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MINI-REVIEW

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Microbial production of poly-D-3-hydroxybutyrate from CO_2

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Abstract This short review covers the biotechnological aspects of the production of poly-D-3-hydroxybutyric acid, P(3HB), from H_2, O_2 and CO_2 by autotrophic culture of the hydrogen-oxidizing bacterium, *Ralstonia eutropha*. Considering the efficiency of utilization of a gas mixture as substrate, a practical fermentation process using *R. eutropha* for the mass production of P(3HB) from CO_2 should be designed on the basis of a recycled-gas, closed-circuit culture system. Also, maintaining the O_2 concentration in a gas phase lower than 6.9% (v/v) is essential to prevent the gas mixture from exploding. Our study, using an explosion-proof fermentation bench plant and a two-stage culture system with a newly designed air-lift fermenter, demonstrated that very high P(3HB) yield and productivity could be obtained while the O_2 concentration was maintained below 6.9%. However, a study on the continuous production of P(3HB) from CO_2 by chemostat culture of *R. eutropha* revealed that the productivity and content of P(3HB) in the cells was considerably lower than by fed-batch culture. It is deduced that the use of the hydrogen-oxidizing bacterium, *Alcaligenes latus*, which accumulates P(3HB) even in the exponential growth phase, will be useful for the effective production of P(3HB) from CO_2.

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Introduction to hydrogen-oxidizing bacteria and biodegradable plastics

A hydrogen-oxidizing bacterium is a chemolithoautotrophic bacterium that can grow using a mixture of H_2, O_2 and CO_2 gasses as substrate. This chemolithoautotrophic bacterium requires only inorganic salts like ammonium sulfate as nutrients in the culture medium. Hydrogen-oxidizing bacteria obtain energy for CO_2 fixation by the oxidation of H_2 gas with O_2 and occur in the natural environment, especially in the soil, ocean, rivers and hot springs. Among hydrogen-oxidizing bacteria, *Ralstonia eutropha*, which was reclassified from *Alcaligenes eutrophus*, (Yabuuchi et al. 1995), is the best characterized species, because its growth rate is much higher than that of other chemolithoautotrophic bacteria. However, the highest specific growth rate (μ=0.68 h^{-1}) was observed in *Hydrogenobacter thermophilus*, which was isolated by Goto et al. (1977a). Many studies on the physiology and basic character of hydrogen-oxidizing bacteria were reported by Goto et al.(1977b), Nishihara et al.(1991), Repaske (1961, 1966), Repaske and Mayer (1976), Repaske and Repaske (1976), Schlegel (1989), Schlegel and Lafferty(1971) and Schlegel et al. (1961). Biotechnological studies on autotrophic cultivation of hydrogen-oxidizing bacteria were mainly carried out by Foster and Litchfield (1964), Heinzle and Lafferty (1980), Kodama et al. (1975b, 1976), Miura et al. (1981, 1982), Ohi et al. (1979a, b), Schuster and Schlegel (1967) and Sonnleitner et al.(1979).

It is known that many hydrogen-oxidizing bacteria, especially *R. eutropha*, accumulate poly-D-hydroxybutyric acid, P(3HB), as a storage material within the cell under nutrient-limited conditions. P(3HB) is a kind of bacterial polyhydroxyalkanoate (PHA) and has potential as a raw material for manufacturing biodegradable thermoplastics. The general formula of PHAs is [-O-CH(*R*)-CH_2-CO-]$_n$, where *R*=CH_3 in P(3HB). Ordinary plastic, which is mainly manufactured from fossil fuel, is nondegradable and therefore it easily accumulates in the en-

vironment. The amount of non-degradable plastics accumulating in the environment is estimated to be 25×10^6 t year^{-1} (Dawes 1990). PHA produced by *R. eutropha* and other bacteria has thermoplastic properties and is decomposed by micro-organisms in the soil, rivers and oceans. Thus, PHA can be a candidate for a biodegradable plastic. In the production of microbial polyester, biomass and/or CO_2 are used as the carbon sources; and this does not threaten the fossil fuel reserves or increase the CO_2 concentration in the atmosphere. CO_2 gas used as a carbon source for the production of PHA can be obtained by the separation of exhaust gasses from factories and/or thermal power stations with a pressure swing adsorption system. At present, it is expected that H_2 gas can be supplied from the process of refining naphtha or generated by electrical dissociation of H_2O with the energy from nuclear power stations.

