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Establishment of gas fermentation platform combined with water electrolysis for production of value-added chemicals from CO_2 and evaluation of engineered *Ralstonia eutropha*

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Introduction

Climate change caused by the increasing atmospheric greenhouse gases and the pollution of natural environments by plastic wastes and microplastics are looming problems that our society is challenging. The use of CO_2 as a feedstock in bioprocesses to produce biodegradable plastics and biofuels could be one of the interesting solutions for these problems.

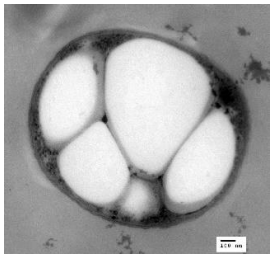

Fig 1: TEM image of *R. eutropha* cell accumulating PHA granules

The Gram-negative, "Knallgas" H_2-oxidizing bacterium *Ralstonia eutropha* (Fig. 1) emerges as a potential candidate as an autotrophic fermentation platform owing to its rapid growth on CO_2, H_2, and O_2 independent of light irradiation. *R. eutropha* has been also known as an efficient polyhydroxyalkanoates (PHAs) producer, and many studies for this bacterium have been done focusing on PHA production from biomass feedstocks. One of the previous works enabled the production of P(3HB-*co*-3HHx), a copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) showing flexible properties, from sugars as the carbon source by the engineered strains of *R. eutropha* [1] (Fig. 2).

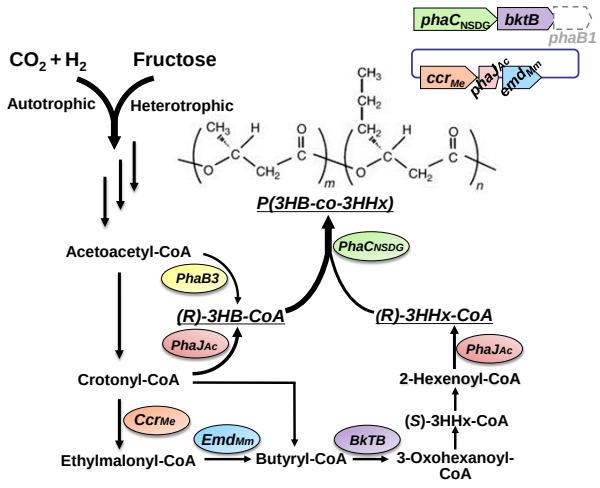

Fig. 2. Biosynthesis of PHA and by *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd from sugars or CO_2 and H_2.

Meanwhile, there has been limited research on aerobic gas fermentation using H_2-oxidizing bacteria, mainly due to safety concerns caused by the explosive property of the gas mixture of H_2 and O_2 (within the range between 4% and 75% in atmospheric air). It has been reported that the wild strain of *R. eutropha* H16 efficiently produced poly(3-hydroxy-butyrate) homopolymer by the circulation of a gas mixture of $H_2/O_2/CO_2$ = 84:6:10 vol% [2]. It should be noted that the *R. eutropha* strains engineered for the biosynthesis of P(3HB-*co*-3HHx) from sugars are able to produce the copolyester also from CO_2 [3].

In order to facilitate the upscaling of aerobic gas fermentation in industrial settings, it is necessary to mitigate the risk of hazardous incidents. Moreover, in the bioproduction from CO_2 using H_2-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 in achieving low-cost production. In this study, the author focused on the production of P(3HB-*co*-3HHx) from CO_2 by using the engineered strains of *R. eutropha* using a novel recycling gas-closed circular (RGCC) culture system combined with water electrolysis. The evaluation of the bioproduction demonstrated the high performance of *R. eutropha* for the fixation of CO_2 by using energy from H_2.

Experimental methods

Strains and plasmids

Previously engineered strains of *R. eutropha* harboring a pBPP-based expression vector for the biosynthesis of P(3HB-*co*-3HHx) were used in this study. Overexpression of *can* gene was done by using pHRPPP-can which was compatible with pBPP-based vectors.

Cultivation and analytical methods

R. eutropha strains were cultivated in a nitrogen-limited mineral salt medium not containing any carbon source, and a gas mixture of $H_2/CO_2/O_2/N_2$ (initial composition 5:12:7:76) was circulated in a 2-L fermenter (working volume 1 L). The bioreactor operated at a constant impeller speed of 400 rpm and a temperature of 30°C. Intracellular PHA was determined by GC after direct methanolysis of the dried cells. Gas volumes in the system were measured by gas detectors, and the broth parameters (temperature, pH, dissolved oxygen) were measured by probes. Every day a small portion of the broth was sampled to measure the OD_{600} and the ammonia concentration.

Results and Discussion

1) Establishment of RGCC culture system combined with water electrolysis

In order to avoid the explosion risk of the gas mixture containing H_2 and O_2, a non-combustible mixture of $H_2/CO_2/O_2/N_2$ was circulated by using a diaphragm pump (Fig. 3). H_2 and O_2 were supplied by using a proton-exchange membrane (PEM)-type water electrolyzer assembled with Pt/C-MnIrO membrane catalyst, and CO_2 was supplied from a gas cylinder. The concentrations of each gas species were monitored by on-line gas analyzers, and the CO_2 flow rate, water electrolysis, and discharge of O_2 were regulated by a feedback loop based on the outputs of the analyzers. An aluminum gas bag was used to avoid pressure changes in the system, and a water trap was positioned after the PEM electrolyzer to avoid eventual water leakages.

