Bioresource Technology 394 (2024) 130266

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Gas fermentation combined with water electrolysis for production of polyhydroxyalkanoate copolymer from carbon dioxide by engineered *Ralstonia eutropha*

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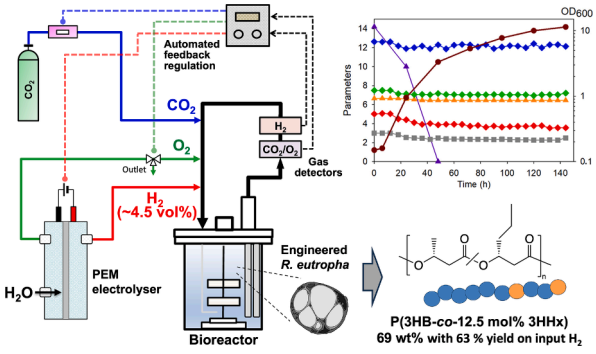
HIGHLIGHTS

- Recycled-gas closed circuit culture system combined with PEM water electrolysis.
- Safe gas fermentation using non-combustible gas mixture with low H_2 concentration.
- Automated feedback regulation of supply of H_2, CO_2, and O_2.
- 69.2 wt% of P(3HB-*co*-3HHx) with 63.1% yield on input H_2 by engineered *R. eutropha*.
- Overexpression of cytosolic carbonic anhydrase increased 3HHx composition.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Gas fermentation
Water electrolysis
Autotrophic production
Ralstonia eutropha
Polyhydroxyalkanoates
Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
Recycled-gas closed-circuit culture system
PEM electrolyzer

ABSTRACT

A recycled-gas closed-circuit culture system was developed for safe autotrophic cultivation of a hydrogen-oxidizing, polyhydroxyalkanoate (PHA)-producing *Ralstonia eutropha*, using a non-combustible gas mixture with low-concentration of H_2 supplied by water electrolysis. Automated feedback regulation of gas flow enabled input of H_2, CO_2, and O_2 well balanced with the cellular demands, leading to constant gas composition throughout the cultivation. The engineered strain of *R. eutropha* produced 1.71 g/L of poly(3-hydroxybutyrate-*co*-12.5 mol% 3-hydroxyhexanoate) on a gas mixture of $H_2/CO_2/O_2/N_2$ = 4:12:7:77 vol% with a 69.2 wt% cellular content. Overexpression of *can* encoding cytosolic carbonic anhydrase increased the 3HHx fraction up to 19.6 mol%. The yields of biomass and PHA on input H_2 were determined to be 72.9 % and 63.1 %, corresponding to 51.0 % and 44.2 % yield on electricity, respectively. The equivalent solar-to-biomass/PHA efficiencies were estimated to be 2.1–3.8 %, highlighting the high energy conversion capability of *R. eutropha*.

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https://doi.org/10.1016/j.biortech.2023.130266
Received 21 November 2023; Received in revised form 24 December 2023; Accepted 25 December 2023
Available online 28 December 2023

1. Introduction

Plastic materials have been intensively utilized in modern society owing to their superior properties such as high functionality and durability as well as low-cost production from petroleum, whereas their usage has resulted in severe accumulation of plastic wastes in natural land and marine environments due to their poor degradability. Biodegradable plastics have been expected to be a promising solution toward the environmental pollution caused by conventional plastics, while offering comparable performance and functionality (Możejko-Ciesielska and Kiewisz, 2016).

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters synthesized by diverse microbes as insoluble storage compounds within the cells. As PHAs are bio-based biodegradable polymers, some of them showing thermoplastic properties have been attracted as potential eco-friendly alternatives to petroleum-based plastics (Możejko-Ciesielska and Kiewisz, 2016). Poly((*R*)-3-hydroxybutyrate) [P(3HB)] is the most abundant kind of PHAs, however, it is not suitable for practical applications due to the high melting temperature (T_m) of 170-180℃ near from the thermal degradation temperature of ~200 °C, as well as the brittle property (~4% elongation to break). Many efforts have been therefore made for microbial production of PHA copolymers exhibitting better physical and mechanical properties than P(3HB) homopolymer. It has been reported that, for example, poly((*R*)-3-hydroxybutyrate-*co*–(*R*)-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] shows lower T_m and higher flexibility owing to the longer side chain in the 3HHx unit when compared with P(3HB) (Doi et al., 1995; Shimamura et al., 1994). In particular, the copolyester composed of 10 mol% 3HHx fraction shows T_m of 136 °C along with ~400 % elongation to break, making this copolyester as one of the most practical PHAs for industrial production.

The β-proteobacterium *Ralstonia eutropha* (*Cupriavidus necator*) has been often used as a useful host for PHA production (Reinecke and Steinbüchel, 2009). Several previous studies have focused on metabolic engineering of *R. eutropha* for biosynthesis of P(3HB-*co*-3HHx) from vegetable oils (Mifune et al., 2010, Arikawa and Sato, 2022) as well as from structurally unrelated sugars (Fukui et al., 2002; Insomphun et al., 2015; Zhang et al., 2019). In the case of using sugars as the carbon sources, the C_6-monomer (*R*)-3HHx-CoA was formed by elongation of the C_4-acyl-CoA intermediates via artificial reverse *β*-oxidation pathway, and then copolymerized with (*R*)-3HB-CoA by PHA synthase. One of the engineered strains produced 2.79 g/L of P(3HB-*co*-12 mol% 3HHx) (3.7 g/L of dry cell weight (DCW), 75 wt% of intracellular PHA content) from glucose (Zhang et al., 2019).

Meanwhile, the alarming rise in global CO_2 emissions and the pressing need to mitigate climate change have brought renewed attention to sustainable alternatives for petroleum-based materials. CO_2, considered as one of major greenhouse gases, is now being explored as a valuable resource for production of diverse compounds containing plastic materials. By harnessing CO_2 as a raw material, researchers aim to develop innovative processes that can simultaneously reduce CO_2 emissions and produce value-added compounds (Hasan et al., 2021; Zhang et al., 2022). This approach not only addresses the issue of waste management but also offers an opportunity to close the carbon cycle and achieve a more sustainable and circular economy.

R. eutropha was originally isolated as a hydrogen-oxidizing facultative chemolithoautotroph capable of growing on gaseous H_2 and CO_2 as energy and carbon sources, respectively. The CO_2 fixation is driven by Calvin-Benson-Bassham cycle, of which enzymes are encoded by two *cbb* operons on the chromosome 2 and megaplasmid pHG1 (Bowien and Kusian, 2002; Pohlmann et al., 2006). H_2 is oxidized by two oxygen-tolerant [NiFe]-hydrogenases (soluble and membrane-bound) and the electrons are utilized to supply energy and reducing equivalents required for the CO_2 fixation and anabolic metabolisms (Schwartz et al., 1998). The H_2-derived reducing equivalents are regenerated to the oxidized form through respiratory electron transfer chain. This bacterium prefers O_2 as the terminal electron acceptor under usual aerobic conditions, and the proton motive force generated by the oxygen respiration drives oxidative phosphorylation to acquire ATP for the autotrophic growth and diverse cellular functions (Cramm, 2009).

