extensively studied for use in wastewater treatment and resource recovery due to their robustness and versatile metabolism, which allow them to use a variety of carbon sources. However, compared to microalgae and cyanobacteria, PPB-based bioproduction has gained less attention due to the availability of genetic tools and genome data. Within the past few years, many studies have demonstrated the use of PPB as a phototrophic production platform for valuable bioproducts, including the heterologous production of plant-derived bioactive compounds

such as pinene (monoterpene) in *R. sphaeroides* [49], valencene (a sesquiterpene aroma compound) in *R. sphaeroides* [50], and botryococcene (a triterpene hydrocarbon) in *Rhodobacter capsulatus* [51].

### Biopolymer production in purple photosynthetic bacteria

PPB can fix CO<sub>2</sub> into cell material through an effective CO<sub>2</sub>-fixing CBB cycle. It has also been proposed as a photoautotrophic platform for biopolymer production. Some PPB strains have been found to naturally accumulate PHA biopolymers during carbon storage. The freshwater PPB *R. rubrum* is a well-studied PPB due to its ability to simultaneously produce PHA. Under phototrophic nitrogen-limited conditions, *R. rubrum* could accumulate PHA up to 50% of its biomass [12]. It has also been extensively studied for its ability to convert synthetic gas (syngas) to PHAs under photoautotrophic conditions. Despite the toxicity of carbon monoxide (CO) to most organisms, *R. rubrum* has the ability to utilize CO from syngas under anaerobic conditions as a sole carbon and energy source [52–54]. When *R. rubrum* is exposed to CO, carbon monoxide dehydrogenase (CODH) and CO-insensitive hydrogenase are induced to catalyze the oxidation reaction of CO into CO<sub>2</sub> and H<sub>2</sub>, and CO<sub>2</sub> is then assimilated into biomass and other metabolites through the CBB cycle. During syngas fermentation with acetate supplementation, *R. rubrum* accumulated PHA up to 20% of its dry weight under both light and dark conditions [53] (Table 1). The engineered *R. rubrum* S1, which harbored heterologous genes encoding the membrane-bound transhydrogenase (*PntAB*) from *E. coli* MG1655 and the *phaB1* gene coding for an NADPH-dependent acetoacetyl-CoA reductase from *Ralstonia eutropha* H16, successfully synthesized the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(HB-HV)] up to 5.1% of its dry cell weight (Table 1), with a 3-hydroxyvalerate (3HV) fraction of 28.3 mol% from syngas [55]. By further introducing genes encoding enzymes responsible for producing mcl-PHA from *Pseudomonas putida* into *R. rubrum* S1, a heteropolymer mainly consisting of 3-hydroxydecanoic acid and 3-hydroxyoctanoic acid [P(3HD-co-3HO)] was successfully obtained in up to 7.1% of its dry cell weight from artificial syngas [56] (Table 1). Moreover, *R. rubrum* was tested for its potential to utilize syngas obtained by microwave pyrolysis of household wastes as feedstock for PHA biosynthesis. By fermenting *R. rubrum* using syngas obtained from microwave-induced pyrolysis with trace amounts of acetate supplementation, PHA was obtained in up to 16% of its dry cell weight (Table 1) with approximately 37% CO conversion efficiency [54].

Apart from the freshwater PPB *R. rubrum*, marine PPB have also been proposed as ideal microbial hosts for sustainable bioproduction due to several potential advantages, such as metabolic versatility and high salt tolerance, which



**Fig. 3** **A** TEM images of the intracellular PHA granules in *R. visakhapatnamense* under nitrogen-limited medium; the figure is adapted from Higuchi-Takeuchi et al., *PLoS One*, 2016 [14]. **B** Production of the MaSp1-(6-mer) artificial spider silk from the marine purple bacterium *R. sulfidophilum* and a representative fiber; the figure is adapted from Foong et al., *Commun. Biol.*, 2020 [13]

