Effects of Rheologic Conditions in the Culture Medium on the Autotrophic Production of Poly-(D-3-hydroxybutyric Acid) in an Air-lift Fermentor

Taga, Naohiko
Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Tanaka, Kenji
Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Ishizaki, Ayaaki
Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

https://doi.org/10.5109/24106

出版情報：九州大学大学院農学研究院紀要. 40 (1/2), pp.197-207, 1995-12. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 40 (1・2),197-207(1995)

Effects of Rheologic Conditions in the Culture Medium on the Autotrophic Production of Poly-(D-3-hydroxybutyric Acid) in an Air-lift Fermentor

Naohiko Taga, Kenji Tanaka and Ayaaki Ishizaki

Department of Food Science and Technology, Faculty of Agriculture,
Kyushu University, Fukuoka 812-81, Japan
(*Received July 31, 1995*)

The effects of rheological changes in the culture medium, caused by addition of sodium carboxymethylcellulose (CMC), on the autotrophic production of poly-(n-3-hydroxybutyric acid) [P(3HB)] by *Alcaligenes eutrophus* was investigated in an air-lift fermentor. Addition of 0.05% CMC increased P(3HB) productivity to twice as in the control culture. The effect of CMC on the volumetric mass transfer coefficient, (K_La) and the relationship between K_La and P(3HB) productivity were investigated. The production of P(3HB) rate was not correlated with K_La, when K_La was measured by the sulfite oxidation method, but it was correlated with K_La, when K_La was measured by the static method. Thus, the sulfite oxidation method was unsuitable for measurements of the K_La of this system.

INTRODUCTION

Polyhydroxyalkanoic acid (PHA) has potential utility as a raw material for the synthesis of biodegradable plastics (Anderson *et al.* 1990). A hydrogen-oxidizing bacterium, *Alcaligenes eutrophus*, is able to grow using hydrogen, oxygen and carbon dioxide under autotrophic culture conditions. The microorganism accumulates poly-(D-3-hydroxybutyric acid) [P(3HB)], which is one type of PHA under nutrient- or oxygen-limited conditions. Production of P(3HB) from carbon dioxide could contribute to the solution of two environmental pollution problems simultaneously: the elevation of the concentration of carbon dioxide in the atmosphere and the disposal of non-degradable plastic waste. However, for the practical application of the production of P(3HB) from carbon dioxide by *A. eutrophus*, the danger of a substrate-gas explosion needs to be eliminated from the culture system. To avoid such a gas explosion, the concentration of oxygen (v/v) in the gas phase should be maintained below 6.9% (Ishizaki *et al.* 1993). Unfortunately, the oxygen transfer rate in culture systems becomes very small at such low concentrations of oxygen, with a serious resultant decrease in biomass productivity. Furthermore, a high concentration of P(3HB) cannot be achieved if the exponential growth of cells ceases at when the concentration of cells is still low as a consequence of oxygen limitation: the P(3HB) production rate decreases with the increased accumulation of P(3HB) in the cells and eventually ceases when the cell content of P(3HB) reaches about 90% by weight. To solve these problems, we developed a two-stage culture method that consists of a heterotrophic culture system for cell growth and an autotrophic culture system for production of P(3HB) (Tanaka *et al.* 1994).

In recent years, the air-lift fermentor has often been used instead of the traditional stirred-tank fermentor. Since the air-lift fermentor does not require mechanical agitation, consumption of energy is very low as compared to that by a stirred-tank fermentor.