The ability of *R. eutropha* to fast grow on CO_2 is recently attracting increasing attention in the viewpoint of direct utilization of CO_2 for production of value-added compounds, such as isopropanol (Garrigues et al., 2020), 2,3-butanediol (Wei et al., 2023), and trehalose (Löwe et al., 2021). PHAs are one kind of the target compounds, because the wild strain H16 has been demonstrated to produce P(3HB) from a mixture gas of H_2, CO_2, and O_2 (Schlegel et al., 1961). The serious concern in the application of aerobic hydrogen-oxidizing bacteria is that gas mixtures of H_2 and O_2 are explosive in a wide range of H_2 concentration (4–75 vol% H_2 in air) (Zabetakis, 1965). The chemoautotrophic cultivation of hydrogen-oxidizing bacteria has been thus done by using gas mixture with H_2 concentration higher than upper explosion limit such as 80 vol%, however, the use of such the gas mixtures still arises a risk of explosion caused by gas leakage. These potential risks of the H_2-O_2 gas mixtures have hampered engineering studies on aerobic gas fermentation so far. The pioneering works were done by Tanaka et al., by which a recycled-gas closed-circuit (RGCC) culture system was developed for autotrophic cultivation of hydrogen-oxidizing bacteria with high cell density (Ishizaki et al., 2001; Ishizaki and Tanaka, 1990, 1991; Tanaka et al., 1995). They achieved efficient production of P(3HB) with 70 g/L (94 g/L of DCW, 74.4 wt% of PHA content) by *R. eutropha* H16 by circulation of a gas mixture of $H_2/O_2/CO_2 = 84:6:10$ vol%. It should be noted that the *R. eutropha* strains engineered for biosynthesis of P(3HB-*co*-3HHx) from sugars are able to produce the copolyester efficiently from CO_2. The production of 5.8 g/L of P(3HB-*co*-11.1 mol% 3HHx) (8.5 g/L of DCW, 68 wt% of PHA content) was obtained by 20 mL flask-scale autotrophic cultivation of the strain MF01ΔB1/pBPP-$ccr_{Me}J_{Ac}$-emd on a gas mixture of $H_2/O_2/CO_2$ = 80:10:10 vol% (Tanaka et al., 2021). Recently, the autotrophic cultivation of the related strain using the RGCC culture system achieved production of 34 g/L of P(3HB-*co*-11 mol% 3HHx) (40.1 g/L of DCW, 84 wt% of PHA content) (Tanaka et al., 2023). Although the PHA production was yet much lower than that obtained by fed-bach cultivation of another engineered strain of *R. eutropha* with palm karnel oil (196 g/L of P(3HB-*co*-3HHx, Arikawa and Sato, 2022), these results open the door for future production of the practical copolyester from H_2 and CO_2.

In order to facilitate the upscaling of chemolithoautotrophic production in industrial settings, it is necessary to mitigate the risk of hazardous incidents. Consequently, the utilization of a non-combustible gas mixture emerges as an optimal strategy for fostering the autotrophic bioproduction of chemicals on a large scale. Recently, safe autotrophic cultivation of *R. eutropha* was reported by using non-combustible gas mixture with H_2 concentration beneath the lower explosion limit (Miyahara et al., 2020; 2022). Continuous supply of a gas mixture of $H_2/CO_2/O_2/N_2$ = 3.8:7.3:13.0:75.9 vol% to *R. eutropha* H16 resulted in autotrophic production of 2.94 g/L of P(3HB) (3.31 g/L of DCW, 89 wt% of PHA) (Miyahara et al., 2022).

In the chemoautotrophic bioproduction using hydrogen-oxidizing bacteria, the raw material cost will be mainly occupied by H_2. This situation suggests that efficient utilization of the input H_2 by bacterial cells will be one of the most important issues to achieve the low-cost production. However, the yields and energy conversion efficiencies on H_2 to the target compound by hydrogen-oxidizing bacteria have not been well discussed so far. In this study, we developed a RGCC culture system using a non-combustible gas mixture with a low H_2 content for safe biosynthesis of P(3HB-*co*-3HHx) from CO_2 by the engineered strains of *R. eutropha*. A proton exchange membrane (PEM)-type water electrolyzer was applied for supply of H_2 and O_2, and automated feedback regulation of the gas flow rates allowed stable operation as well as evaluation of the bacterial performance. The results demonstrated high capability of *R. eutropha* for autotrophic growth and PHA production from CO_2 and electricity.

2. Materials and methods

2.1. Bacterial strains and cultivation

Bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* H16-derived strains were routinely cultivated at 30°C in a nutrient rich (NR) medium containing 1 % (w/v) of bonito extract (Kyokuto, Tokyo, Japan), 1 % (w/v) of polypeptone, and 2 % (w/v) of yeast extract in tap water. *E. coli* strains were grown at 37°C on a Lysogeny broth (LB) medium for general gene manipulation and trans-conjugation. Kanamycin (100 μg/mL for *E. coli* and 250 μg/mL for *R. eutropha* strains), tetracycline (12.5 μg/mL for *E. coli* and for *R. eutropha* strains), and/or ampicillin (100 μg/mL for *E. coli*) was added into the medium when necessary.

2.2. Construction of recombinant plasmids and strains

DNA manipulations were carried out according to standard procedures, and PCR reactions were performed with variants of KOD DNA polymerase (Toyobo, Osaka, Japan). The sequences of oligonucleotide primers used for PCR amplification are shown in supplementary material. pHRPPP, a broad host range plasmid based on pHRP309 (Parales and Harwood, 1993) harboring pSF1010 ori, was used as an expression vector compatible with a pBBR1-based vectors in *R. eutropha*. This was constructed by replacement of the *lacZ* region in pHRP309 by a *phaP1* promoter-multi cloning sites-T_{rrnB} region amplified from pBPP (Fukui et al., 2011), and replacement of the gentamicin resistance gene by a tetracycline resistance gene derived from pJB866 (Blatny et al., 1997). The *can* gene (*h16_A0169*), encoding one of carbonic anhydrases in *R. eutropha*, was amplified from the genomic DNA and then inserted into pHRPPP at the NdeI-BamHI restriction sites. The resulting pHRPPP-can was used to transform the P(3HB-*co*-3HHx)-producing strain of *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd (Zhang et al., 2019) by transconjugation through *E. coli* S-17 as described previously (Mifune et al., 2010).

2.3. Bioreactor and autotrophic cultivation

The *R. eutropha* cells pre-cultivated in an NR medium at 30°C for 15–16 h were subjected to chemolithoautotrophic PHA production in a nitrogen-limited minimal salt medium (MB) composed of 9 g/L (w/v) of $Na_2HPO_4{\cdot}12H_2O$, 1.5 g/L (w/v) of KH_2PO_4, 0.5 g/L (w/v) of NH_4Cl, 0.2 g/L (w/v) of $MgSO_4{\cdot}7H_2O$, and 1 mL/L of trace element solution (Kato et al., 1996) in deionized water along with appropriate antibiotic(s).

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or reference
Escherichia coli		
S17-1	*thi pro hsdR recA*; chromosomal RP4; Tra$^+$; Tmp^r Str/Spc^r	[Simon et al., 1983]
Ralstonia eutropha		
H16	Wild type	DSM 428
MF01	H16 derivative, Δ*phaC*::*phaC*$_{NSDG}$, Δ*phaA*::*bktB*	[Mifune et al., 2010]
MF01ΔB1	MF01 derivative; Δ*phaB1*	[Insomphun et al., 2015]
Plasmids		
pBBR1-MCS2	pBBR ori, *mob*, P_{lac}, *lacZα*, Kan^r	[Kovach et al., 1995]
pBPP-ccr$_{Me}$J$_{Ac}$-emd	pBBR1-MCS2 derivative, P_{phaP1}-*ccr*$_{Me}$-*phaJ*$_{Ac}$-*emd*$_{Mm}$, T_{rrnB}	[Zhang et al., 2019]
pHRP309	pSF1010 ori, *mob*, *lacZ*, Gm^r	[Parales and Harwood, 1993]
pHRPPP	pHRP309 derivative, P_{phaP1}, T_{rrnB}, Tet^r	This study
pHRPPP-can	pHRPPP derivative, *can*	This study

Ac, *Aeromonas caviae*; Me, *Methylorubrum extorquens*; Mm, *Mus musculus*.