can eliminate biological contamination during large-scale cultivation [57]. Marine PPB have been studied for their ability to naturally biosynthesize and accumulate PHA as intracellular granules [14, 58] (Fig. 3A). Among the tested strains, *Rhodovulum euryhalinum*, *Rhodovulum imhoffii*, *Rhodovulum visakhapatnamense*, and *Rhodovulum sulfidophilum* were reported to naturally accumulate some PHA under photoautotrophic culture conditions [14, 15]. In the group of marine PPB, *R. sulfidophilum* is the most studied for its ability to biosynthesize PHA and for its potential to be developed as a host for biopolymer production. Improving culture conditions to enhance PHA accumulation in *R. sulfidophilum* has been extensively studied. Salt and iron concentrations in culture have been studied in terms of their influence on PHA accumulation [59, 60]. The optimum iron concentration ( $\sim 1\text{--}2\ \mu\text{M}$ ) was found to be essential to promote cell growth and led to the highest PHA accumulation with a dry biomass content of 32.4 wt.% [59] (Table 1). Furthermore, a salinity of 4.5% was found to be the best stress-growth condition to reach the highest concentration of PHA in an *R. sulfidophilum* culture (820 mg/L) with a PHA dry biomass fraction of 33% [60] (Table 1). The *R. sulfidophilum* strain has been recently improved by employing genome-wide mutagenesis coupled with high-throughput screening [61]. With this method, the improved

strain showed a 1.7-fold increase in PHA production while also accumulating PHA faster than the original strain [61] (Table 1).

However, PHA production by *R. sulfidophilum* under photoautotrophic cultivation conditions remains highly challenging since photoheterotrophic conditions are preferable for PHA accumulation. The recent application of engineered nanoparticles to facilitate microbial cell factories is a fascinating approach. This newly developed cultivation method implementing the use of engineered nanogel particles in *R. sulfidophilum* photoautotrophic cultivation has been demonstrated [62]. This technique successfully enhanced PHA accumulation up to 157-fold compared to the control without nanogel particle supplementation [62] (Table 1). The benefit of using the engineered nanogel particle strategy is to enable PHA bioproduction where the genetic use of modified microorganisms for practical production is not allowed or is difficult due to biosafety regulations. However, this strategy is not meant to replace other strategies (such as genetic modification to engineer metabolic pathways) but rather to complement them to synergistically further enhance biorefinery efficiency.

### Genetic modification approaches toward biopolymer production in purple photosynthetic bacteria

A genetic modification strategy has also been employed to improve PPB strains in targeting the bioproduction of valuable compounds. Metabolically engineered *R. sphaeroides*, which disrupted a poly(3-hydroxyalkanoate) depolymerase (*phaZ*) gene along with overexpression of the PHB biosynthesis genes in specific isoforms (acetyltransferase, *phaA2*; acetoacetyl coenzyme A reductase, *phaB2*; and poly(3-hydroxyalkanoate) polymerase, *phaC1*), improved the PHB production yield up to  $1.88 \pm 0.08\ \text{g L}^{-1}$  ( $\sim 79\%$  of its biomass weight, Table 1) under nitrogen-limited conditions [16]. Furthermore, the highly efficient Cas9-based genomic DNA targeting system has been successfully employed to elucidate the essential genes in the PHB production pathway in the freshwater PPB *R. sphaeroides*. The developed tool was employed to elucidate genetic information in terms of PHB production in *R. sphaeroides* and to construct mutant strains with dramatically reduced PHB production capacity that did not affect growth [63]. This is one possible strategy to redirect the metabolic flow from PHB to other desired metabolites. In addition to the PHA carbon polymer, a spider silk protein polymer (Fig. 3B) was also successfully produced by introducing the MaSp1 gene from *Nephila clavipes* into *R. sulfidophilum* under photoautotrophic growth conditions [13] (Table 1). This study expands the possibility of using PPB to produce heterologous biopolymers other than those naturally synthesized.