Moreover, an air-lift fermentor has no sealed junction so that it is easy to avoid contamination. Air-lift fermentors are now used for production of antibiotics in some cases. During fermentation by filamentous fungi that produce antibiotics, the medium sometimes becomes very viscous (König et al. 1982). The effects of high-viscosity medium on the characteristics of air-lift fermentors have been reported (Kennard et *al*. 1991). In the present study, carboxymethylcellulose (CMC) was used to increase the viscosity of the medium. Addition of CMC causes drastic changes in the parameters of a culture system, such as the flow pattern, mixing time, gas hold-up, and/or mass transfer of oxygen. In particularly, many authors have reported that the gas hold-up is increased by the addition of CMC (Schumpe et *al*. 1982, Kennard et *al*. 1991 and Deckwer et al. 1993). Since the increase in gas hold-up is expected to increase the mass transfer of substrate gasses, P(3HB) productivity during autotrophic fermentation might be expected to be increased.

In this study, we investigated the use of an air-lift fermentor for P(3HB) production by *A. eutrophus* under autotrophic condition. The effects of rheological changes in the culture medium caused by addition of CMC on P(3HB) productivity in the air-lift fermentor were also investigated.

MATERIALS AND METHODS

Microorganism

Alcaligenes eutrophus ATCC 17697^T was used throughout this study.

Compositions of media

The medium used for the stock culture, stored at -80°C, and the refreshed culture consisted of meat extract, **7.0 g;** polypeptone, 10.0 g; and NaCl, 5 g in 1 *l* of tap water. The pH was adjusted to 6.8 with 1 N NaOH. The medium for the preculture consisted of $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 5.0 mg; fructose 10.0 g and 1 ml of a solution of trace elements in 1 *l* of distilled water. The pH was adjusted to 6.8 with 1 N KOH. The solution of trace elements consisted of $CoCl_2$, 119 mg; $FeSO_4 \cdot 7H_2O$, 16.2 g; $NiCl_2 \cdot 6H_2O$, 118 mg; $CrCl_3 \cdot 6H_2O$, 153 mg; $CuSO_4 \cdot 5H_2O$, 156 mg and citric acid, 15.6 g in 100 ml of 1 N HCl. For autotrophic cultures, the mineral medium containing without a source of organic carbon was used.

Cultivation system

Figure 1 shows a schematic diagram of the cultivation system used for this study. The culture system was a based on the recycled-gas closed-circuit culture system (Ishizaki et *al.*, 1990). For maintainance of a low concentration of oxygen, to eliminate the danger of a gas explosion, we used the substrate-gas feeding system with a gas composition controller that has been developed by the Research and Development Institute of Saibu Gas Co. Ltd., (Fukuoka, Japan). The system consisted of two gas cylinders with high pressure tolerance. The two gas cylinders contained 99.9% hydrogen and 50% oxygen plus 50% carbon dioxide, respectively, at pressures above 2.0×10^3 kPa. The pressure in the cylinders was reduced to about 30 kPa by a pressure regulator, then

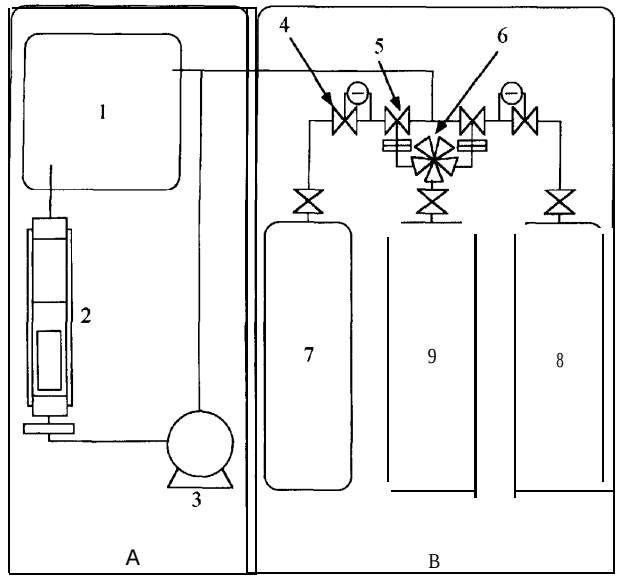

Fig. 1. Schematic diagram of the autotrophic culture system. A: Recycled-gas closed-circuit culture system. B: Substrate-gas feeding system with gas composition controller. 1: Gas reservoir. 2: Air-lift fermentor. 3: Blower. 4: Pressure regulator. 5: Valve driven by pressured nitrogen gas. 6: Five-direction valve with timer control. 7: Gas cylinder containing 99.9% hydrogen. 8: Gas cylinder containing 50% carbon dioxide and 50% oxygen. 9: Gas cylinder containing 99.9% nitrogen.