Useful reviews on the general features of PHAs, PHA-producer strains and the fermentation process have been reported by many researchers (Anderson and Dawes 1990; Barham et al. 1992; Bonthrone et al. 1992; Brandl et al. 1990; Braunegg et al. 1998; Byrom 1994; Choi and Lee 1997, 1999a; Cox 1994; Doi 1990; Doi et al. 1994; Inoue and Yosie 1992; Lafferty et al. 1988; Lee 1996; Madison et al. 1999; Sasikala and Ramana 1996; Steinbuchel 1991; Steinbuchel and Fuchtenbusch 1998; Steinbuchel et al. 1992). Most of these works were carried out with heterotrophic cultures, using organic substrate such as sugars, methanol and/or organic acids; and the effective production of PHAs at high concentration with high productivity was attained only in fed-batch cultures (Ahn et al. 2000; Kim et al. 1994, 1996; Rhee et al. 1993; Ryu et al. 1997; Suzuki et al. 1986; Tsuge et al. 1999; Wang and Lee 1997a, b; Yamane et al. 1996). In contrast, attempts at producing PHA from CO_2 using hydrogen-oxidizing bacteria are still rare, because autotrophic culture of the bacteria is accompanied by many difficult problems.

The problems in autotrophic culture of hydrogen-oxidizing bacteria

The substrate for autotrophic culture of hydrogen-oxidizing bacteria is a mixture of the gasses H_2, O_2 and CO_2. The composition of this gas mixture which attains sufficient cell growth in flask and plate cultures usually has a ratio of H_2:O_2:CO_2=7:1:1. However, such a gas composition is completely within the gas-explosion range and therefore can easily explode. The low solubility of H_2 and O_2 gasses is another serious problem. Most of the studies on autotrophic culture of hydrogen-oxidizing bacteria have been carried out using conventional fermenters, through which the substrate gas is fed continuously and the exhaust gas is discharged outside the fermentation system. The discharge of the exhausted gas constitutes a loss in substrate and therefore the efficiency of gas utilization is decreased. To prevent the waste of substrate gas, two basic concepts for practical culture system of hydrogen-oxidizing bacteria were suggested. One was the dead-end culture system by Bongers (1970) and the other was the recycled-gas culture system by Schlegel and Lafferty (1971). The dead-end culture system exhibits some disadvantages in gas mass-transfer, due to the lack of continuous feeding of substrate gas, while the recycled-gas, closed-circuit culture system attains high gas mass-transfer by recycling the substrate gas. Autotrophic cultivation of hydrogen-oxidizing bacteria using the recycled-gas culture system was only carried out by Kodama et al. (1975a), Repaske (1966) and the present authors, because the system was difficult to operate and needed a large gas reservoir. However, the authors obtained much biotechnological information on the autotrophic culture of *R. eutropha*, using the recycled culture system with a 200-ml jar fermenter (Ishizaki and Tanaka 1990a, b, 1991, 1992; Ishizaki et al. 1997; Sugimoto et al. 1999; Taga et al. 1997; Takeshita et al. 1993a, b; Tanaka and Ishizaki 1994; Tanaka et al. 1992, 1993, 1995).

Autotrophic fermentation characteristics of *R. eutropha*

The features of cell growth and PHA accumulation in autotrophic culture of *R. eutropha*, which were demonstrated by the authors, are summarized as follows:

1. The specific growth rate of *R. eutropha* was considerably affected by the concentration of dissolved oxygen. The specific growth rate decreased with the increase in dissolved oxygen concentration. The maximum specific growth rate of 0.42 h^{-1} was obtained at a concentration of dissolved oxygen of 5.0 kPa. *R. eutropha* could not grow at a dissolved oxygen concentration higher than 30 kPa (Ishizaki and Tanaka 1990a).
2. When the concentration of dissolved oxygen decreased below 3.2 kPa, oxygen became a limiting factor in the culture. Cell growth followed linear growth kinetics under oxygen-limited condition (Ishizaki and Tanaka 1990a, b).
3. Under oxygen-limited conditions, *R. eutropha* accumulated polyester in the cells, while the formation of cell protein and nucleic acids almost stopped. NMR analysis revealed that the polyester was the homopolyester of D-3-hydroxybutyric acid, P(3HB). In autotrophic culture, the production rate of P(3HB) by ammonium limitation, which is usually used as the best regime to promote P(3HB) accumulation, was lower than that by oxygen limitation (Ishizaki and Tanaka 1991).
4. The stoichiometric formulae for the formation of exponentially growing cells and P(3HB) were determined to be as follows (Ishizaki and Tanaka 1990a, 1991) for cell mass:

$$21.36H_2 + 6.21O_2 + 4.09CO_2 + 0.76NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76}$$