The gas fermentation was carried out at 30℃ using a 2 L jar fermenter model BMZ linked with a digital controller DPC-3. The working volume and constant agitation using an impeller were set to 1 L and 400 rpm, respectively. Temperature, dissolved oxygen concentration, and pH of the medium were continuously measured by specific on-line probes (Able-Biott Co.,Ltd, Tokyo, Japan). Optical density of the culture broth was monitored using a turbidity probe iS-PRB-220 (Marubishi Co.,Ltd, Tokyo, Japan). A gas mixture of $H_2/CO_2/O_2/N_2$ with initial composition of 5:12:7:76 vol% (typically) in the 1 L-headspace was circulated using a diaphragm pump FD-2S equipped with a speed controller (IBS Co.,Ltd, Tokyo, Japan) and sparged back into the medium at a flow rate of 600 mL/min (0.6 VVM) set by using a rotameter (Kofloc Co.,Ltd, Aichi, Japan). The gas flow lines were assembled with hybrid gas-barrier tubes HGB (Aoi Co.,Ltd, Tokyo, Japan) and stainless steel fitting (model es-fit, Aoi). The gas concentration inside the system was continuously monitored with an $CO_2.O_2$ analyzer (galvanic cell sensor for O_2 and non-dispersive infrared absorption sensor for CO_2) model OFF-gas Jr. DEX-2562A (Able-Biott) and H_2 analyzer specifically developed based on semiconductor gas sensor (Able-Biott), both on bypass lines. The flow rates to the CO_2-O_2 and H_2 analyzers on the bypass lines were set to 300 mL/min and 100 mL/min, respectively, allowing rapid responses of the sensors. These detectors were calibrated by using air and a mixture gas of 6 vol% H_2, 20.6 vol% CO_2, and 73.3 vol% N_2.

CO_2 (99.9 % purity) was supplied into the system directly from a gas cylinder using a mass flow controller model 2000 (Fcon Co., LTD, Kouchi, Japan) with setting the maximum flow rate of 1.3 mL/min and the lower limit of 0 or 0.3 mL/min. O_2 and H_2 were supplied by a PEM electrolyzer (FC-R&D, Kanagawa, Japan) with a membrane electrode assembled with a platinum/carbon (Pt/C) cathode and manganese-iridium composite oxide (MnIrOx) anode (Nakamura et al., 2023). The electricity for the water splitting was supplied using a power supply model PMX18-5A (Kikusui Electronics, Kanagawa, Japan), where the maximum current density was set to 56 mA/cm^2. The excess O_2 was discharged by a three-way solenoid valve USG3-6-1-J (CKD Co.,Ltd, Aichi, Japan). A 20 L aluminum bag (GL Science, Tokyo, Japan) was used for avoiding an eventual accumulation of gas and maintaining the constant internal pressure of the system.

2.4. Analytical procedures

A small portion of the fermentation broth was subjected to measurement of the optical density at a wavelength of 600 nm (OD_{600}). 50 or 100 mL of the culture broth directly taken from the jar was centrifuged (8,000 *g*, 4℃, 10 min), and the harvested cells were washed with ice-cold milli-Q water and then freeze-dried. The cellular PHA content and composition were determined by gas chromatography (GC) after direct methanolysis of the dried cells in the presence of 15 % sulfuric acid (Kato et al., 1996). GC-2014 (Shimadzu, Kyoto, Japan) equipped with a capillary column InertCap 1 (30 m × 0.25 mm I.D., df = 0.4 μm, GL Science) and a flame-ionization detector was used for the analysis, as described previously (Kato et al., 1996). The gas composition was determined by GC analysis using GC-2014 equipped with a stainless packed column SHINCARBON-ST (8 m × 3 mm I.D.) (Shinwa Chemical Industries, Kyoto, Japan) and a thermal conductivity detector. The temperature range for the analysis started from 40°C and increased until reaching 200°C. The ammonium concentration in supernatant of the culture broth taken every 24 h was determined using an ammonia colorimetric assay kit (Fujifilm Wako Pure Chemical Co., Osaka, Japan).

2.5. Calculation of yields and energy conversion efficiency

The yields of cellular biomass and PHA on H_2 were calculated by the following formula with stoichiometry of the product determined from the ratios of the amounts of supplied gas corresponding to the gas consumed by the bacteria in the desired time interval,

$$Yield(\%) = \frac{Biomass\ or\ PHA\ (mmol) \times H_2\ coefficient\ in\ stoichiometry}{H_2\ (mmol)} \times 100$$

The thermodynamic electricity-to-biomass/PHA efficiency (η_{elec}) was calculated based on standard reaction Gibbs free energy (Δ_rG^o) of the product and input electric power as described by Liu et al. (2016).

$$\eta_{elec}(\%) = \frac{\Delta_rG^0\ gain\ from\ CO_2\ to\ biomass\ or\ PHA}{charge\ passed\ through\ (C) \times applied\ voltage\ (V)} \times 100$$

The Δ_rG^o value per mole of 1,916 kJ/mol for biomass is based on the previous literature (Grosz and Stephanopoulos, 1983). 1,903 kJ/mol for P(3HB) formation is corresponded to enthalpy of combustion for 3HB unit, and 3,213 kJ/mol for 3HHx is approximated from the enthalpy of combustion for 3HB unit and 2-carbon extension of saturated alkyl chain.

3. Results and discussion

3.1. RGCC culture system combined with water electrolysis and automated regulation of gas flow

The set-up of a closed-gas circulation system for chemo-lithoautotrophic fermentation of *R. eutropha* is illustrated in Fig. 1. A non-combustible gas mixture of H_2:CO_2:O_2:N_2 with the H_2 composition lower than 4.5 vol% was circulated with 0.6 VVM. The input flow rate of CO_2 determined by the mass flow controller was regulated by feedback loop based on output of the CO_2 analyzer to maintain constant CO_2 concentration at 12 vol% in the circulated gas mixture.

H_2 was supplied by water splitting using a PEM electrolyzer implementing a membrane electrode assembly (MEA). The MnIrOx anode in MEA was prepared as described by Nakamura et al. (2023), in which the low Ir content allows cost-effective utilization of the catalyst. The energy conversion efficiency (electricity-to-H_2) of the PEM electrolyzer used here has been determined to be as high as 70 %. The H_2 concentration was controlled to never exceed the upper limit of 5 vol% by feedback regulation of the electric current using the output signal of the H_2 analyzer.

O_2 was initially supplied by using an air compressor, however this led to an increase in volume of the aluminum bag set for stabilizing the system pressure due to N_2 in the atmospheric air. We thus introduced O_2 evolved at the anode of the PEM electrolyzer. Several preliminary operations suggested that the amount of O_2 demanded by the bacterial cells was less than half amount of H_2, namely, direct supply of all of oxygen from the electrolyzer was too much for the cells. The O_2 supply was therefore controlled by a 3 way-solenoid valve, by which excess O_2 was discharged to the outside of the system. The regulation of open/close of the 3-way valve was also regulated by feedback loop with the O_2 analyzer to maintain the constant O_2 concentration of 7 vol%.