the substrate gasses were supplied to the fermentor. The ratio of the supplied gasses could be controlled by changing the time, set by a control timer for opening and closing the valves. The valves were operated by nitrogen at high pressure to avoid a fire due to electric sparks in the substrate-gas feeding line. Figure 2 shows the air-lift fermentor and its dimensions are given in Table 1. To obtain high mass transfer via formation of small bubbles, a sintered stainless steel sparger (pore size, 10 μ m; diameter, 12 mm; length, 20 mm) was installed at the bottom of the reactor (Okabe *et al.* 1993).

Cell culture

The cells were cultured by a two-stage method (Tanaka et *al.* 1994). First, one loop of the stock culture was inoculated into 5 ml of nutrient-rich medium in a test tube and incubated at 30°C for 12 h with shaking. Two milliliters of the culture broth were then inoculated to 20 ml of fructose medium in a 500-ml shaking flask and incubated at 30 °C for 6 h. Ten ml of the resultant broth were inoculated into 100 ml of fructose medium in a 200-ml stirred jar-fermentor and cultivation was continued until the carbon source was exhausted. They were then pelleted by centrifugation and washed twice with the fresh mineral medium. The cells were resuspended in 150 ml of mineral medium for

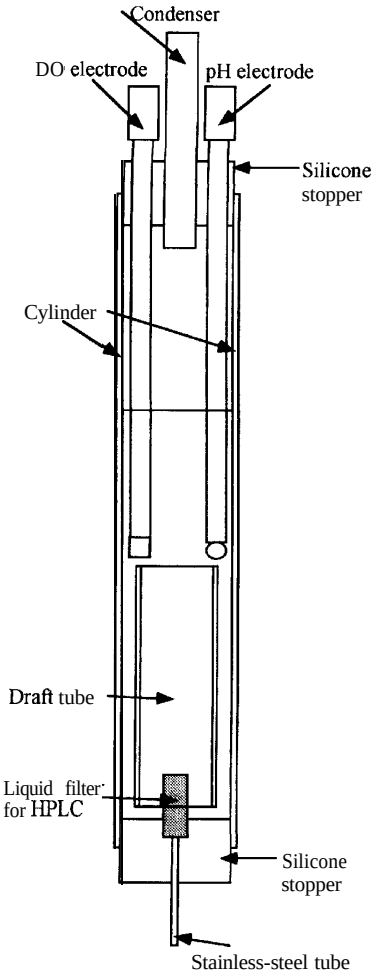

Fig. 2. Schematic diagram of air-lift fermentor.

Table 1. Dimensions of the air-lift fermentor

Reactor:		
	toral volume	350 ml
	total length	350 mm
Cylindrical part:		
	length	300 mm
	inner diameter	40 mm
	diameter of draft tube	30mm
	length of draft, tube	100 mm
Sparger:		
	pore size	10μm

autotrophic culture in the air-lift fermentor.

Culture conditions

The working volume of the air-lift fermentor was 150 ml. The pH of the culture liquid was maintained at 6.8 by automatic addition of a 2.5% solution of ammonia water and the temperature was kept at 30 "C. The feeding rate of the substrate gas mixture was 2 *l*/min, which was equivalent to a superficial gas velocity of 2.62 cm/s. The composition of the substrate mixture of carbon dioxide, hydrogen and oxygen was maintained at 10 : 85 : 5 (v/v) by the substrate gas feeding system with the gas composition controller.

