Environment and Ecology Research 4(4): 231-236, 2016
DOI: 10.13189/eer.2016.040407 http://www.hrpub.org

Autotrophic Growth of *Paracoccus denitrificans* in Aerobic Condition and the Accumulation of Biodegradable Plastics from CO_2

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Abstract The cell growth on H_2 and O_2 as the energy source and CO_2 as the sole carbon source in the autotrophic culture condition was tested for the gram-negative bacteria *Paracoccus* spp. The aerobic growth in the autotrophic condition was only observed in *Paracoccus denitrificans* NBRC13301 and *P.pantotrophus* NBRC102493. Both strains were sensitive to O_2 in particular the growth of *P.pantotrophus* was completely inhibited at the concentrations above 2% O_2. *P.denitrificans* grew until 15% O_2 however the optimum O_2 concentration was 5%. The growth characteristic of *P.denitrificans* was investigated by pH-controlled batch culture with supplying the gas mixture of H_2, O_2 and CO_2. The specific growth rate was 0.12 h^{-1} at 5% O_2, 30°C and pH7.0. The growth was much slower than other hydrogen-oxidizing bacteria however *P.denitrificans* accumulated biodegradable plastic, polyhydroxybutyrate, PHB with the content of 57.3% w/w in the cell under nitrogen limitation. Under DO limitation, the cell concentration increased to 25 g/L without accumulating PHB if NH_3 solution as the nitrogen source was fed sufficiently.

Keywords Polyhydroxyalkanoates, *Paracoccus*, Hydrogen-oxidizing Bacterium

1. Background

Paracoccus spp. is the gram-negative bacteria possessing the ability for denitrification (reduction of NO_3^- to N_2). They play an important role in the nature. Especially, *P.denitrificans* is employed in the biotreatment of waste water. Genome of this bacterium was sequenced in 2004 [1]. *Paracoccus* spp. accumulates poly-β-hydroxybutyrate (PHB) or the copolyester of β-hydroxybutyrate and other hydroxyalkanoate (PHA) in their cells and the polyester is expected as biodegradable plastics [2]. Some strains of *Paracoccus* spp. grow with CO_2 as carbon source, and H_2 or thiosulfate as energy source in autotrophic condition. Many researchers have been reported about *Paracoccus* spp., however in most of the researches for the autotrophic culture using the inorganic gasses as the substrate was studied only for denitrification in anaerobic condition, and the accumulation of PHB or PHAs was studied only in heterotrophic culture using organic compounds as the substrate, for example feeding mixed substrate of acetic acid and valeric acid [3], glycerol [4], methanol and *n*-amyl alcohol [5]. We studied the growth characteristics of *Paracoccus* spp. in the autotrophic condition using the gas mixture of H_2, O_2 and CO_2 as the substrate, and high cell density culture and the accumulation of PHB from CO_2.

2. Methods

The bacteria used in this study were *Paracoccus denitrificans* NBRC13301, *P.alcaliphilus* JCM7364^T, *P.pantotrophus* NBRC102493, *P.thiocyanatus* NBRC14569, *P.aminophilus* NBRC16710, *P.aminovorans* NBRC 16711 and *P.versutas* NBRC14567. All the strains were purchased from NBRC (NITE Biological Resource Center, National Institute of Technology and Evaluation, Tokyo) or JCM (Japan Collection of Microorganisms, RIKEN, Tsukuba, Japan). These bacteria were subcultured every three weeks on BY medium or PYG medium agar plates. The composition of BY media was meat extract 5.0g, peptone 5.0g, NaCl 2.5g, K_2HPO_4 0.1g, $MgSO_4 \cdot 7H_2O$ 0.2g, yeast extract 5.0g and agar 15g per 1 L of distilled water (pH7.2); and that of PYG medium was peptone 5.0g, yeast extract 5.0g and glucose 5.0g per 1 L (pH7.0).