3.2. Autotrophic production of P(3HB-co-3HHx) by R. eutropha MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd

The previously engineered strain of *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd (Insomphun et al., 2015) was subjected to the autotrophic cultivation using the RGCC system. The P(3HB-*co*-3HHx) biosynthesis pathway is shown in Fig. 2. In this strain, the C_4 monomer (*R*)-3HB-CoA was formed from acetyl-CoA through the conventional three step reactions, and the C_6 monomer (*R*)-3HHx-CoA was formed by elongation of the C_4 intermediate (reverse β-oxidation). BktB, β-ketothiolase with broad substrate specificity translated from the inherent gene and additional copy in the *pha* operon, catalyzed condensation of butyryl-CoA with acetyl-CoA as well as that of two acetyl-CoA molecules. The resulting C_6-intermediate was converted to (*R*)-3HHx-CoA, followed by copolymerization with (*R*)-3HB-CoA by PhaC$_{NSDG}$, the N149S/D171G double mutant of PHA synthase from *Aeromonas caviae*. The pivotal reaction in this artificial pathway is the conversion of crotonyl-CoA to

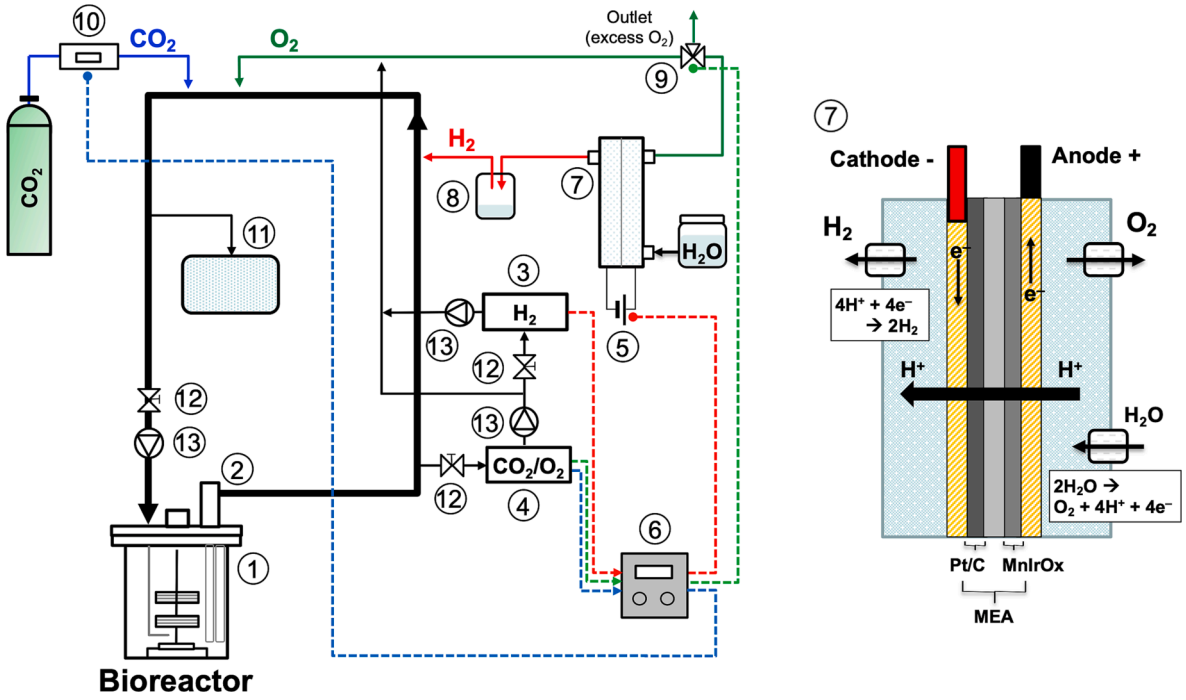


Fig. 1. Recycled-gas closed-circuit (RGCC) culture system for autotrophic cultivation of *R. eutropha*. Left: (1) 2 L (1 L headspace) glass jar fermenter, (2) condenser, (3) H_2 analyzer, (4) CO_2.O_2 analyzer, (5) power supply, (6) fermentation controller, (7) PEM water electrolyzer, (8) water trap, (9) three-way solenoid valve, (10) mass flow controller, (11) 20 L aluminum bag, (12) rotameters, (13) diaphragm pumps. Solid lines show gas flow, and dotted lines show signal transmission. Right: Details of the PEM water electrolyzer. MEA, membrane electrode assembly; Pt/C, platinum/carbon cathode for hydrogen evolution reaction; MnIrOx, manganese-iridium composite oxide anode for oxygen evolution reaction.