Analytical methods

The composition of the gas phase was analyzed with a gas chromatograph (GC-8A; Shimadze Seisakusyo Co. Ltd., Tokyo, Japan) equipped with a column (4 mm i.d.× 6 m) into which molecular sieve 5A and Porapack Q has been packed (Morinaga et *al.* 1978). Dissolved oxygen (DO) tension in the culture broth was determined with a membrane electrode (S-Type; Able Co. Ltd., Tokyo, Japan). The concentration of cells was measured by converting the absorbance of the broth at 562 nm to dry cell weight (DCW) by reference to a previously prepared calibration curve. Protein in the culture broth was quantitated by the Biuret method (Herbert et al. 1971) with bovine serum albumin (SIGMA Co. Ltd., St. Louis, U.S.A.) as the standard. The concentration of P(3HB) was determined by gas chromatography (Braunegg et *al.* 1978).

Measurements of viscosity, gas hold-up and volumetric mass transfer coefficient

The viscosity of media was measured by Ostwald's method (Tanford et al. 1956). The gas hold-up in the air-lift fermentor was measured as described by Russel et al. (1993). The gas hold-up was determined by measuring the height of the aerated liquid and calculating the hold up according to the following equation:

$$\varepsilon = (H_D - H_L) / H_D$$

where ε = overall gas hold up (-); H_D = height of gas-liquid dispersion (m); and H_L = ungassed liquid height (m).

Volumetric mass transfer coefficients (K_La) were measured by using the sulfite oxidation method (Cooper et al. 1944) and the static method (Wise 1951).

RESULTS AND DISCUSSION

Effects of the addition of CMC on viscosity and gas hold-up

The viscosity of the medium is an important physical property in the air-lift fermentor, in particular because it has a considerabe effect on K_La, the mixing time and the gas hold-up. In general, very high viscosity has diverse effects on the mass transfer of the substrate gasses into a culture system. The viscosity of the medium can be increased by the addition of CMC. Figure 3 shows the changes in the viscosity of the present

medium at various concentrations of CMC. The results show that addition of a small amount of CMC increased the viscosity considerably. The gas hold-up is an important parameter related to the gas-supply capacity of the air-lift fermentor. The gas hold-up is equivalent to the reaction area of the gas-liquid phase in the fermentor. Figure 4 shows the effects of the concentration of CMC on the gas hold-up in the air-lift fermentor. At a superficial gas velocity of 2.62 cm/s, the gas hold-up was increased by addition of CMC up to 0.1% and then it decreased with further increases in levels of CMC. From this result, we expected that addition of CMC up to 0.1% would increase P(3HB) productivity.

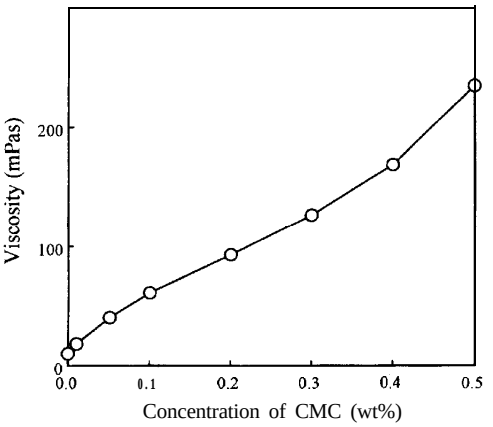

Fig. 3. Effects of the concentration of CMC on the viscosity of the medium at 30°C

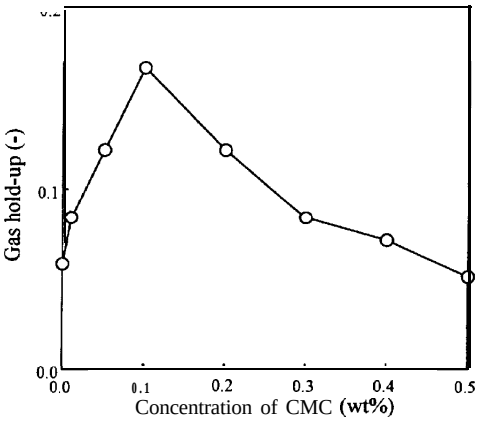

Fig. 4. Effects of the concentration of CMC on gas hold-up in the air-lift fermentor at 30 ℃ at a superficial gas velocity of 2.62 cm/s.

