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Batch Culture of *Alcaligenes eutrophus* ATCC 17697^T Using Recycled Gas Closed Circuit Culture System

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Batch culture of *Alcaligenes eutrophus* ATCC 17697^T using the recycled gas closed circuit culture system was done with the intention of developing a practical fermentation system for industrial culture of autotrophs. The gas phase of the culture system consisted of substrate gas so that gases in this culture system could be recycled forever as long as the amount of the gas consumed would be refilled. All gasses supplied into this system could be completely used without any loss as exhaust. Thus, this system assures high gas usage efficiency as well as operation safety. Studies on the effect of oxygen concentration showed the high oxygen concentration in substrate gas mixture suppressed the specific growth rate while a low oxygen concentration promoted the growth rate. This introduced the possibility of development of an explosion-free fermentation system with a high growth rate if appropriate system design and fermentation conditions were given. Since this system is closed, material balance and elementary analysis provide the ultimately accurate stoichiometry of the autotrophic culture of this bacterium.

Nowadays, carbon dioxide is suspected to be a serious pollutant that causes the greenhouse effect to raise the atmospheric temperature resulting in abnormal weather and elevation of sea water. The approach to tackle this problem is therefore very urgent for mankind and a feasible technology has not yet been established. Although there are many strategies to approach this task, the culture of hydrogen-oxidizing bacteria is a matter worth considering as a possible tool. Although autotrophic culture of this microorganism has been studied almost completely (1-3), the fermentation technology for this microorganism with industrial aspects is still incomplete due to many difficulties. One is detonation and the other is the low gas usage efficiency if the fermentor lets exhaust gas flow out. Two types of closed culture systems were reported; one is the dead-end culture reported by Bongers (4) and the other is the recycled gas culture reported by Schlegel (5) and Kodama *et al.* who used the system for the isolation of strain 9-5 (6). The dead-end culture system must have disadvantages in gas mass-transfer due to lack of aeration. The recycled gas closed culture system can make high gas mass-transfer with recycling of substrate gas to use it up with no loss. In addition, this system allows many other benefits such as operation safety for detonation and continuous operation. According to recent progress, the autotrophic culture of *Alcaligenes eutrophus* gives the possibility of fermentative production of PHB (7), which would be a raw material for biodegradable plastic (8-9). This paper deals with the bioengineering aspects of recycled gas closed circuit culture for industrial application to the culture of hydrogen-oxidizing bacteria.

MATERIALS AND METHODS

Microorganism The microorganism used was *A. eutrophus* ATCC 17697^T.

Media The medium for the refreshment of the working culture consisted of: yeast extract, 5.0 g; Polypepton, 10.0 g; NaCl, 5.0 g; and glucose, 10.0 g in 1 *l* of tap water, adjusted to pH 7.0 with NaOH. The medium for autotrophic growth, both for inoculum preparation in a shaking flask and for main culture in a jar fermentor consisted of: $(NH_4)_2SO_4$, 5.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and $CaSO_4$, 4.0 mg in 1 *l* of tap water, adjusted to pH 7.0 with NaOH. Twenty mg of separately sterilized $FeSO_4 \cdot 7H_2O$ were added to 1 *l* of the medium. The method for feeding gaseous material (CO_2, H_2, and O_2) was described in a separate paragraph. Hydrogen gas of 99.9% purity was provided from a 7,000-*l* cylinder and 99.9% pure oxygen was provided from a 1,500-*l* cylinder. Liquid carbonic acid in a 30 kg cylinder was 99.5% pure.

Cultivation system The flask culture for inoculum preparation was done in a 500-ml shaking flask with a rubber stopper to which an glass tube with a piece of silicone tube sealed with a screwed sealer was inserted. The gas mixture, $H_2 : CO_2 : O_2 = 7 : 1 : 1$, prepared through a simple manifold glassware pipework was introduced into the flask, which was evacuated to 760 mm Hg by a pump. The recycled gas closed circuit culture system was the same as the system reported by Kodama *et al.* (6) using a mini-jar fermentor (total volume 200 ml, Able Co., Ltd., Tokyo) with instrumentation to control culture conditions. The gas mixture was prepared in the gas chamber (15 *l*) to which the gas from the cylinder was introduced. The gas composition was estimated by reading a vacuum gauge during gas filling and finally confirmed by gas chromatography. The gas consumption during fermentation was known by the level change of saline measured from a ruler attached on the wall of the bottle. The whole system should be strictly sealed from any gas leak, otherwise hydrogen introduced in the system will be replaced with air. After inoculation, the cells will grow by consumption of the gas which is recycled between the fermentor and gas chamber. Thus the gas as substrate for this fermentation can be used without any loss because no exhaust gas is produced in this fermentation system.