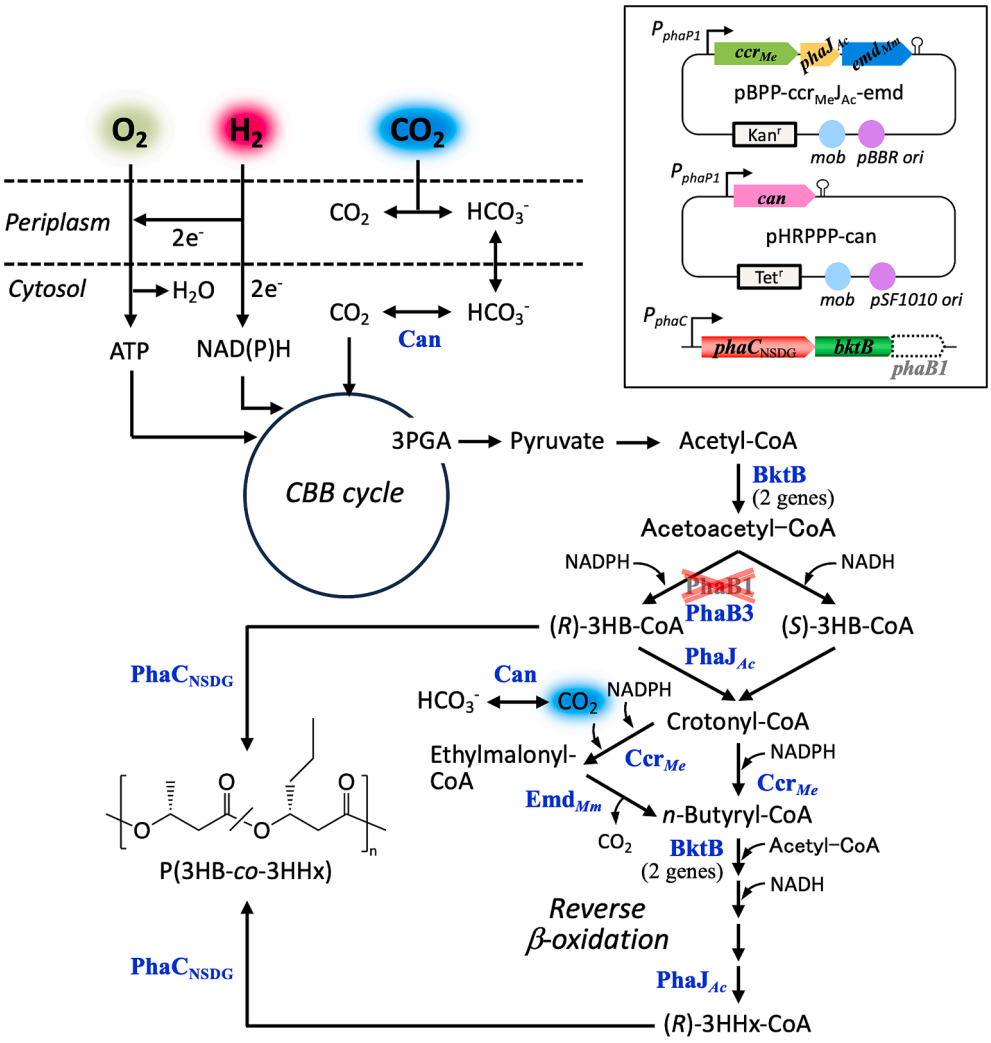


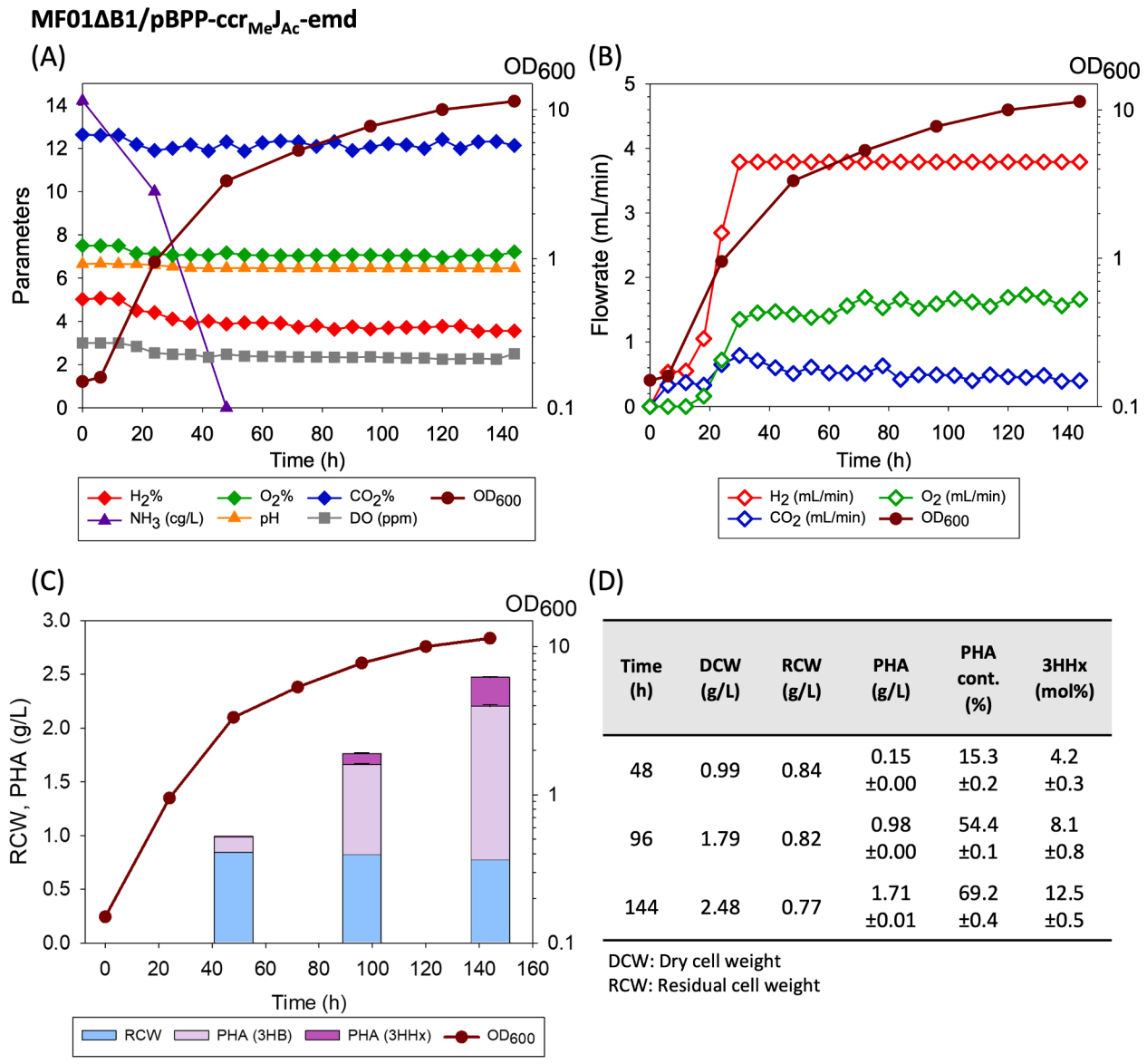
Fig. 2. Pathway for P(3HB-*co*-3HHx) biosynthesis from CO_2, H_2 and O_2 by engineered strains of *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd (/pHRPPP-can). Can, cytoplasmic carbonic anhydrase; Ccr$_{Me}$, crotonyl-CoA carboxylase/reductase from *Methylorubrum extorquens*; Emd$_{Mm}$, ethylmalonyl-CoA decarboxylase from *Mus musculus*; BktB, β-ketothiolase (encoded by inherent gene and additional copy in *pha* operon); PhaB1 and PhaB3, NADPH-acetoacetyl-CoA reductases; PhaC$_{NSDG}$, N149S/D171G mutant of PHA synthase from *A. caviae*; PhaJ$_{Ac}$, (*R*)-enoyl-CoA hydratase from *Aeromonas caviae*.

butyryl-CoA directly by reductase activity of Ccr$_{Me}$ or *via* ethylmalonyl-CoA by combination of Ccr$_{Me}$ and Emd$_{Mm}$ encoded in the plasmid (Insomphun et al., 2015). The deletion of *phaB1* in the *pha* operon, encoding major NADPH-acetoacetyl-CoA reductase, led to relative enhancement of the (*R*)-3HHx-CoA formation, as described in detail in the previous report (Zhang et al., 2019).

The autotrophic cultivation was conducted in a nitrogen-limited medium for induction of PHA biosynthesis by nitrogen deficiency. The initial gas compositions were set to 12 vol% CO_2, 7 vol% O_2 and 3.5–4.5 vol% H_2, and the cultivation results are shown in Fig. 3. The OD_{600} values of the culture broth was rapidly increased during the initial 48 h without significant lag phase, further increased gradually after 48 h, and then reached to plateau of 11.4 at 144 h (Fig. 3A). Considering the consumption of ammonium in the medium, the period around 48 h was thought to be the transition phase between the growth phase and non-growth associated PHA production phase after nitrogen exhaustion. The gradual increase in OD_{600} after 48 h was due to accumulation of PHA within the cells. Indeed, the cells at 48 h contained only 15 wt% of PHA, whereas the PHA contents increased up to 54.4 wt% and 69.2 wt% at 96 h and 144 h in the production phase, respectively, without increase in the residual cell weight (Fig. 3C and 3D). The synthesized PHA was actually P(3HB-*co*-3HHx) copolymer, demonstrating that the artificial PHA biosynthesis pathway from CO_2-derived acetyl-CoA was functional even under the condition of low H_2 concentration. A total of 1.71 g/L of the copolymer composed of 12.5 mol% 3HHx was obtained by the cultivation for 144 h.

The time-dependent changes of the gas flow rates are shown in Fig. 3B. The individually automated feedback regulation for supply of the three gas species enabled constant gas composition throughout the cultivation. From the start of the operation, the gas supply increased gradually until 35 h, which was a result of response to the increasing demand of the gas substrates by the growing cells. The current density in the PEM electrolyzer was regulated to increase from 0 to 31 mA/cm^2 during the initial 30 h. In the transition and PHA production phases after 30 h, the current density of 56 mA/cm^2, corresponding to 3.79 mL/min of H_2 evolution with the voltage of 2.0 V, kept the constant H_2 concentration of 3.6–4.0 %. The dissolved oxygen concentration (DO) was constant in the range of 2.3–3.0 ppm with 400 rpm agitation, and pH was also constant at 6.5–6.7 without any control. These profiles of growth and PHA production as well as gas-flow rates showed high reproducibility in the second independent operation (see supplementary material).

While the intracellular PHA content reached up to ~70 wt%, the total cell weight and consequent PHA production (~2.5 g/L and ~1.7 g/L, respectively) were lower than those of the same strain grown with high concentration of H_2 (6.93 g/L of DCW in flask and 26.7 g/L of DCW



Time (h)	DCW (g/L)	RCW (g/L)	PHA (g/L)	PHA cont. (%)	3HHx (mol%)
48	0.99	0.84	0.15 ±0.00	15.3 ±0.2	4.2 ±0.3
96	1.79	0.82	0.98 ±0.00	54.4 ±0.1	8.1 ±0.8
144	2.48	0.77	1.71 ±0.01	69.2 ±0.4	12.5 ±0.5

DCW: Dry cell weight
RCW: Residual cell weight

Fig. 3. Autotrophic production of P(3HB-*co*-3HHx) by *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd using the RGCC culture system combined with PEM electrolyzer. (A) Time-courses of OD_{600}, gas concentrations (vol%), ammonium concentration (cg/L), pH, and dissolved oxygen (ppm). (B) Time-courses of OD_{600} and gas flow rates (mL/min). (C) Residual cell weight (g/L) and PHA (g/L) (bars) along with time-course of OD_{600} (closed circle). PHA(3HB) and PHA (3HHx) shows amounts of 3HB and 3HHx units in P(3HB-*co*-3HHx) copolymer (g/L). (D) Summary of the cell growth and PHA production at different time points. The results of PHA analysis are presented as the means of technical triplicates.

by the RGCC culture system (Tanaka et al., 2021; 2023)). This was thought to be due to low mass transfer of H_2 in the medium at the low H_2 partial pressure in the gas phase. Further optimization of cultivation condition to support better cell growth, such as agitation condition or improvement of sparger to increase availability of dissolved H_2 for the cells, should be investigated to achieve more efficient production without any explosion-proof equipment.