Experimental cultures

Figure 5a shows the time course of the autotrophic culture of *A. eutrophus* without addition of CMC, as a control culture. The concentration of dissolved oxygen decreased to below the lower limit for detection by the DO electrode after 6 h. However, the protein concentration gradually increased until 33 h of cultivation and then remained at 10 $g \cdot l^{-1}$. After 33 h, DCW and the concentration of P(3HB) increased linearly to reach 60.0 $g \cdot l^{-1}$ and 49.2 $g \cdot l^{-1}$, respectively, by 120 h of cultivation. During the P(3HB) accumulation phase from 33 h to 120 h, the P(3HB) productivity was 0.550 $g \cdot l^{-1} \cdot h^{-1}$. The final P(3HB) content of the cells was 82.0 % (w/w).

We confirmed that our strain of *A. eutrophus* could not utilize CMC as a carbon source (data not shown). Figure 5b shows the time course of fermentation with 0.05% CMC in the culture medium. After 9 h of cultivation, the extent of the decrease in the dissolved oxygen concentration was lower than that in the control culture. The protein concentration increased slowly as compared that in the control culture until 42 h of cultivation. After 42 h, DCW and the concentration of P(3HB) increased linearly to 69.3 $g \cdot l^{-1}$ and 56.4 $g \cdot l^{-1}$, respectively, at 92 h of cultivation. In the P(3HB) accumulation phase from 42 h to 92 h, the P(3HB) productivity was 1.02 $g \cdot l^{-1} \cdot h^{-1}$. The final P(3HB) content of the cells was 81.4% (w/w).

Figure 5c shows the time course of fermentation in the presence of 0.1% CMC. After 3 h of cultivation, the decrease in dissolved oxygen concentration was greater than that in the control culture. The protein concentration slowly increased as compared to the control culture until 60 h of cultivation. The result of this last experiment was different from those of the first two culture experiments and in that the accumulation of P(3HB) started simultaneously with cell growth. After 30 h, DCW and the concentration of P(3HB) increased linearly to 58.8 $g \cdot l^{-1}$ and 46.2 $g \cdot l^{-1}$, respectively, until 84 h. During the

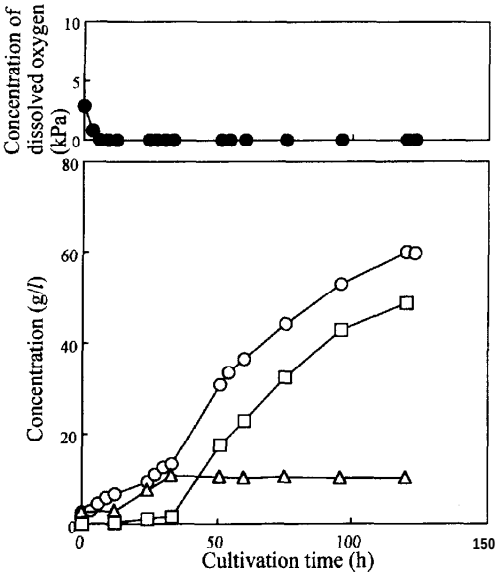

Fig 5a.