## Natural chemolithoautotrophic hosts

As an alternative to light, chemolithoautotrophs use reduced inorganic compounds ( $H_2$  or  $NH_3$ ) as an energy source. One of the most promising candidates is the “Knallgas” bacterium *Capriavidus necator* (formerly *R. eutropha*), which is a hydrogen-oxidizing bacterium that can grow using a mixture of  $H_2$ ,  $O_2$ , and  $CO_2$  gases as substrates [64]. Under aerobic autotrophic growth conditions, *C. necator* fixes  $CO_2$  via the CBB cycle (Fig. 1A, B) and is capable of growing under ambient  $CO_2$  concentrations with a doubling time of 21 h [65]. It is considered a model chemolithoautotroph due to the relatively high availability of genetic engineering tools, which have been implemented to develop a biotechnological platform for commodity chemical production. *C. necator* can naturally accumulate an excess of the carbon-dense biopolymer poly[(R)-3-hydroxybutyrate] (PHB), which is a storage polymer and bioplastic precursor, in an amount equal to 70% of its biomass weight [33]. The carbon flux can be redirected by metabolic engineering to produce several value-added chemicals, such as branched-chain alcohols, isoprenoids, 1,3-butanediol, sucrose, and modified PHBs, along with a variety of commodity chemicals [66–69].

Another promising group of chemolithoautotrophs is the anaerobic acetogens, which are capable of growing anaerobically using  $H_2$  and  $CO_2$ . All acetogens fix  $CO_2$  using the reductive acetyl-CoA or Wood–Ljungdahl (WL) pathway (Fig. 1A, C). Unlike the CBB cycle, the Wood–Ljungdahl pathway is a noncyclic carbonic fixation pathway that forms acetyl-CoA from  $CO_2$ , and acetyl-CoA is ultimately converted into acetate [70] (Fig. 1C). Their high autotrophic flux to generate acetyl-CoA makes them attractive candidates for the autotrophic production of value-added chemicals. Apart from acetate, some acetogens naturally accumulate fermentative end products of industrial interest, such as ethanol, butanol, and 2,3-butanediol [71, 72]. Among the acetogens, *Acetobacterium woodii* and acetogenic *Clostridium* spp. such as *C. ljungdahlii* and *C. autoethanogenum*, are considered the most relevant candidates for further development as bioproduction platforms since genetic engineering tools are becoming more available for these genera than others [73]. This section reviews the recent developments and advances in the bioengineering of both *C. necator* and acetogens related to biopolymer production via an autotrophic metabolism. We also provide current insights into metabolic engineering for further biotechnological applications in chemolithoautotrophic hosts.

## Recent development with *C. necator* as an autotrophic cell factory and its use for biopolymer production

*C. necator* has been used for polyhydroxyalkanoate (PHA) production in the past few decades. The native biosynthetic

capacity of *C. necator* yields up to 70% of the dry cell weight under autotrophic conditions [74–76]. However, the growth of *C. necator* is relatively slow under ambient  $CO_2$  [65], which is the limiting factor for developing it as an autotrophic production host. Thus, research has been focused on improving the  $CO_2$  fixation efficiency by engineering the CBB cycle of *C. necator*. Using the combination approaches that introduced endogenous GroES/EL chaperons into the heterologous cyanobacterial Rubisco and fine-tuned the hydrogenase module in *C. necator*, both the autotrophic growth efficiency and PHB production were improved, with increases of 93.4% and 74.7%, respectively, compared to its parental strain [77] (Table 1).

The CBB cycle requires a high amount of ATP, which limits the biomass and production yields [18]. Recent research has proposed the replacement of the CBB cycle with the reductive glycine pathway (rGlyP), allowing *C. necator* to incorporate formate in an ATP-efficient route for  $C_1$  assimilation. By integrating heterologous rGlyP into *C. necator*, the growth yield was comparable to that of the native CBB cycle, demonstrating the practicability of using the heterologous inorganic carbon assimilation pathway in *C. necator* [65]. Although *C. necator* lacks the  $CO_2$  concentrating mechanism (CMM), it expresses four carbonic anhydrase-like enzymes to provide a sufficient amount of bicarbonate ions in the cytoplasm and a relatively  $CO_2$ -specific Rubisco variant [78]. This supports the strategy of improving  $CO_2$  bioconversion yield by transplanting a heterologous CCM into *C. necator*. Recently, heterologous expression of CCM has been accomplished in heterotrophs. The functional  $\alpha$ -carboxysome from *Halothiobacillus neapolitanus* was successfully transplanted into *E. coli* [79]. Furthermore, this design was used to develop a strategy for oxygen-sensitive enzyme encapsulation to construct a robust nanoreactor for hydrogen production in *E. coli* [80].