3.3. Effects of overexpression of *can* on autotrophic growth and PHA production

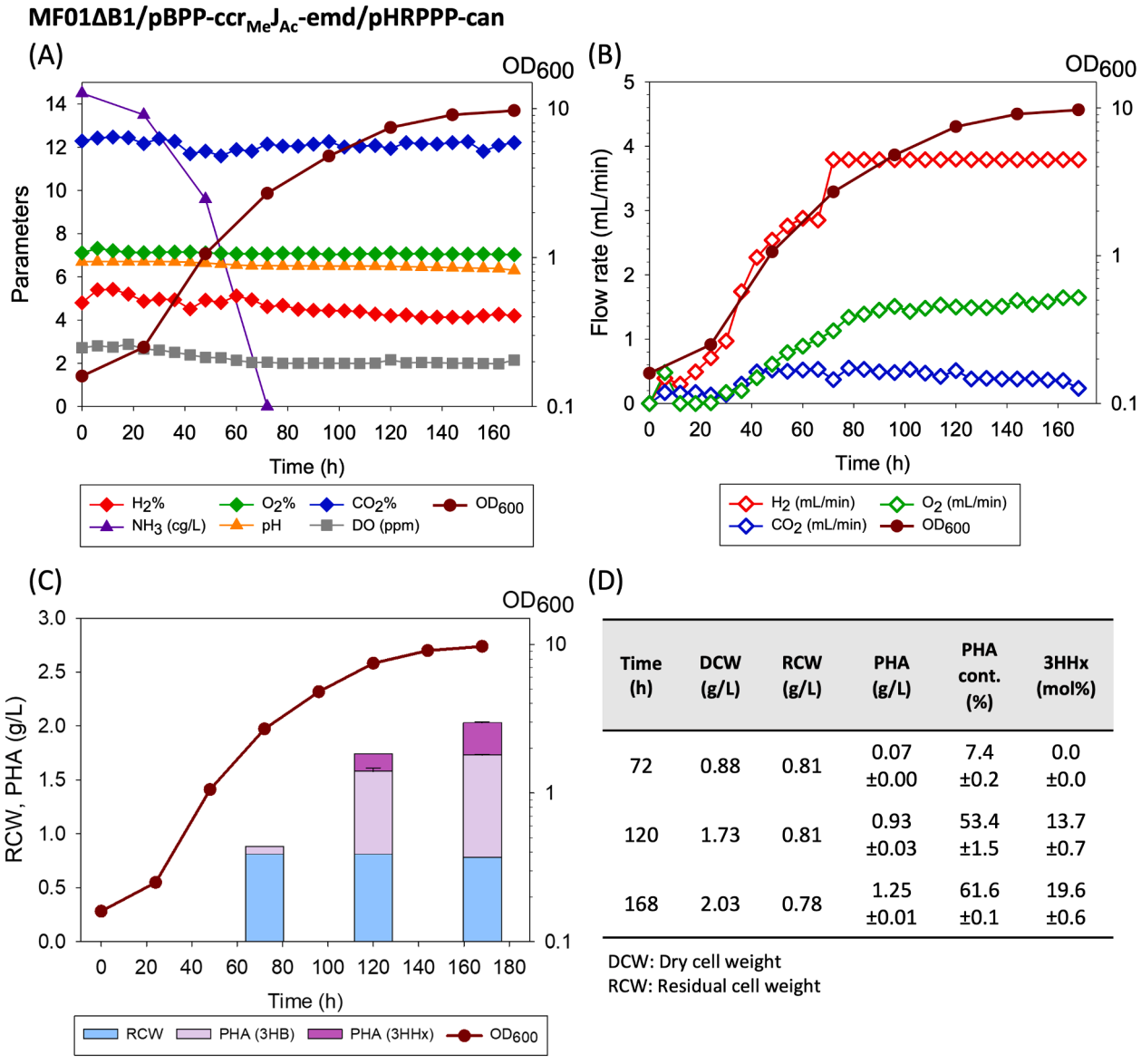
Carbonic anhydrases (CAs) catalyzing the interconversion of CO_2 and HCO_3^- play important roles in various physiological functions involving dissolved inorganic carbons, especially for transport and fixation of CO_2 by autotrophic organisms, even though the high rate of non-enzymatic hydration/dehydration of CO_2/HCO_3^- (Smith and Ferry, 2000). *R. eutropha* possesses 4 genes annotated as putative CAs, which are *can* and *can2* encoding β-class CAs, *caa* encoding periplasmic α-CA, and *cag* encoding γ-like-CA/acetyltransferase (Gai et al., 2014). Previous gene deletion analyses demonstrated that Caa and Can were important enzymes for the growth of *R. eutropha*. Can is essential to *R. eutropha* for growing at ambient CO_2 concentration on various carbon source, possibly accelerating conversion of HCO_3^- to CO_2 in the cytoplasm (Gai et al., 2014, Kusian et al., 2002). Although individual overexpression of the CAs in *R. eutropha* wild strain gave neither significant change in growth nor P(3HB) production under heterotrophic conditions (Gai et al., 2014), the introduction of *can*-expressing vector increased cellular P(3HB) content from 44 wt% to 68 wt% in *R. eutropha* autotrophically grown on a mixture gas of H_2:CO_2:O_2:N_2 with low hydrogen concentration in the flask cultivation (Thorbecke et al., 2021).

We here investigated the effects of overexpression of *can* on the autotrophic biosynthesis of P(3HB-*co*-3HHx) copolymer. The expression vector pHRPPP-can was constructed by inserting the *can* gene at downstream of the strong constitutive *phaP1* promoter (Fukui et al.,

2011). When the resulting strain *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd/pHRPPP-can was subjected to usual heterotrophic cultivation on fructose, the PHA production and 3HHx composition were slightly decreased when compared with those by the parent strain harboring the empty vector (see supplementary material).

In the gas-recycling fermentation with the settings described in the section 3.1, the strain harboring the plasmid-borne *can* gene exhibited a lag phase in the initial 24 h (Fig. 4A). The fermentation properties after the lag phase were similar to those of the parent strain, namely, the ammonia source was exhausted at 72 h and the cells were then transferred to PHA production phase accompanied with gradual increase in OD_{600}. The cells at 72 h contained 7.4 wt% of PHA, and the PHA content subsequently increased up to 53.4 wt% at 120 h. While the residual cell weights of ~0.8 g/L were almost the same as those of the strain not harboring the second plasmid, the final PHA content of 61.6 wt% was slightly lower resulting in less PHA production of 1.25 g/L than the parent strain (69.2 wt% and 1.71 g/L, respectively). It should be noted that the 3HHx composition in the accumulated copolyester was increased up to 19.6 mol% (Fig. 4C and 4D). Even in the presence of initial lag phase, the individual supply of the gas species was well managed by the feedback regulation, thus the gas composition was constant throughout the cultivation, as shown in Fig. 4A and 4B. DO and pH values were again stable, although pH reached a slightly lower value of 6.4 in the final hours of the cultivation. The reproducibility of these results was also assessed by independent run carried out the same culture conditions except for the minimum CO_2 flow rate of 0.3 mL/min. This caused slight accumulation of excess CO_2 during the growth phase, whereas the cell growth and PHA production were comparable to those obtained by the operation with the minimum flow of 0 mL/min shown in Fig. 4 (see supplementary material).