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Culture conditions The working volume of fermentor was 100 ml. The pH was maintained at 7.0 by automatic addition of 4% ammonia water and the temperature was kept at 30°C. The agitation speed was 1400 rpm and the gas feeding rate was 200 ml/min (equivalent to 2 vvm).

Assurance of no gas leakage Not only for the safety of the operation but also for measurement of gas consumption and kinetics, no gas should be leaked from this fermentation system. All stoppers and connections were carefully sealed by packing materials and bond. To confirm the sealing, the jar and bottle with the stoppers were evacuated and the pressure change was observed for a few hours. After the piping between the jar, bottle, and other instruments was checked, the mixed gas used for fermentation was put into the system without inoculum. Changes in gas composition were looked for to indicate the leakage of the mixed gas.

Analysis The gas composition was analyzed by a Shimadzu Type GC-8A gas chromatograph using a column 4 mm × 6 m into which a molecular sieve 5A and a Porapack Q were packed (10). Dissolved oxygen tension in the culture broth was measured by a membrane electrode S-Type (Able Co. Ltd., Tokyo). Cell growth was monitored by measuring the optical absorbance at 562 nm of the culture broth with the spectrophotometer (Uvidec 320, Jas. Co., Ltd., Tokyo). Cell concentration was converted into dry cell weight according to a calibration curve prepared earlier. Protein in the cells was measured by the Biuret method (11). Analysis of elemental composition of the cell was entrusted to the Kyushu University analysis center.

RESULTS AND DISCUSSION

Batch culture profile of gas recycled system Batch culture was done using the recycled gas closed fermentation system and a typical course of cell growth with the variation of the partial pressure of oxygen of recycled gas and of dissolved oxygen in culture liquid is shown in Fig. 1. After a lag phase, the microorganisms grew with a constant specific growth rate (maximum specific growth rate at the given culture conditions) until the partial pressure of dissolved oxygen (P_L) approached the critical value. When the dissolved oxygen electrode indicated nearly zero, the cell growth was repressed and followed linear growth kinetics to reach approximately 30 g/l of cell concentration, which is higher than the concentration using the ordinary aeration agitation fermentation system reported by Repaske *et al.* (12). The decrease of oxygen concentration in gas phase was continued after P_L indicated nearly zero. Therefore the culture was eventually affected by oxygen limitation.

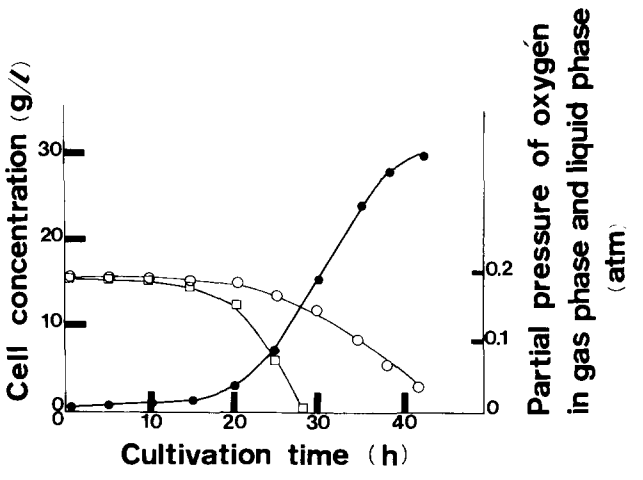

FIG. 1. Autotrophic growth curve of *A. eutrophus* under the gas composition $H_2 : O_2 : CO_2 = 73.0 : 10.0 : 11.0$ in recycled gas closed circuit culture system. Symbols: cell concentration (●), partial pressure of oxygen in gas phase (○), and in liquid phase (□).

Gas consumption in terms of individual substrate gasses *vs.* fermentation time is shown in Fig. 2. The data plotted were restricted to the exponential growth phase and the gas consumption rate for individual substrate gasses during exponential growth phase was maintained constant. Thus, the yield factors for the substrate gasses remained constant for the exponential growth phase.