Table 1 List of methods for the autotrophic, high-density cultivation of hydrogen-oxidizing bacteria

Strain	Culture method	Cell concentration ($g\ l^{-1}\cdot h^{-1}$)	Cell productivity ($g\ l^{-1}\cdot h^{-1}$)	Reference
Ralstonia eutropha	Batch	25.0	1.00	Repask and Meyer 1976
Pseudomonas hydrogenovora	Batch	24.0	0.50	Goto et al. 1977b
Ralstonia eutropha	Continuous	–	0.40	Morinaga et al. 1978
Pseudomonas hydrogenothermophila	Continuous	–	3.00	Igarashi 1986
Alcaligenes hydrogenophilus	Continuous	–	0.33	Miura et al. 1982

and for P(3HB):

$$33H_2 + 12O_2 + 4CO_2 \rightarrow C_4H_6O_2 + 30H_2O$$

5. The amount of H_2 required for the fixation of CO_2 during the P(3HB) accumulation phase was 1.5 times larger than that during the exponential growth phase. However, according to the result of the analysis of ATP pool level in the cells, it was deduced that the ATP yield for the oxidation of 1 mol of H_2 was almost the same in both culture phases. It was suspected that the increase in the consumption of H_2 during P(3HB) accumulation under oxygen-limited condition was due to a decrease in the efficiency of ATP utilization in P(3HB) synthesis (Tanaka et al. 1992).
6. The relationship between the overall volumetric coefficient of mass transfer for hydrogen, $(K_La)_{H2}$, and that for oxygen, $(K_La)_{O2}$, was expressed by the following formula:

$$(K_La)_{H2}=0.280(K_La)_{O2}^{1.29}$$

The critical concentration of dissolved hydrogen for *R. eutropha* was also determined to be 11.6 kPa, using an on-line dissolved-hydrogen sensor (Takeshita et al. 1993a, b).

The biotechnological information described above was essential for designing a practical culture system of hydrogen-oxidizing bacteria to attain a high cell density with high cell productivity for the mass production of P(3HB) from CO_2.

Strategy to ensure high-density culture and to prevent the gas mixture from exploding

In autotrophic cultivation of hydrogen-oxidizing bacteria, it is not easy to attain a high cell concentration and/or high cell productivity, because the substrate gas mixture is insoluble in the culture medium and is very explosive. As shown in Table 1, in only a few cases were relatively high concentrations and productivities of cells obtained. In the autotrophic culture of hydrogen-oxidizing bacteria, the productivity of cell mass depends on the mass transfer rate of substrate gasses in the culture medium. Especially, P(3HB) accumulation is remarkably inhibited if dissolved hydrogen in the culture system is limited (Ishizaki and Tanaka 1991). Only an improvement in the coefficient of gas-mass transfer of the fermenter can enable an increase in the yield and productivity of cell mass. The improvement of gas-mass transfer is particularly important in the fermentation process to prevent the gas mixture from exploding. For a practical application of the autotrophic cultivation of hydrogen-oxidizing bacteria for the production of P(3HB) from CO_2, it is absolutely essential to eliminate the potential of gas explosion. The strategy to prevent explosion is to maintain the oxygen concentration in the gas phase below the lower limit for explosion [6.9% (v/v)]. However, in such a low oxygen concentration, the productivity of the cell mass remarkably decreases. Therefore, a fermenter attaining a high value of K_La is essential for the effective production of PHA from CO_2, employing hydrogen-oxidizing bacteria.

The work to prevent gas explosion in an autotrophic high-cell-density culture of hydrogen-oxidizing bacteria for the production of P(3HB) from CO_2 was mainly carried out by Tanaka and his colleagues (Sugimoto et al. 1999; Taga et al. 1997; Tanaka et al. 1993, 1995). They designed an explosion-proof fermentation bench-plant which was installed with several security devices and countermeasures; and they investigated P(3HB) production using *R. eutropha*, while maintaining the oxygen concentration in the gas phase below the lower limit for explosion [6.9% (v/v)]. A fermenter equipped with a specially designed, doughnut-shaped agitation system (K_La=2,970 h^{-1}) was used to obtain a high oxygen transfer rate at such a low oxygen concentration. They obtained 91.3 g cells l^{-1} and 61.9 g $3HB\cdot l^{-1}$ after 40 h of cultivation, while the oxygen concentration in the gas phase was maintained below 6.0% (v/v). The overall productivities of cells and P(3HB) were 2.28 $g\ l^{-1}\cdot h^{-1}$ and 1.55 $g\ l^{-1}\cdot h^{-1}$, respectively.