Although the detailed reason for the initial lag phase has been unclear, this may be related to fitness cost for retaining the second plasmid and/or the effect of additional antibiotic on the cells. It was because, under the usual heterotrophic condition on fructose, the strain



Time (h)	DCW (g/L)	RCW (g/L)	PHA (g/L)	PHA cont. (%)	3HHx (mol%)
72	0.88	0.81	0.07 ±0.00	7.4 ±0.2	0.0 ±0.0
120	1.73	0.81	0.93 ±0.03	53.4 ±1.5	13.7 ±0.7
168	2.03	0.78	1.25 ±0.01	61.6 ±0.1	19.6 ±0.6

DCW: Dry cell weight
RCW: Residual cell weight

Fig. 4. Autotrophic production of P(3HB-*co*-3HHx) by *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd /pHRPPP-can using the RGCC culture system combined with PEM electrolyzer. (A) Time-courses of OD_{600}, gas concentrations (vol%), ammonium concentration (cg/L), pH, and dissolved oxygen (ppm). (B) Time-courses of OD_{600} and gas flow rates (mL/min). (C) Residual cell weight (g/L) and PHA (g/L) (bars) along with time-course of OD_{600} (closed circle). PHA(3HB) and PHA (3HHx) shows amounts of 3HB and 3HHx units in P(3HB-*co*-3HHx) copolymer (g/L). (D) Summary of the cell growth and PHA production at different time points. The results of PHA analysis are presented as the means of technical triplicates.

Table 2
Stoichiometries, yields and efficiencies for biomass and polymer production during specific time intervals.

Phase	Product	H_2/CO_2 (mol/mol)	O_2/CO_2 (mol/mol)	Biosynthesis formula	Yield $(H_2)^b$	Yield (Elec.)[c]	Yield (Equiv. solar)[d]	η^e_{elec}	η^f_{solar}
R. eutropha MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd									
Growth (12–36 h)	Biomass	4.56	1.49	4.0 CO_2 + 18.2 H_2 + 6.0 O_2 + 0.96 NH_3 → $C_{4.0}H_{7.09}O_{1.96}N^a_{0.96}$ + nH_2O	72.9	51.0	10.2	19.1	3.8
PHA production(48–96 h)	P(3HB-*co*- 8.9 mol% 3HHx)	7.22	2.94	4.18 CO_2 + 30.2 H_2 + 12.3 O_2 → $C_{4.18}H_{6.35}O_2$ + nH_2O	63.1	44.2	8.8	10.7	2.1
PHA production (48–144 h)	P(3HB-*co*- 13.4 mol% 3HHx)	7.82	3.30	4.27 CO_2 + 33.4 H_2 + 14.1 O_2 → $C_{4.27}H_{6.53}O_2$ + nH_2O	65.2	45.7	9.1	10.3	2.1
R. eutropha MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd/pHRPPP-can									
Growth (24–60 h)	Biomass	4.45	1.24	4.0 CO_2 + 17.8 H_2 + 5.0 O_2 + 0.96 NH_3 → $C_{4.0}H_{7.09}O_{1.96}N^a_{0.96}$ + nH_2O	61.7	43.2	8.6	16.1	3.2
PHA production (72–120 h)	P(3HB-*co*- 15.0 mol% 3HHx)	7.63	2.93	4.30 CO_2 + 32.8 H_2 + 12.6 O_2 → $C_{4.30}H_{6.60}O_2$ + nH_2O	70.3	49.3	9.9	11.4	2.3
PHA production (72–168 h)	P(3HB-*co*- 20.7 mol% 3HHx)	8.89	3.54	4.41 CO_2 + 39.2 H_2 + 15.6 O_2 → $C_{4.41}H_{6.83}O_2$ + nH_2O	56.6	39.6	7.9	8.0	1.6

a) Cellular composition based on the previously reported empirical formula (Grosz and Stephanopoulos, 1983).
b) e) Calculated as described in Materials and Methods.
c) Electricity-to-"biomass/PHA" yield calculated with electricity-to-H_2 efficiency of 70% by the PEM electrolyzer.
d) Equivalent solar-to-"biomass/PHA" yield calculated with solar-to-electricity effciency of 20% by general high-performance solar panels.
f) Equivalent solar-to-"biomass/PHA" thermodynamic efficiency calculated with solar-to-electricity efficiency of 20%.

MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd/pHRPPP-can exhibited significantly lower growth and PHA production when compared to the strain not harboring the second vector (see supplementary material). In addition, the enhanced activity of Can may cause growth retardation in the initial phase of the autotrophic growth. It has been reported that bicarbonate is necessary to avoid a long lag phase of *R. eutropha* under autotrophic conditions (Repaske et al., 1971). As the Can-mediated interconversion between CO_2 and HCO_3^- oriented to CO_2 formation, the overexpression of *can* may reduce HCO_3^- concentration within the cells leading to the lag phase prior to the autotrophic growth. Interestingly, the can-overexpressing strain produced P(3HB-*co*-3HHx) with higher 3HHx fraction than the parent strain during the autotrophic cultivation, although this effect was not seen under the heterotrophic condition. As introduced above, the combination of Ccr_{Me} and Emd_{Mm} is the key function in P(3HB-*co*-3HHx) biosynthesis from acetyl-CoA, where Ccr accepts CO_2 as the substrate for the carboxylation reaction (Stoffel et al., 2019). It was supposed that higher concentration of cytosolic CO_2 attributed with the *can* overexpression enhanced the butyryl-CoA formation by the carboxylase activity of Ccr_{Me} cooperated with Emd_{Mm}, that would increase the provision of 3HHx-CoA monomer.

3.4. Yields and energy conversion efficiencies

The constant composition of the circulated gas, achieved by the automated feedback regulations of H_2-O_2 evolution, discharge of excess O_2, and flow rate of CO_2, indicated that the supply of each the gas species was well balanced with the cellular demands on the gas substrates. This was supported by the fact that the inside pressure of the circulated gas was always atmospheric, and no accumulation of excess gas was observed in the 20 L aluminum bag set in the circulation line. The amounts of the supplied gases could be readily calculated from logs of the feedback regulation, and they were considered to correspond to amounts of the gases consumed by the bacterial cells under the condition of the well-balanced gas supply. Namely, the ratio of the consumed amounts of H_2 or O_2 to CO_2 during the early and late phases reflected stoichiometric ratios of the gases in the autotrophic production of biomass (*R. eutropha* cells not containing PHA) and P(3HB-*co*-3HHx), respectively, because the cells grew in the early phase without PHA synthesis, then accumulated PHA without growth in the late phase as described above. In the case of *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd, the gas consumption rates were determined to be 6.93 mmol/h for H_2, 2.26 mmol/h for CO_2, and 1.52 mmol/h for O_2 with biomass production rate of 1.11 mmol/h, while they were 9.29 mmol/h for H_2, 3.78 mmol/h for CO_2, and 1.28 mmol/h for O_2 with PHA production rate of 0.19 mmol/h.

The H_2/CO_2 and O_2/CO_2 consumption ratios (mol/mol) observed here are shown in Table 2. The two examined strains both showed a lower H_2/CO_2 ratio during the growth phase (4.6–4.5) than those during the mid-phase of PHA accumulation (7.2–7.6). It has been reported that *R. eutropha* strain H in the growth phase showed H_2/CO_2 consumption ratio of 2.5–3.3 in high pressure (3 atm)-batch cultivation (Yu et al., 2013) and the ratio of 3.1 in gas-flow cultivation (Lu and Yu, 2017) both with high H_2 concentration of 70–85 vol%. Instead, the ratio in the late phase of the strain H in the gas-flow cultivation (probably PHA accumulation phase) was 6.4–8.2 (Lu and Yu, 2017), and those in production phases of engineered *R. eutropha* for autotrophic synthesis of 2,3-butandiol and isopropanol were reported to be 7.9 and 8.1, respectively (Bommareddy et al., 2020). These facts suggested that, regardless the cultivation conditions, *R. eutropha* (as well as the related strain) is able to fix one molecule of CO_2 by using energy conserved from approximately 3–4 molecules of H_2 for the growth, but the efficiency was reduced as requiring approximately 7–8 molecules of H_2 for the CO_2 fixation after the growth.