Effects of oxygen concentration Cultures with different oxygen concentrations in the gas mixture were done to examine the effects of the partial pressure of oxygen in the gas phase. The results are shown in Fig. 3 and the data showed that in the range of very low oxygen concentrations, the specific growth rate increased with increasing the oxygen concentration, but after the maximum

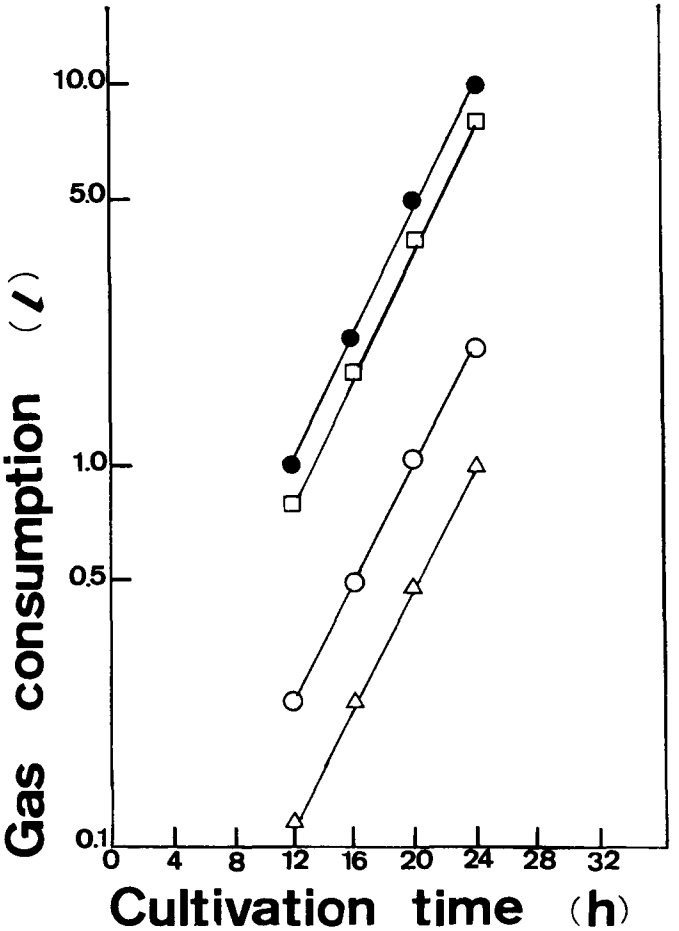

FIG. 2. Linear relationship of gas consumption increase during culture. Symbols: hydrogen (□), oxygen (○), carbon dioxide (△), and gas mixture (●).

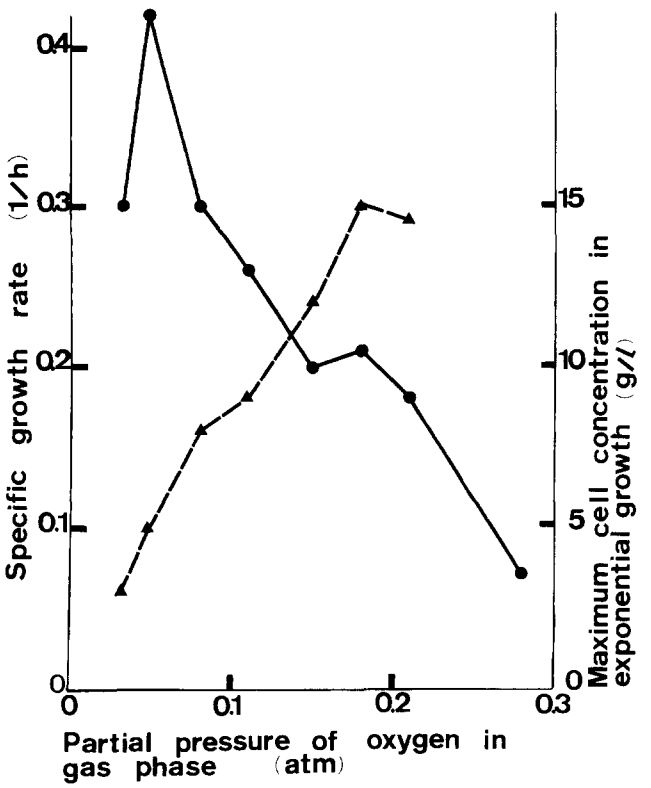

FIG. 3. Effects of partial pressure of oxygen in gas phase on specific growth rate (●) and maximum cell concentration (▲) in exponential growth phase.