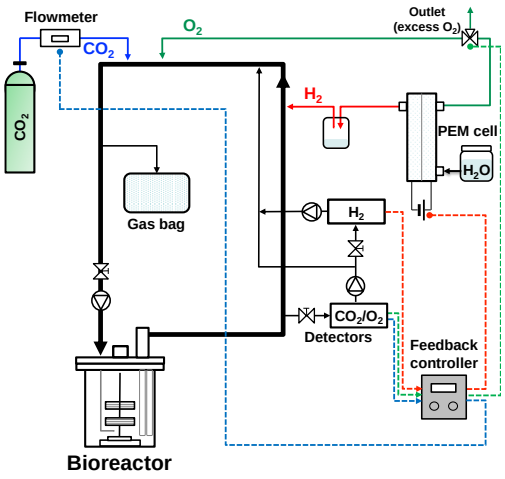

Fig. 3. RGCC culture system with PEM water electrolyzer.

2) Autotrophic production of P(3HB-*co*-3HHx)

The engineered strain of *R. eutropha* MF01ΔB1/pBPP-ccr$_{Me}$J$_{Ac}$-emd produced 1.71 g/L of P(3HB-*co*-12.5 mol% 3HHx) on the gas mixture of $H_2/CO_2/O_2/N_2$ = 4:12:7:77 vol% with 69 wt% of the cellular content. As shown in Fig. 4, the gas composition was constant throughout the cultivation owing to the feedback regulation, indicating gas supply well balanced with the bacterial demands to the gas substrates. The PHA yields on H_2 and electricity were calculated to be 63.1 % and 44.2 %, respectively, while the thermodynamic

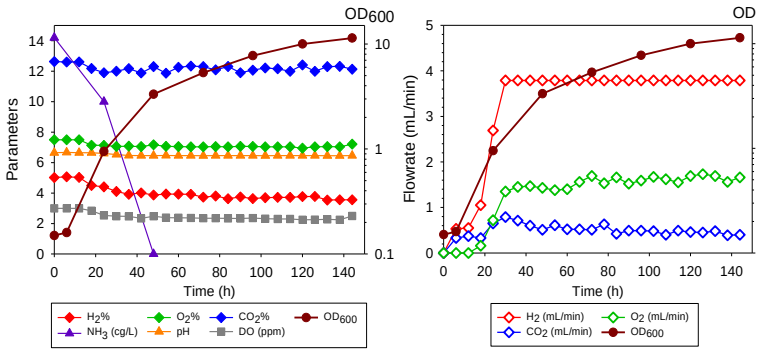

Fig. 4. Autotrophic production of P(3HB-*co*-3HHx) by *R. eutropha* engineered strain using the RGCC culture system combined with PEM electrolyzer.

electricity-to-PHA efficiency (η_{elec}) was 10.7 %. The equivalent solar-to-biomass/ PHA efficiencies were estimated to be 2.1-3.8 %, highlighting the high energy conversion capability of *R. eutropha* (Table 1).

3) Effect of overexpression of carbonic anhydrase gene on the autotrophic PHA biosynthesis

Carbonic anhydrases (CAs), catalyzing the interconversion of CO_2 and HCO_3^-, play important roles in various physiological functions involving dissolved inorganic carbons. A previous research showed that, among 4 genes encoding CAs in *R. eutropha*, overexpression of *can* encoding a cytosolic CA enhanced P(3HB) production from CO_2 by *R. eutropha* [4]. Here, the effects of the overexpression of *can* on yield and composition of P(3HB-*co*-3HHx) synthesized from CO_2 by the engineered *R. eutropha*. The strain harboring the plasmid-borne copy of *can* was constructed and subjected to gas fermentation using the established RGCC culture system. Although a lag phase for about 24 h was observed before the growth, the *can*-overexpressing strain produced P(3HB-*co*-3HHx) composed of higher 3HHx molar fraction up to 19.6% with high yield and efficiency when compared to the parent strain (Table 1). Considering the artificial biosynthesis pathway, it was supposed that the overexpression of *can* increased the availability of CO_2 for crotonyl-CoA carboxylase/reductase (Ccr), a crucial enzyme in the formation of the C_6-monomer, (*R*)-3HHx-CoA.

Conclusion

The results demonstrated not only the high potential of the engineered *R. eutropha* for production of P(3HB-*co*-3HHx) from H_2 and CO_2, but also the usefulness of the present system for the evaluation of the potential of hydrogen-oxidizing bacteria and the effects of genetic modifications. Further investigations will be needed to increase biosynthesis efficiency using CO_2 as a carbon source, by further engineering in the cellular carbon and energetic metabolisms, as well as fermentation engineering for increasing availability of the gaseous substrates with low solubility in the medium.

Table 1. P(3HB-*co*-3HHx) production from CO_2 and H_2 (electricity) by engineered *R. eutropha* by the RGCC culture system.

Strain	RCW (g/L)	Titer (g/L)	Yield (H_2)	Yield (elec)	η (elec)
Parent strain	0.77	1.71	63.1	44.2	10.7
Can-over-expressing strain	0.78	1.25	56-70	49.3	11.4

RCW: Residual cell weight
elec: electricity
η: Thermodynamic efficiency

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