Since a very high performance agitation system was essential in the fermentation plant, the authors developed a new culture method to obtain a relatively high productivity of cells and P(3HB), using a conventional jar fermenter (K_La=340 h^{-1}). The new culture method was named the two-stage culture method (Tanaka and Ishizaki 1994). In this culture method, *R. eutropha* was heterotrophically grown in a fructose/mineral medium with aeration; and the cells were then cultivated in an inorganic medium for autotrophic P(3HB) accumulation by feeding the gas mixture with an oxygen concentration below 6.9%. Using 10, 20 and 30 $g\ l^{-1}$ fructose medium in the first stage, an average P(3HB) productivity of 0.56 $g\ l^{-1}\cdot h^{-1}$ was obtained in the autotrophic stage. The P(3HB) contents in the cells were 55.7–82.1% (w/w).

P(3HB) production from CO_2 by chemostat culture

Continuous culture of hydrogen-oxidizing bacteria in autotrophic conditions were reported by several researchers (Ammann et al. 1968; Miura et al. 1982; Nishimura et al. 1991), although they were not focused on the production of P(3HB) but cell protein. In the study of an autotrophic chemostat culture of *R. eutropha* carried out by Morinaga et al. (1978), cell productivity was 0.2 g $l^{-1}\cdot h^{-1}$ and the percentage of P(3HB) in the cells was 20% (w/w) under oxygen-limited conditions. According to the report by Siegel and Ollis (1984), the P(3HB) content in the cells in autotrophic chemostat culture was 20% (w/w) at maximum.

We also investigated the continuous production of P(3HB) from CO_2 by two-stage culture, using an air-lift fermenter in the autotrophic stage (Taga 1996). An air-lift fermenter does not require mechanical agitation; and thus the energy consumption is lower than that of a stirred-tank fermenter. In this culture method, heterotrophic chemostat culture in the first stage was carried out by feeding fructose medium at a working volume of 100 ml and at the dilution rate of 0.1 h^{-1}. The culture broth from the jar fermenter was transferred into the air-lift fermenter by a micro-tube pump; and chemostat culture was carried out for autotrophic P(3HB) accumulation. The working volume in the air-lift fermenter was 1.0 l and the dilution rate in the second stage was then 0.01 h^{-1}. When 10 g fructose medium l^{-1} was fed in the first stage, the P(3HB) content in the cells reached 57.6% (w/w). However, the highest P(3HB) productivity was only 0.025 g $l^{-1}\cdot h^{-1}$. The lower productivity in chemostat culture, compared to the batch culture, was thought to be due to the limited mass transfer of oxygen in the second stage (the value for $K_L a$ of the air-lift type fermenter was determined to be only 100 h^{-1}).

Hence, we used a newly designed air-lift fermenter with a draft tube and an HPLC filter as sparger; and we added a slight amount of carboxymethylcellulose (CMC) to culture medium to increase the $K_L a$ (Taga et. al. 1997). Some kinds of surface-active reagents are known to increase the oxygen transfer rate (Bailey and Ollis 1986; Pirt 1975). Many researchers have reported that the gas hold-up increases in the presence of CMC in the air-lift fermenter (Deckwer and Schumpe 1993; Kennard and Janekeh 1991; Schumpe and Deckwer 1982). The rheological change in the culture liquid in air-lift fermenters after adding surface-active reagents like CMC affects bubble formation, flow pattern and the mass transfer coefficient of oxygen (Kennard and Janekeh 1991). In our study, the $K_L a$ value of the air-lift fermenter, which was measured by a static method, increased to 375 h^{-1} by the addition of 0.05% (w/w) CMC to the culture medium, while the $K_L a$ value without CMC addition was ca. 250 h^{-1}. Actually, after the addition of 0.05% (w/w) CMC into the culture medium, the concentration and productivity of P(3HB) increased to 56.4 g l^{-1} and 1.02 g $l^{-1}\cdot h^{-1}$, respectively. The content of P(3HB) in the cells increased to 81.4% (w/w). The fermentation result was superior than those of any previous reports for autotrophic P(3HB) production using conventional stirred-tank fermenters (Ishizaki and Tanaka 1991; Morinaga et al. 1978; Siegel and Ollis 1984; Tanaka and Ishizaki 1994; Vollbrecht et al. 1979).