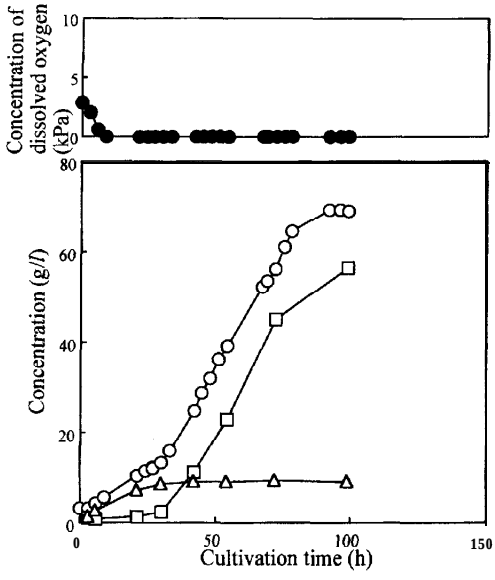

Fig. 5b.

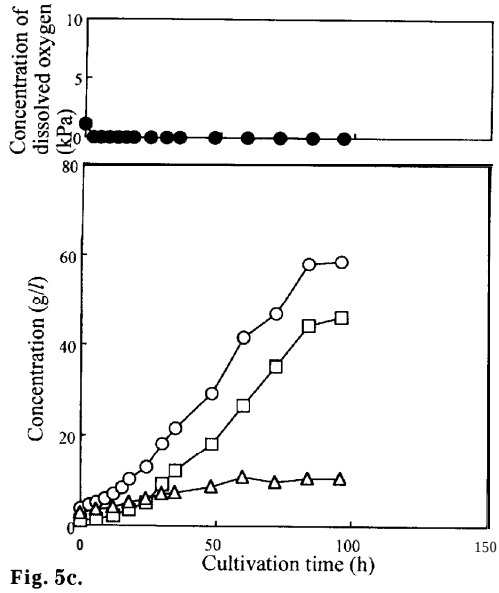

Fig. 5c.

Fig. 5. Time course of an autotrophic culture of A. *eutrophus* in the air-lift fermentor. a, 0% CMC medium as control; b, 0.05% CMC medium ; c, 0.1% CMC medium. Symbols: ○, DCW; □, P(3HB); △, Protein.

P(3HB) accumulation phase from 30 h to 84 h, the P(3HB) productivity was 0.743 $g \cdot l^{-1} \cdot h^{-1}$. The final P(3HB) content of the cells was 78.6% (w/w). The P(3HB) productivity during cultivation with 0.05% CMC was about twice that in the control culture. Although the gas hold-up with the addition of 0.1% CMC was higher than that with 0.05 % CMC, P(3HB) productivity of the culture with 0.1% CMC was only about 1.4 times that of the control culture. The following experiment was carried out to investigate the effects of CMC in further detail.

Volumetric mass transfer coefficient

During autotrophic cultivation, P(3HB) productivity depends on the volumetric mass transfer coefficient of the fermentor and the concentration of oxygen in the gas phase. Figure 6 shows the effect of the concentration of CMC on values of K_La measured by the sulfite oxidation method and the static method. K_La measured by the sulfite oxidation method decreased with increases in the concentration of CMC. With measurements by the static method, the maximum K_La was obtained at 0.05% CMC. In the range of CMC concentrations from 0 to 0.05%, the K_La measured by the sulfite oxidation method was somewhat higher than that measured by the static method. It is generally known that certain surfactants, such as CMC, can affect sulfite oxidation. Figure 7 shows the relationship between the P(3HB) production rate in the above cultivation experiments and values of K_La measured by the sulfite oxidation method and the static method. The correlation between the P(3HB) production rate and the K_La measured by the static

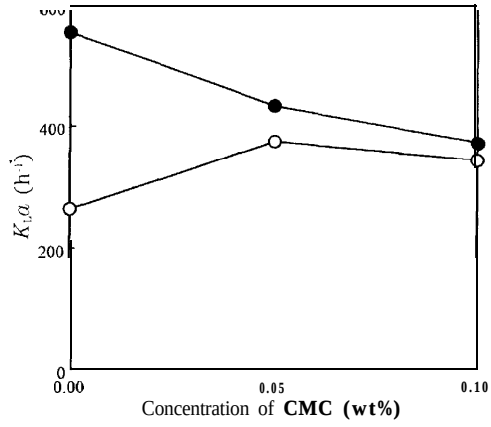

Fig. 6. K_La of air-lift fermentor at various concentrations of CMC. Symbols: ○, static method; ● , sulfite oxidation method.