The stoichiometric formula of the PHA synthesis by the strain MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd during 48–96 h was determined based on the ratios of the consumed gas amounts, as below:

$4.18CO_2 + 30.2H_2 + 12.3O_2 \rightarrow C_{4.18}H_{6.35}O_2$ [P(3HB-*co*-8.9 mol% 3HHx)] + nH_2O

The PHA yields could be obtained from the amounts of supplied gas in the late phase and the stoichiometry during the selected time intervals, and thermodynamic efficiency (η) of electricity-to-PHA was calculated based on standard reaction Gibbs free energy (Δ_rG^0) of PHA over input electric power as described in Materials and Methods. The results were summarized in Table 2. The PHA yield on H_2 by the strain was determined to be 63.1 %, which corresponded to 44.2 % yield on electricity by considering 70 % evolution of H_2 on the input electrons by the PEM electrolyzer used in this study. The η_{elec} value for the PHA production during the 48–96 h period was calculated to be 10.7 %.

The stoichiometry, yield of cellular biomass, and thermodynamic electricity-to-biomass efficiency in the growth phase were calculated in the same way by applying the cellular composition of $C_{4.0}H_{7.09}O_{1.96}N_{0.96}$ previously reported (Grosz and Stephanopoulos, 1983). The stoichiometric formula of the cell growth of MF01ΔB1/pBPP-$ccr_{Me}J_{Ac}$-emd during 12–36 h was obtained as below:

$4.0CO_2 + 18.2H_2 + 6.0O_2 + 0.96NH_3 \rightarrow C_{4.0}H_{7.09}O_{1.96}N_{0.96} + nH_2O$

The biomass amount of *R. eutropha* not containing PHA was estimated from the ΔOD_{600} values with the coefficient of 0.37 g-cell/OD_{600}, which had been determined in advance by using a PHA-negative mutant strain PHB$^-$4. The biomass yield on input H_2 and thermodynamic electricity-to-biomass efficiency during the 12–36 h period were calculated to be 72.9 % and 19.1 %, respectively, both were higher than those for PHA production (Table 2). These formulae of the cell proliferation and PHA synthesis determined here were well agreed with those of growth and P(3HB) production by *R. eutropha* wild strain H16 with high H_2 concentration previously reported by Ishizaki and Tanaka (1990; 1991). This implied that the autotrophic metabolisms of carbon and energy in *R. eutropha* were not so affected by H_2 concentration in the gas phase.

In the case of the *can*-overexpressing strain MF01ΔB1/pBPP-$ccr_{Me}J_{Ac}$-emd/pHRPPP-can, the range of 62–70 % yields on H_2 was similar to those observed by the parent strain (Table 2). The decrease in the yield and efficiency during 72–168 h period was mainly due to unexpected deceleration of PHA accumulation after 120 h. The results of the independent operations of the parent and *can*-overexpressing strains are summarized in supplementary material.

Taking into consideration that feedstock cost for production of value-added compounds from CO_2 and H_2 will be mainly occupied by supplementation of H_2, the yield and conversion efficiency of the target compound on H_2 are important parameters for the cost-effective production. Hitherto, the studies on autotrophic cultivation of hydrogen-oxidizing bacteria have often dealt with growth rate and final titer of the product, thus the yield of the target product on input H_2 (not on consumed H_2) has been not well considered, because the yield was hard to be determined when re-fill of the gas mixture in the head space and/or gas reservoir is needed in batch cultivation or excess H_2 not consumed by the cells is wasted in continuous gas-flow cultivation. The present grasp of the supplied gas amount corresponding to the consumption by the cells, enabled by the automated feedback regulation of gas supply, gave useful insight into evaluation of growth and production performances of hydrogen-oxidizing autotrophs focusing on input H_2. We here obtained 63–73 % yields for the cell biomass and P(3HB-*co*-3HHx) on input H_2 based on the calculated stoichiometry. These yields were thought to be rather high, but about one-third of input H_2 was lost. A possible cause for the loss of H_2 would be unexpected leakage of H_2 during the circulation before the utilization by the cells, even with the use of multilayer gas-barrier tubes. Enhancement of the gas barrier properties of the system would be one of effective points to obtain higher production yield.

When considering that the electricity for water splitting is supplied by high-performance solar panels (~20 % solar-to-electricity efficiency), the equivalent η values of solar-to-biomass and solar-to-PHA by the present gas fermentation system were estimated to be 3.8 % and 2.1 %, respectively, with the strain MF01ΔB1/pBPP-$ccr_{Me}J_{Ac}$-emd. These efficiencies were higher than photosynthetic solar-to-biomass efficiencies of ~0.2 % by land plants (annual average) and 1.5–1.8 % by agricultural crop (growing season), indicating high capabilities of *R. eutropha* for energy conversion and CO_2 fixation. However, we here observed that the energetic efficiencies on electricity (19 % for growth and 10 % for PHA synthesis) were much lower than the yields of 44–51 % on electricity. This might be due to yet insufficient energy conservation or the presence of some energy-consuming metabolisms, resulting in requirement of more H_2 for CO_2 fixation, especially in the production phase as described above. A high thermodynamic efficiency on electricity of 36 % (6.4 % on equivalent solar) was reported for cell growth and P(3HB) biosynthesis from CO_2 by *R. eutropha* H16 with a hybrid microbial-water splitting system, in which H_2 was supplied by *in situ* water splitting catalyzed by a cobalt-phosphorus alloy cathode and cobalt phosphate anode in the medium (Liu et al., 2016). The gradual evolution of H_2 on the cathode surface might be favorable for the cellular uptake of H_2 and the following energy metabolisms, although this hybrid system showed rather low productivity of cell biomass plus P(3HB) of 0.70 g/L for 6 days. Further investigations will be needed to understand the H_2-dependent autotrophic metabolisms in *R. eutropha*. The improvement of H_2-supplementation condition/method as well as enhancement of cellular ability for utilization of H_2 by genetic modifications would contribute to achieve both higher efficiency and productivity of the RGCC bioreactor suitable for production of not only PHA but also other chemicals from CO_2.

4. Conclusion

This study developed the recycled-gas closed-circuit culture system combined with PEM water electrolysis for production of PHA copolymers from CO_2 and H_2 by engineered *R. eutropha*. The feedback regulation of the gas supply allowed to determine yields and efficiencies of biomass and PHA on the input H_2. The results demonstrated not only the high potential of *R. eutropha* for autotrophic bioproduction but also usefulness of the system for evaluation of gas fermentation. Further investigations are expected to serve as a cornerstone toward the development of sustainable, efficient, and safe autotrophic processes for utilization of CO_2 as a feedstock.

CRediT authorship contribution statement

Gabriele Di Stadio: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Izumi Orita:** Writing – review & editing, Methodology. **Ryuhei Nakamura:** Writing – review & editing, Methodology. **Toshiaki Fukui:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

Acknowledgements

GDS and TF would like to acknowledge the Support for Pioneering Research Initiated by the Next Generation (SPRING) program by Japan Science and Technology Agency (Grant No. JPMJSP2106). The authors

gratefully thank Risa Kato and Ryota Inoguchi (Tokyo Institute of Technology) for construction of the expression vector pHRPPP, and Takahiro Muramatsu (Able-Biott Co., Ltd) for suggestion and support in establishment of the gas flow system.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130266.

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