Conclusion

Many attempts at the effective production of PHA with very high yield, coupled with high productivity, from organic carbon sources have been attained using only fed-batch culture. In most of the studies on continuous culture, the productivity and yield of PHA were found to be much lower than the values for fed-batch culture. As shown in Tables 2 and 3, the highest productivity of PHA obtained by heterotrophic continuous culture is about 1.0 g $l^{-1}\cdot h^{-1}$, but the value is considerably lower than those obtained by fed-batch cultures. Also, in continuous autotrophic culture by our two-stage method, the productivity of P(3HB) was about 1.0 g $l^{-1}\cdot h^{-1}$. In continuous cultures, which were carried out without regard to autotrophic or heterotrophic conditions, the productivity of PHA was lower than that in fed-batch culture. The reason why the productivity in continuous culture was lower than in fed-batch culture can be explained by the mechanism of accumulation of PHA in

Table 2 Polyhydroxyalkanoate (PHA) production from organic carbon sources by heterotrophic continuous culture. *3HD* D-3-Hydroxydecanoate, *3HDD* D-3-hydroxyhexadecanoates, *3HDDE* D-3-hydroxydodecenoate, *3HHx* D-3-hydroxyhexanoate, *3HO* D-3-hydroxyoctanoate, *3HTDE* D-3-hydroxytetradecenoate, *3HV* D-3-hydroxyvalerate

Bacterium used	Carbon source	Type of PHA	PHA productivity (g $l^{-1}\cdot h^{-1}$)	Reference
Ralstonia eutropha	Fructose and petanonic acid	3HV-*co*-3HV copolymer	0.31	Koyama and Doi 1995
Pseudomonas putida KT2442	Long chain fatty acids	3HHx-*co*-3HO-*co*-3HD-*co*-3HDD-*co*-3HDDE-*co*-3HTDE	0.69	Huiberts and Eggink 1996
P. oleovorans	*n*-Octane	Copolymer of 3-hydroxyalkanoates	0.58	Preusting et al. 1993
P. oleovorans	*n*-Octane	Medium-chain-length copolymer	1.06	Jung et al. 2001
P. oleovorans	*n*-Octane	Long-side-chain copolymer	0.18	Ramsay et al. 1991
R. eutropha	Lactate	P(3HB)	0.38	Henderson and Jones 1997

Table 3 PHA production from organic carbon sources by heterotrophic fed-batch culture

Bacterium used	Type of PHA	PHA yield ($g\ l^{-1}$)	PHA productivity ($g\ l^{-1}\cdot h^{-1}$)	Reference
Protomonas extroquens	P(3HB)	136.0	0.78	Suzuki et al. 1986
R. eutropha	P(3HB)	121.0	2.42	Kim et al. 1994
R. eutropha	3HB-*co*-3HV copolyner	90.4	1.81	Lee et al. 1995
Recombinant *Escherichia coli*	P(3HB)	98.7	4.94	Wang et al. 1997b
Alcaligenes latus	P(3HB)	68.4	4.00	Yamane et al. 1996
Recombinant *E. coli*	3HB-*co*-3HV copolymer	158.8	2.88	Choi and Lee 1999b

the cells: Most of the PHA-producing bacteria accumulate PHA under growth-limited conditions, due to a shortage of some nutrient, but hardly accumulate any PHA in the exponential growth phase. Therefore, in continuous culture, a high PHA content in the cells can be obtained only at a very low dilution rate, resulting in a low PHA productivity. Ramsay et al. (1990) pointed out that *A. latus* would be suited for polymer production in single-stage continuous culture, because it accumulates PHA in the cell to a high content, even during the exponential growth phase. This bacterium is also a hydrogen-oxidizing bacterium. If *A. latus* is used as the producer strain for P(3HB) production from CO_2 by chemostat culture, the productivity will be further improved at the higher dilution rate, with higher P(3HB) content. However, information on the growth characteristics of *A. latus* in autotrophic culture condition is very limited (the growth rate is thought to be apparently lower than that of *R. eutropha*). The biotechnological investigation on autotrophic culture of *A. latus* for PHA production from CO_2 will be a future task.

Although the cost of polyolefins is less than U.S. \$1 kg^{-1} (Poirier et al. 1995), the price of PHA is much higher than that of synthetic plastics. However, recent calculations suggest that the price of PHA will be further reduced by an advanced fermentation strategy. Lee et al. (1999) reported that P(3HB) and medium-chain-length PHA can be produced at a cost of ca. U.S. \$2 kg^{-1} by an effective production strategy to attain a productivity greater than 4 $g\ l^{-1}$ at a content of 80% of dry cell weight with a cheap carbon source. The improvement of the polyester content in the cell and productivity is the key for the practical application of P(3HB) production from CO_2. Industrial applications for *A. latus* and other hydrogen-oxidizing bacteria for PHA production from CO_2 are expected in the near future.

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