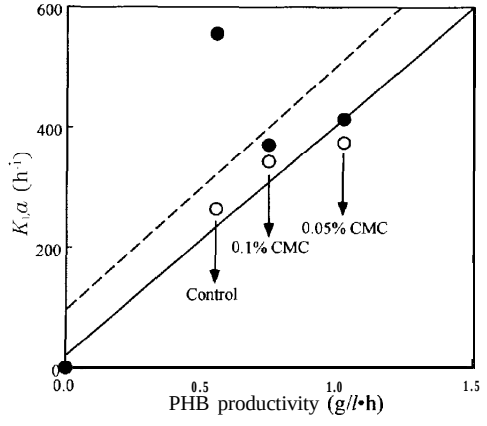

Fig. 7. Correlation between P(3HB) productivity and K_La. Symbols: ○, static method; ●, sulfite oxidation method. Functions for correlations: static method, solid line, y = 22.352+385.21x R^2=0.953; sulfite oxidation method, dotted line, y = 98.601+409.45x R^2=0.554.

method was very high. However, the correlation between the P(3HB) production rate and the K_La measured by the sulfite oxidation method was low. The flow characteristics of the aerated culture liquid with sodium sulfite were different from those of the culture medium that contained no sodium sulfite. The bubbles in the culture liquid with sodium sulfite seemed very small and the flow was homogeneous. Therefore, the gas hold-up in the presence of sodium sulfite was larger than that in the absence of sodium sulfite.

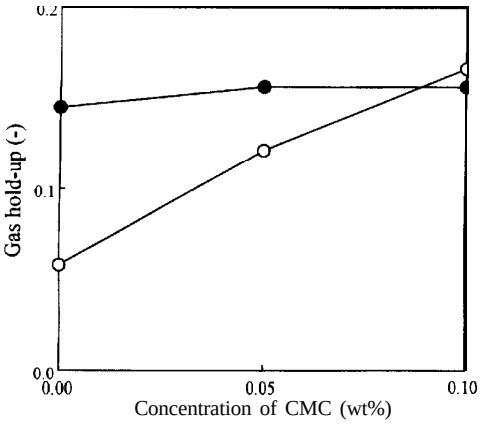

Fig. 8. Effects of addition of sodium sulfite on the gas hold-up in the air-lift fermentor. Symbols: ○, medium without sodium sulfite; ●, medium with sodium sulfite.

Figure 8 shows the effects of the presence of sodium sulfite on the gas hold-up. Measurements of K_La by the static method were made under the same gas hold-up conditions using the liquid without sodium sulfite. The results showed that the static method is more suitable for measurements of K_La than the sulfite oxidation method in our system. However, there was a weak relationship between the K_La measured by the static method and the gas hold-up. This result cannot be easily explained at present. It is possible that the size of bubbles in the liquid increased with the addition of CMC and that these large bubbles increased the gas hold-up. As the size of bubbles increases, the gas-liquid surface would become smaller, and then the K_La measured by the static method might be reduced. However, since it is very difficult to determine the mean size of bubbles, it is hard to validate this assumption.

In conclusion, a laboratory-scale air-lift fermentor was used for autotrophic culture of *A. eutrophus*. The P(3HB) production rate was increased by addition of 0.05% CMC. The P(3HB) production rate was not correlated with the K_La measured by the sulfite oxidation method but was correlated with the K_La measured by the static method. We are now investigating a culture system with higher P(3HB) productivity as a consequence of an increase of the protein concentration during the first stage of the two-stage culture system.