Basic composition of mineral medium for autotrophic

culture was $(NH_4)_2SO_4$ 3.0g, KH_2PO_4 4.0g, $NaHPO_4$ 0.8g, $NaHCO_3$ 1.0g, $MgSO_4 \cdot 7H_2O$ 0.2g and 1 L distilled water. The pH was adjusted to 7.0 with 1 M NaOH then it was autoclaved 20min at 120°C (Mg salt was autoclaved separate from other salts). After cooling, 0.1 mL of filter-sterilized trace elements solution was added to the medium. Composition of the trace elements solution was $CoCl_2$ 119mg, $FeSO_4 \cdot 7H_2O$ 16.2g, $NiCl_2 \cdot 6H_2O$ 118mg, $CrCl_2 \cdot 6H_2O$ 133mg, $CuSO_4 \cdot 5H_2O$ 156mg, $CaCl_2 \cdot 2H_2O$ 10.3g and citric acid 15.6g in 100 mL of 1 M HCl, which was originally used for a hydrogen-oxidizing bacterium, *Ralstonia eutropha* [6].

Autotrophic culture experiment was carried out carefully using the explosive gas mixture of H_2, O_2 and CO_2 as described below [7]. The bacteria were refreshed on the BY or PYG agar plate, and further inoculated into 20mL of the mineral liquid medium in 300-mL Erlenmeyer flask. The flask was plugged with a rubber stopper which was inserted with a glass tube connected to a silicone tube and a sterile membrane filter (pore size, 0.2 μm). The inside of the flask was vacuumed and then it was filled with H_2, O_2 and CO_2 from each gas cylinder. Basic composition of the substrate gas mixture was H_2 80%, O_2 10%, and CO_2 10%. Cultivation was carried at a temperature of 30°C and a reciprocal shaking of 200 rpm. High cell density culture experiment was carried out using a glass jar fermenter (total volume 1000 mL; working volume 600 mL) equipped with a pH controller, PHC-2201 and a dissolved oxygen (DO) meter, DJ-1033 (Biott Co., Ltd., Tokyo). Batch culture was performed with a recycled-gas closed-circuit culture system. The substrate gas mixture in the gas chamber was supplied to the fermenter at a flow rate of 0.5vvm and the exhausted from the fermenter was returned to the chamber. The agitation speed was kept at 1200 rpm. As the cell concentration increases, the foaming of culture liquid becomes vigorous and it causes the flow out of culture liquid from the fermenter. Then, a defoaming agent (Einol, Biott Co. Ltd., Tokyo) was used in high cell density culture.

Cell growth was monitored by measuring turbidity (OD_{600nm}) of the culture liquid. The composition of the substrate gas mixture in the flask and the fermentation system was measured by a gas chromatograph (Shimadzu type GC-8A) using a column 4mm x 6m into which a molecular sieve 5A and a Porapack Q were packed. Total ammonia (NH_4-N) in culture liquid was determined by indophenol blue reaction. PHB accumulated in the cells was determined according to the method using gas chromatography [8]. The lyophilized cells, a mixture of 2 mL of methanol acidified with 3%(w/v) H_2SO_4 and 2 mL of chloroform were added into a screw cap vial then it was heated at 100°C for 3.5 h for degradation of PHB, and esterification of hydroxybutyric acid and methanol. After cooling, 1 mL of H_2O was added into the vial then the suspension was shaken well for 10min. After two phases were allowed to separate, the organic phase containing the methyl ester was applied to gas chromatography.

3. Results

3.1. Growth of *Paracoccus* Strains

Growth of *Paracoccus* spp. in the autotrophic condition was tested by flask culture. The head space inside the culture flask was filed with the gas mixture with a ratio $H_2/O_2/CO_2$ = 8:1:1 before the start of cultivation. After 7 days cultivation, the increase in the turbidity of culture liquid and the consumption of substrate gas (a decrease in pressure of head space gas inside the flask) were observed only in *P.denitrificans* NBRC13301. There was no growth in the other *Paracoccus* spp. It is known that many strains of hydrogen-oxidizing bacteria are sensitive to O_2 although they are aerobic bacteria. Then, the culture test was carried out again with lowering O_2 concentration in the substrate gas mixture (Table). The cell growth was observed in *P.denitrificans* NBRC13301 and *P.pantotrophus* NBRC102493. *P.denitrificans* grew from 5 to 15% O_2 and *P.pantotrophus* did only at 2% O_2.

Table 1. Growth of *Paracoccus* spp. at various concentrations of O_2 in autotrophic culture condition. The increase in cell growth (OD_{600}) after 48 h from the inoculation was compared (n=2).