Furthermore, recent research has used genetic engineering to generate more tailored and versatile PHA-copolymers produced from  $CO_2$  and  $H_2$  to extend the use of *C. necator* for bioplastic production. Using approaches that combined the overexpression of engineered thioesterases (TEs) to produce fatty acids, fine-tuning the native PHA synthase PhaC and the addition of acrylic acid (a  $\beta$ -oxidation inhibitor) into *C. necator* showed the potential to genetically control the PHA-copolymer composition [69]. Apart from PHA, *C. necator* has been successfully used as an autotrophic host to produce the amino acid polymer cyanophycin. With heterologous expression of the cyanophycin synthetase gene (*cphA*) from *Synechocystis* sp. PCC6308 in *C. necator*, cyanophycin was successfully produced under autotrophic conditions with up to 5.5% of its dry cell weight [81] (Table 1). The *C. necator* mutant that is unable to biosynthesize PHB has been used for the synthesis of many commodity chemicals due to the large pool of acetyl-CoA

and pyruvate precursors. Recently, 1,3-butanediol (1,3-BDO), an essential precursor for synthetic rubber, was successfully produced autotrophically by an engineered *C. necator*. Constructing the (*R*)-3-hydroxybutyraldehyde-Co-(*R*)-3-hydroxybutyryl-CoA-dependent and pyruvate-dependent pathways in PHB-abolished *C. necator* together with reducing the flux through the tricarboxylic acid cycle enabled the engineered strain to produce up to 2.97 g/L 1,3-BDO from CO<sub>2</sub> [66] (Table 1).

### Recent acetogen autotrophic cell factory developments and their use in biopolymer production

Acetogens have great potential as a cell biofactory to convert C<sub>1</sub> gases, such as CO<sub>2</sub> and CO, into value-added chemicals using the WL pathway. However, their slow growth and low autotrophic production rate limit their industrial applications [82]. Research has focused on improving the C<sub>1</sub>-fixation efficiency of the WL pathway in acetogens to overcome these limitations. Overexpression of the four tetrahydrofolate-dependent enzymes, which have been considered a catabolic bottleneck of H<sub>2</sub>/CO<sub>2</sub> in the WL pathway [83], resulted in an increase in CO<sub>2</sub> fixation efficiency and enhanced acetate production of up to 50 g/L in *A. woodii* [84]. Due to the limitations of genetic tools, adaptive laboratory evolution has been implemented to improve the growth and tolerance of *E. limosum* under CO [85]. The resulting strain that evolved under CO over 150 generations significantly increased the microorganism growth rate and production of the target metabolite acetoin by 1.44- and 1.34-fold, respectively [85]. Moreover, a recent report showed the connection between the WL pathway and the glycine synthase-reductase (GSR) pathway in the CO<sub>2</sub> fixation activity of *Clostridium drakei* [86]. Functional cooperation of these pathways has been employed to enhance growth and the CO<sub>2</sub> consumption rate in the acetogen *Eubacterium limosum* under autotrophic conditions, which enhanced the growth rate by up to 1.4-fold and the acetate production rate by up to 2.1-fold compared to the parental strain [86].

Acetogens have been successfully employed as autotrophic hosts for the heterologous production of chemicals of industrial interest, such as acetone, isopropanol, butanol, and isoprene [87–90]. Moreover, acetogenic *C. coskatii* and *C. ljungdahlii* have been successfully used as hosts for PHB production [91]. PHB was produced up to 1.2% of the dry cell weight (Table 1) under autotrophic conditions in recombinant *C. coskatii* and *C. ljungdahlii* that expressed the synthetic PHB pathway containing the genes *thlA* (encoding thiolase A), *hbd* (encoding 3-hydroxybutyryl-CoA dehydrogenase), *crt* (encoding crotonase), *phaJ* (encoding (*R*)-enoyl-CoA hydratase), and *phaEC* (encoding PHA synthase) [91]. The efficient coproduction of three valuable chemicals,

isopropanol, ethanol, and 3-hydroxybutyrate (3-HB; a metabolic precursor of PHB biosynthesis), using synthesis gas (CO<sub>2</sub>/CO) was successfully engineered in autotrophic *C. ljungdahlii* [92]. By constructing the artificial isopropanol-producing pathway, which included the *thlA* (encoding thiolase A), *ctfAB* (encoding CoA transferase subunits A and B), *adc* (encoding acetoacetate decarboxylase), and *sadh* (primary/secondary alcohol dehydrogenase) genes, fine-tuned acetic acid reassimilation in *C. ljungdahlii* could be used to autotrophically produce up to 13.4, 3.0, and 28.4 g/L isopropanol, 3-HB, and ethanol, respectively, during gas fermentation [92] (Table 1).