ACKNOWLEDGMENTS

The authors are grateful to professor Mitsuyasu Okabe of Shizuoka University for his guidance on the air-lift fermentor. We express our appreciation to the members of the Research and Development Institute of Saibu Gas Co. Ltd., for their assistance and advice related to development of the substrate-gas feeding system with the gas composition

controller employed in this study.

REFERENCES

Anderson, A. and E.A. Dawes 1990 Occurrence, metabolic role, and industrial use of bacterial polyhydroxyalcanoates. *Microbiol.Rev.*, 54: 450-472

Braunegg, G., B. Sonnleitner and R.M. Lafferty 1978 A rapid gas chromatographic method for the determination of poly-b-hydroxybutyric acid in microbial biomass. *Eur. J. Appl. Microbiol.*, 6: 29-37

Cooper, C.M., G.A. Fernstrom and S.A. Miller 1944 Performance of agitated gas-liquid contacters. Ind. *Eng.Chem.*, 36: 504-509

Deckwer, W.D. and A. Schumpe 1993 Improved tools for bubble column reactor design and scale-up. *Chem.Eng.Sci.*, 48: 889-911

Herbert, D., J. Phipps and R.E. Strange 1971 Chemical analysis of microbial cells: determination of protein. *In* "Methods in Microbiology", Vol. 5A, ed. by J. R. Norris and D. W. Ribbons, Academic Press, London and New York, pp. 243-249.

Ishizaki, A. and K. Tanaka 1990 Batch culture of *Alcaligenes eutrophus* ATCC 17697" using recycled gas closed circuit culture system. *J.Ferment.Bioeng., 69:* 170-174

Ishizaki, A., K. Tanaka, T. Takeshita, T. Kanemaru, T. Shimoji and T. Kawano 1993 Equipment and operation for fermentative Production of P(3HB) using gaseous substrate to guarantee safety from explosion. *J.Chem.Eng.Japan.*, 26: 224-226

Kennard, M. and M. Janekeh 1991 Two- and three-phase mixing in a concentric draft tube gas-lift fermentor. *Biotechnol. and Bioeng.*, 38: 1261-1270

Kodama, T., Y. Igarashi and Y. Minoda 1975 Isolation and culture conditions of a bacterium grown on hydrogen and carbon dioxide. *Agr.Biol.Chem.*, 39: 77-82

König, B., K. Schügerl and Ch. Seewald 1982 Strategies for penicillin fermentation in a tower-loop reactor. *Biotechnol.Bioeng.*, 24: 259-280

Morinaga, Y., S. Yamanaka, A. Ishizaki and Y. Hirose 1978 Growth characteristics and cell composition of *Alcaligenes eutrophus* in chemostat culture. *Agr. Biol.Chem.*, 42: 439-444

Okabe, M., N. Ohta and Y.S. Park 1993 Itaconic acid production in an air-lift bioreactor using a modified draft tube. *J. Ferment.Bioeng., 76:* 117-122

Russel, A.B., C.R. Thomas and M.D. Lilly 1993 The influence of vessel height and top-section size on the hydrodynamic characteristics of airlift fermentors. *Biotechnol. Bioeng.*, 43: 69-76

Schumpe, A. and W.D. Deckwer 1982 Gas holdups, specific interfacial areas, and mass transfer coefficients of aerated carboxymethylcellulose solutions in a bubble column. *Ind. Eng.Chem.* Process *Des.Dev.*, 21: 706-711

Tanaka, K. and A. Ishizaki 1994 Production of poly-r-3-hydroxybutyric acid from carbon dioxide by a two-stage culture method by Alcaligenes eutrophus ATCC 17697T. *J.Ferment. Bioeng.*, 77: 425-427

Tanford, C. and J.G. Buzzell 1956 The viscosity of aqueous solutions of bovine serum albumin between pH 4.3 and 10.5. *J.Phys.Chem.*,

W i s W.S. 1951 The measurement of the aeration of culture media. *J.Gen. Microbiol.*, 5: 167-177