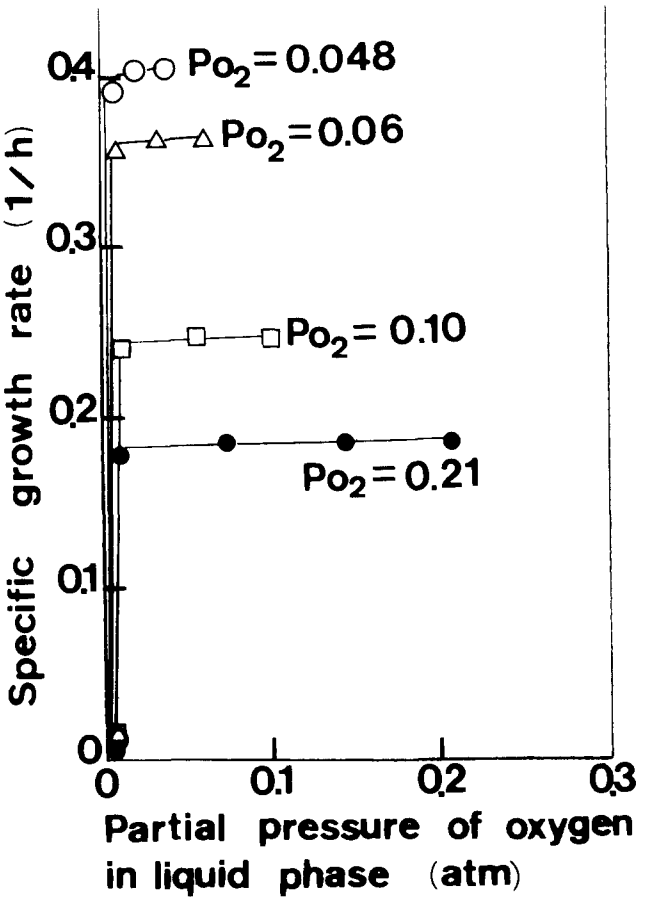

FIG. 4. Effects of partial pressure of oxygen in liquid phase on specific growth rate.

specific growth rate was attained at about 0.05 atm of oxygen partial pressure, the growth rate decreased with increases in the oxygen concentration. When the oxygen concentration was beyond approximately 0.3 atm, the cell growth almost ceased. On the contrary, the maximum cell mass concentration reached at the end of the exponential growth phase was increased in proportion to the increasing in the oxygen concentration. The results observed were similar to the behavior of *Pseudomonas hydrogenovora* reported by Goto *et al.* (13).

There are a number of previous reports regarding the effects of oxygen concentration on the culture of hydrogen-using bacteria (10, 14-16). Figure 4 plots the specific growth rate against the partial pressure of oxygen in the culture liquid (P_L) in same operation. Data for four batches with different initial oxygen concentrations in the substrate gas mixture were plotted and the data for the same batch were indicated by the same symbols in one line. As shown in Fig. 4, the specific growth rate above the critical value of dissolved oxygen concentration depended upon the initial oxygen concentration of the substrate gas mixture and the highest specific growth rate was observed at the low initial oxygen concentration of 0.048 atm. It was observed that when the initial oxygen concentration in gas phase was lower than 25%, the hydrogen concentration in the gas phase increased with decreasing oxygen concentration, while the hydrogen concentration in gas phase decreased with increasing oxygen concentration if the initial oxygen concentration in the gas phase was higher than 25%. Thus, as long as initial oxygen concentration in gas phase was lower than 25%, the culture never reached hydrogen limitation but only oxygen limitation. Since mass-transfer of carbon dioxide was greater than oxygen (17-18), the culture condition given in the experiment shown in Fig. 4 should not reach hydrogen and carbon dioxide limitation. It is also interesting that formation of low P_L by cell growth did not promote the specific growth rate and cell growth was shifted to linear growth from the maximum specific growth rate which was depending upon the initial oxygen concentration in the gas phase.

Thus, the growth of *A. eutrophus* ATCC 17697^T was restricted by oxygen concentration in both culture systems. For a high growth rate, lower oxygen concentration was preferable and for high cell mass concentration, a high oxygen concentration was required. Figure 5 confirmed that a high cell mass concentration was obtained by applying the high oxygen concentration after oxygen limitation was formed.

The fact that low oxygen concentration is favorable for a high growth rate gives great advantages to the industrial application of autotrophic culture of hydrogen-oxidizing bacteria. However, the fermentor for such culture must have a high oxygen transfer efficiency and such a fermentor installed in a recycled gas closed circuit system will provide high cell mass concentration with high growth rate below the lower limit of oxygen concentration for detonation.