Species	O_2 concentration (%) in substrate gas mixture					
	0	2	5	10	15	20
P. denitrificans NBRC13301	0	1.21	4.85	3.89	2.93	0
P. alcaliphilus JCM7364	0	0	0	0	0	0
P. pantotrophus NBRC102493	0	1.19	0	0	0	0
P. thiocyanatus NBRC14569	0	0	0	0	0	0
P. aminophilus NBRC16710	0	0	0	0	0	0
P. aminovorans NBRC 16711	0	0	0	0	0	0
P. versutas NBRC14567	0	0	0	0	0	0

These two strains, in particular *P.pantotrophus* was much more sensitive to O_2 than other hydrogen-oxidizing bacterium like *Ralstonia eutropha* and *Alcaligenes latus*, *Ideonella dechloratans* [9] and *A.hydrogenophilus* [10].

3.2. Growth Condition for *P.dentrificans*

P.denitrificans NBRC13301 showed the best growth among the *Paracoccus* spp. in the autotrophic condition. Hence, the growth characteristics of *P.denitrificans* were investigated in detail. As a result, optimum temperature and pH were 30°C and pH 7.0, respectively. Among the nitrogen sources of nitrate, ammonium salts and urea, the best growth was obtained with $(NH_4)_2SO_4$. There was no growth with urea (the data are not shown). Figure 1 shows a fermentation time course of *P.denitrificans* NBRC13301 in flask culture.

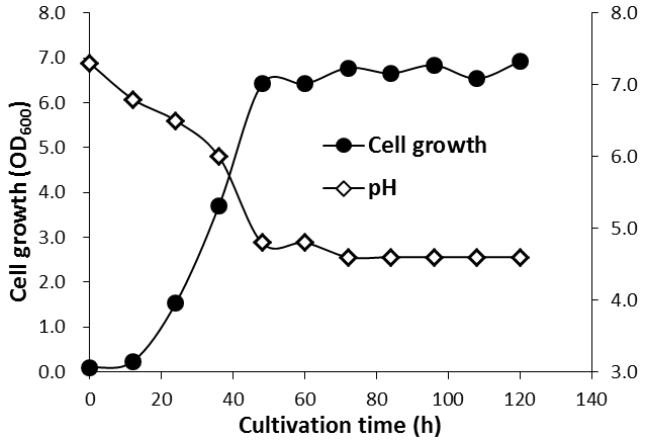

Figure 1. Time course for flask culture of *P. denitrificans* NBRC13301 in autotrophic condition using the substrate gas mixture with the ratio of $H_2/O_2/CO_2$= 85:5:10.

The head space substrate gas in the flask was refiled several times during the cultivation however the cell concentration was not over 8.0 in OD_{600} with any medium composition. The OD_{600}= 8.0 was almost equivalent to about 3.2 g/L of dry cell weight. It was considered that the cell growth was inhibited by the decrease in pH of culture liquid.

3.3. High Cell Density Culture of *P.dentrificans* and PHB Accumulation from CO_2

High cell density culture of *P.denitrificans* NBRC13301 was examined using a pH-controller and a glass jar fermenter. The recycled-gas closed-circuit culture system was used because the high performance of gas utilization. The pH of culture liquid in the fermenter was maintained around 7.0±0.2 by feeding 4% w/v NH_3 solution or 1M NaOH automatically. Cell concentration was determined by measuring OD_{600} of the culture liquid and dry weight of the centrifuged cells. The substrate gas mixture was supplied into the culture medium from a gas chamber (total volume, ca.15 L) using an airtight air pump. The exhausted gas from the fermenter was returned to the gas chamber for reuse. The inner pressure of the gas chamber was maintained at almost constant level because saturated saline water was voluntarily introduced according to the decrease in the volume of substrate gas mixture [7]. Figure 2 (a) shows the fermentation time course of the strain NBRC13301 at pH7.0 with feeding 4% w/v NH_3 solution. Cultivation was started by recycling the substrate gas with the volume ratio of $H_2/O_2/CO_2$= 85:5:10 within the culture system. As the fermentation proceeds, the gas composition in the culture system changed, especially O_2 concentration sharply decreased, the gas mixture in the chamber was exchanged several times to reset to the initial composition. The cells multiplied at the specific growth rate of approximately 0.12 h^{-1} during the exponential growth phase. After 42 h from the start of cultivation, DO decreased to almost 0ppm (the data is not shown), the exponential growth ceased, then the cell concentration increased almost linearly with the time. When the amount of cells increased to the dry weight concentration of 8.12 g/L after 120 h, we stopped the cultivation because it was too difficult to maintain O_2 concentration of gas phase in the culture system at 5%. The cell concentration increased to about 2.5 times as that of the flask culture. Accumulation of PHB in the cells was slight throughout the cultivation when feeding 4% w/v NH_3 solution. The culture liquid at the end of cultivation contained sufficient amount of NH_4^+ and PO_4^{3-} for vigorous multiplication of the cell. Figure 2(b) shows the fermentation time course with feeding 1M NaOH instead of NH_3 solution for pH control. When NH_4^+ was exhausted after 45 h, the cells began to accumulate PHB. The PHB content of the cells increased to 57.3% w/w at the end of cultivation. It is known that PHB generally accumulates in nitrogen- or DO-limited culture condition. Our results indicate that in case of *P.denitrificans*, PHB accumulation is not stimulated under DO limitation but nitrogen limitation.