To unlock the potential of acetogens for bioproduction and overcome the limitations of genetic tools, genome editing technology such as the CRISPR-Cas system has been utilized [93]. Recently, CRISPR interference (CRISPRi), a derivative of the CRISPR-Cas system that uses nuclease-deficient Cas9 (dCas9) instead of Cas9 to block the transcription of a target gene, has been used to downregulate phosphotransacetylase (*pta*) to redirect acetyl-CoA flux to enhance the production of 3-HB in *C. ljungdahlii* [94]. A similar approach was developed using the CRISPR-Cas12a system for efficient gene deletion and regulation in *C. ljungdahlii* by downregulating *adhE1*, an essential ethanol dehydrogenase gene responsible for ethanol synthesis, to restore the acetyl-CoA flux to boost the autotrophic production titer of butyric acid (a metabolic precursor of PHA biosynthesis) [95].

### Summary and perspectives

The rapidly changing climate due to greenhouse gas emissions necessitates new and renewable resources for commodity chemical production. The ability of autotrophic microorganisms to harness energy from nature, such as sunlight, to fix atmospheric CO<sub>2</sub> is an exciting avenue for future sustainable bioproduction to support a carbon-negative bioeconomy. This review comprehensively presents recent research regarding the use of autotrophic microorganisms to produce biopolymers. However, these processes/technologies are still in their infancy and need additional attention to revolutionize mass production for industry. Indeed, further research to identify the fundamental bottleneck of these autotrophic microorganisms using a synthetic biology approach will aid in the development of manipulations to enhance their abilities. One critical and challenging issue is the development of a high-density culture system, since cell growth and polymer accumulation always reduce the light transmission of cells and the exposure conditions of cell culture. For further biotechnological advances, we need to develop such technology.

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## Compliance with ethical standards

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Pisane Srisawat received her B.Sc. in Microbiology and M.Sc. in Biotechnology from Chulalongkorn University in Thailand, majoring in microbial biotechnology under the supervision of Professor Sirirat Rengpipat. She then received a MEXT scholarship to pursue M.Eng and Ph.D. degrees under the supervision of Professor Toshiya Muranaka from Osaka University in 2019, majoring in plant specialized metabolites and metabolic engineering. She worked as a post-doctoral researcher in the RIKEN CSRS in 2019 with Professor Keiji Numata, researching the development of a photoautotrophic production platform in photosynthetic purple bacteria. In January 2022, she moved to join Symbiobe Inc. as a research scientist and currently working to apply her knowledge in photosynthetic bacteria for large-scale production technology development. She enjoys various research topics in microbiology and is deeply interested in metabolic pathway engineering for the production of beneficial compounds to contribute her knowledge directly to society.



Mieko Higuchi-Takeuchi received her Ph.D. in Integrated Biosciences from the University of Tokyo. From 2005 to 2020, she was a postdoctoral researcher at RIKEN. From 2018 to 2020, she was a group leader of JST ERATO program. Her research interests are the biopolymer production utilizing photosynthetic microorganisms and the development of peptide-based transformation system for microorganisms. She especially focused on marine purple photosynthetic bacteria as host for biopolymer production and developed peptide-based transformation system.



Dr. Numata earned his Ph.D. (2007) with a thesis centered on enzymatic degradation and synthesis with hydrolases of biopolymers. He worked as a JSPS Postdoctoral Fellow for Research Abroad at Tufts University (USA). Dr. Numata moved to RIKEN as a Senior Scientist in 2010 to start up the Laboratory for Enzyme Research and also to investigate biosyntheses and material design of structural proteins and polypeptide. He has been a Team Leader (PI) of the lab since 2012 and Research Director, JST-ERATO Numata Organelle Reaction Cluster Project since 2016. Until now, he published about 200 international articles and received several awards including *ACS Macro Letters/Biomacromolecules/Macromolecules* Young Investigator Award, American Chemical Society (2020).