Elementary composition of cells and stoichiometry Cells cultivated under different compositions of gas mixture

TABLE 1. Yield factor and recovery rate of carbon and nitrogen in constant with different initial gas compositions

Initial gas composition (%)			Yield facter (g-cells/g-substrate)			Recovery rate of carbon and nitrogen (%)	
H_2	O_2	CO_2	YH_2	YO_2	YCO_2		
73.0	6.0	11.0	1.91	0.62	0.51	90.4	79.9
72.0	8.3	8.3	2.28	0.56	0.55	98.0	95.5
55.0	14.5	9.5	2.43	0.49	0.53	94.7	100.4
59.3	18.5	10.5	2.29	0.41	0.54	96.6	95.6
55.0	24.0	10.0	2.43	0.55	0.55	98.3	95.8

were harvested and the elementary composition analyzed. All the cells harvested were from the exponential growth phase. The elementary composition of the cells of *A. eutrophus* ATCC 17697^T at exponential growth phase was indicated as the formula of $C_{4.09}H_{7.13}O_{1.89}N_{0.76}$, which is a fairly good approximation to the data for the strain 9-5 obtained by Kodama *et al.*(19) using the flask culture. Since this fermentation system is independent of the outside and the substrate gases are recycled in the closed system, all substrates except the chemicals fed as the liquid form of the medium must be retained in the fermentation system. Table 1 shows the recovery rate of all the elements supplied to the fermentation system. As shown in this table, recovery of all the elements was approximately 100% and it was proved that all the elements supplied in the gaseous form were converted into cell mass but no other by-product was formed in the culture liquid under these culture conditions. The stoichiometry of autotrophic culture of *A. eutrophus* ATCC 17697^T was roughly estimated and discussed by Bongers using the dead-end culture method (4). Our results introduced the stoichiometry indicated as the following formula.

$$21.36H_2+6.21O_2+4.09CO_2+0.76NH_3 \longrightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76}+18.7H_2O$$

The results support the estimation proposed by Bongers (4) but are very different from the data reported by Kodama *et al.* (19) on the strain 9-5 and Ohi *et al.* on *A. hydrogenophilus* N34 (20). Provided hydrogen and oxygen are supplied by electrolysis of water, the above stoichiometry can be simplified as;

$$2.43H_2O+4.09CO_2+0.76NH_3+\text{energy} \longrightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76}+4.36O_2$$

where energy is required for electrolysis of water. This equation tells that carbon dioxide fixation by the hydrogen-oxidizing bacteria *A. eutrophus* is similar to photosynthesis which generate equal molecules of oxygen to assimilated carbon dioxide. If energy for electrolysis of water is not provided by a fossil fuel source, the autotrophic culture of hydrogen-oxidizing bacteria may be a feasible method to solve the environmental difficulty caused by carbon dioxide.

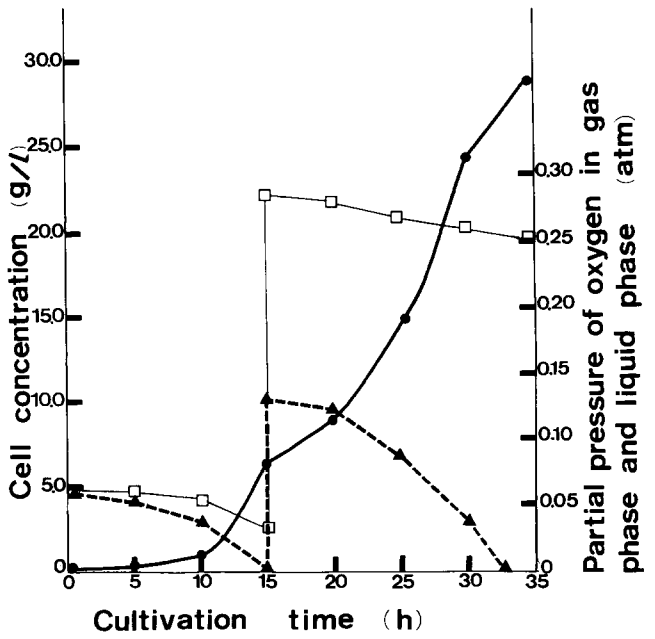

FIG. 5. Effects of gas composition change on microbial growth. Symbols: cell concentration (●), partial pressure of oxygen in gas phase (□), and in liquid phase (▲).

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