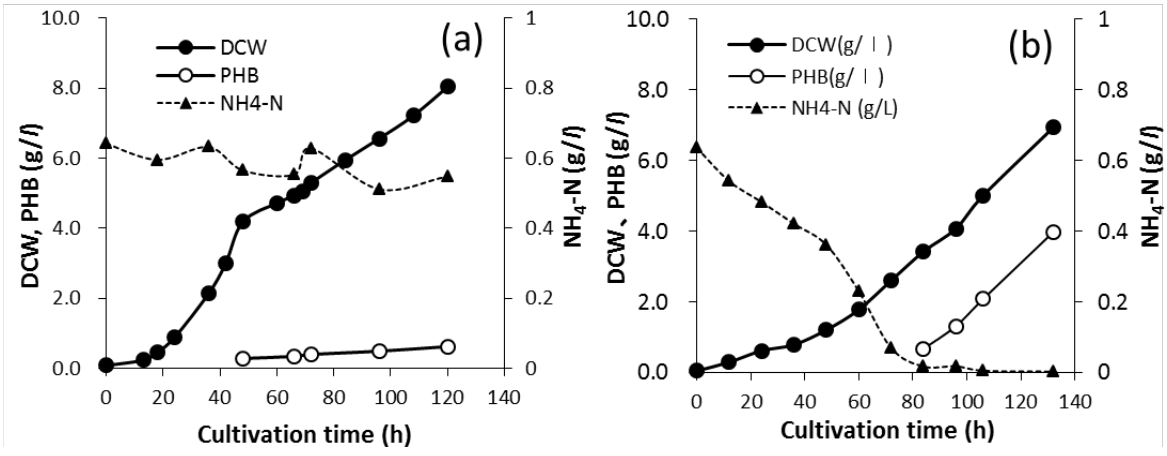

Figure 2. Time course for pH-controlled autotrophic culture of *P.denitrificans* NBRC13301 using jar fermenter and recycled-gas closed-circuit culture system. pH was controlled during the cultivation by feeding 4% NH_3 solution (a) and 1MNaOH (b)

Figure 3 shows the fermentation time course with exchanging the substrate gas mixture in the gas chamber every 12 h during cultivation. The pH was controlled by feeding 4% w/v NH_3 solution. The gas mixture with the volume ratio of $H_2/O_2/CO_2$ = 85:5:10 was used from the start of cultivation until 50h, then the ratio was changed to $H_2/O_2/CO_2$ = 80:10:10. After 80h, the volume ratio of the newly prepared gas mixture was changed to $H_2/O_2/CO_2$ = 75:15:10 and the ratio was used until the end. As a result, DO was almost kept at 0ppm after 40 h (the data is not shown) however the cells continued to increase for the long DO limitation. When the cell concentration increased to about 25 g/L after 140 h, we stopped the cultivation because foaming of the culture liquid was so vigorous that we could not prevent the culture liquid from flowing out of the fermenter. The PHB accumulation was very small because the nitrogen source in the culture liquid was sufficient throughout the cultivation by feeding NH_3 solution.

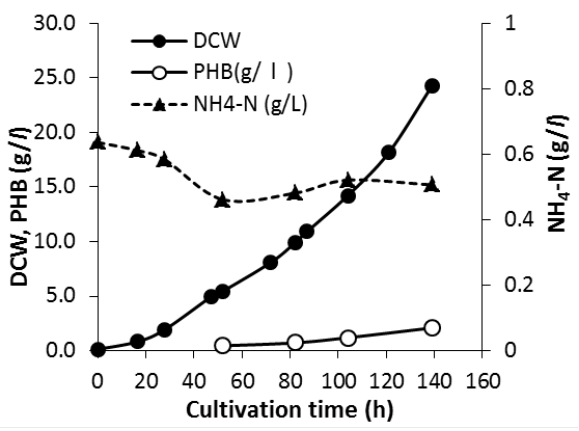

Figure 3. Time course for pH-controlled autotrophic culture of *P.denitrificans* NBRC13301 using jar fermenter and recycled-gas closed-circuit culture system. The O_2 concentration in the substrate gas mixture was raised from 5% at the start to 10% after 50h then to 15% after 80h. pH was controlled during the cultivation by the feeding 4% NH_3 solution.

3.4. Discussion

Our research showed that among the 7 strains of *Paracoccus* spp. tested, *P.denitrificans* NBRC13301 and *P.pantotrophus* NBRC102493 grow in the autotrophic culture condition using the gas mixture of H_2, O_2 and CO_2 as the substrate. However, both strains were very sensitive to O_2 especially *P.pantotrophus* did not grow with the gas mixture containing O_2 above 2%. On the other hand, *P.denitrificans* grew at O_2 concentrations up to 15% and the optimum O_2 concentration for the cell growth was 5%. The specific growth rate of *P.denitrificans* in the autotrophic condition was 0.12 h^{-1} at 5% O_2. The growth of *P.denitrificans* was much slower than other hydrogen-oxidizing bacteria, *R.eutropha*, *A.latus*, *I.dechloratans* [9] and *A.hydrogenophilus* [10]. The specific growth rate of *R.eutropha* at 5% O_2 was 0.42 h^{-1} [7]. It is reported that in case of *R.eutropha* (*A.eutrophus*), the soluble NAD^+-reducing hydrogenase was inactivated by O_2 when H_2 is available as electron donor [11].

PHB and the copolyester of D-3-hydroxybutric acid and other hydroxyalkanoic acid, PHA are expected as the biodegradable "Green plastics". However, most of the researches for microbial production of PHAs are investigated on organic acids, fatty acid and plant oil as the substrate, especially by using recombinants of *R.eutropha* or *Escherichia coli* with improved the intracellular content and the monomer composition of polyester in recent years [12-15]. Biosynthesis of copolymer from "unrelated carbon source" like glucose is also studied using engineered strains [16-22]. There are only several researches reported for the autotrophic production from CO_2 [23]. We have been studied the production of PHB from CO_2 employing several strains of hydrogen-oxidizing bacteria [9, 24-27]. In our study, 60g/L of the cells with 36g/L of PHB were obtained in autotrophic culture of *R.eutropha* with the conventional type jar fermenter in 60 h [24]. Furthermore, the use of a basket-type agitator with very high performance for oxygen transfer (k_La, 2970 h^{-1}) enabled to produce 91.3 g/L of the cells with 61.9 g/L of PHB in 40 h in spite of supplying the substrate gas mixture with the lower O_2 concentration below the "lower explosion limit" was supplied [25]. PHB can be produced from CO_2 even in the presence of high concentration of carbon monoxide (CO) by employing the CO tolerant hydrogen-oxidizing bacterium *Ideonella* sp.O-1 that we isolated [9]. Volova et al. also reported the production of a copolymer containing β-hydroxybutyrate more than 99 mol% from CO_2 in model gas mixture containing CO by employing *R.eutropha* B5786 [28].

In this study, it was shown that the cell growth of *P.denitrificans* in autotrophic condition is more sensitive to O_2 and slower than other hydrogen-oxidizing bacteria. However, PHB accumulated in the cells with the content of 57.3% w/w under nitrogen limitation while it did not under DO limitation. In the practical fermentation process for the production of PHB employing hydrogen-oxidizing bacteria in commercial scale, O_2 concentration of the substrate gas mixture must be maintained lower than 6.9% to prevent the explosion of H_2 [25]. The condition does not exclude *P.denitrificans* from the candidate strains for PHB producer. It will be possible to achieve high PHB yield from CO_2 employing *P.denitrificans* if the fermenter like the basket-type agitator to give high performance for oxygen transfer. On the other hand, some strains of this species are already used for the commercial production other useful substances for instance vitamin B_{12} (cyanocobalamin) from methanol. The ability of *P.denitrificans* to continue the cell growth without PHB accumulation under DO limitation will be beneficial for production of commercially useful substances except PHAs from CO_2.

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