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Effect of Oxygen Composition on Polyhydroxybutyrate Synthesis by *Alcaligenes eutrophus* at Various Pressures

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*Alcaligeneus eutrophus*에 의한 Polyhydroxybutyrate의 합성에 관한 산소효과

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Abstract : Poly-β-hydroxybutyrates(PHB) are well known intracellular biopolymer which are completely degraded to carbon dioxide and water in the landfill. The pressurized fermentation method was used to increase the cell growth of *Alcaligenes eutrophus* and productivity of PHB. The experimental data were analyzed in terms of carbon source and gas composition effects at various elevated pressures and temperatures. The results reveal that the flow rate of hydrogen gas of 0.0075vvm for the culture is better PHB production than the no hydrogen flow when fructose was used as a sole carbon source. The higher yields and productivities of PHB biosynthesized by *A. eutrophus* were obtained when the oxygen composition was changed from 2% to 8% at 6atm and 30℃.

요약 : Poly-β-hydroxybutyrates(PHB)는 토양에서 완전히 이산화탄소와 물로 분해되는 세포 내에 생성되는 고분자 물질로 잘 알려져 있다. *Alcaligenes eutrophus*의 세포 성장과 PHB의 생산성을 증가시키기 위해서 가압배양법이 사용되었다. 실험 데이타를 다양한 가압과 온도하에서 탄소 공급원과 기체성분의 영향에 관해서 분석한 결과 0.0075vvm의 기체수소를 공급한 배양이 수소를 공급하지 않은 배양에 비해 더 나은 PHB 생산성을 나타내었고, 6atm, 30℃에서 산소의 성분비를 2%에서 8%로 변화시켰을 때 더 높은 수율과 PHB의 생산성을 얻을 수 있었다.

Key words : Poly-β-hydroxybutyrates, PHB, *Alcaligenes eutrophus*, **hydrogen**, biopolymer.

1. Introduction

The organism *Alcaligenes eutrophus* was previously known as *Hydrogenomonas eutrophus*. In 1970, Davis *et al.*[1] proposed to abandon the genus

Hydrogenomonas and to reassigned the genus to *Alcaligenes*. The hydrogen bacteria uses H_2 as an energy source and, without exception, they are facultative chemoautotrophs, growing well on a wide range of simple organic compounds. *H. eutrophus* is a gram-negative, motile coccobacillus, with the size of the cells being 0.7 by 1μm.[2] Schlegel *et al.*[3] observed the PHB accumulation in Hydrogenomonas H16 when the cells grown in the exponential phase, were collected and subjected to one atmosphere of a gas mixture containing 60% hydrogen, 30% oxygen, and 10% carbon dioxide in the absence of a source of nitrogen. Repaske[4] studied the nutritional requirements for *H. eutrophus* and found that nitrogen can be supplied in the form of ammonium, nitrate, or urea, not as nitrite, and optimal growth of the organism occurred at pH 6.4 to 6.8 at 30℃ in an atmosphere containing 15 to 25% oxygen, 10% carbon dioxide, and 65 to 75% hydrogen. Morinaga *et al.*[5] studied growth characteristics and the cell composition of *A. eutrophus* in a continuous culture and reported that the highest PHB yield was obtained when the organism was cultured with a supply of nitrogen(1 gram of $(NH_4)_2SO_4$ per liter) and a gas mixture composed of 68% hydrogen, 23% oxygen, and 9% carbon dioxide. In that study[5], no more than 25% of the dry cell weight was detected as PHB. In this research, the new pressurized fermentor system was applied to increase the productivity of PHB and the carbon source effect. Oxygen composition effect at different gas mixtures of oxygen and nitrogen(0.5~21mol% O_2) for explaining the oxygen toxicity in the cell growth also considered using elevated pressures and temperatures.

The use of hydrogen and carbon dioxide mixture are one of the solutions for the commercial production of bacterial PHB.[6] Up to date, several investigators experimented with the idea of using the gas mixture for the PHB production.[7~10] This process, however, has possessed safety problems in handling the explosive gas mixture of hydrogen and oxygen. To avoid the explosion, O_2 concentration in the gas phase should maintain below 6.9% by volume[11], but such a low oxygen concentration may cause lower O_2 transfer. In addition, the problem of wasting the gas mixture under the continuous sparging and aeration may be another factor for connection with the industrial scale production. To overcome these drawbacks, two systems have been proposed. One was a closed culture systems which the gas mixture was confined in the head space as reported by Bongers.[12] In this system what was called "dead-end culture system", supply of gas mixture was intermittently conducted as the depletion of a gas occurred during fermentation. The other system was "recycled gas closed-circuit culture system" initially reported by Schlegel and Lafferty[13], and later Kodama *et al.*[14] and Ishizaki and Tanaka.[10] The dead-end culture system has disadvantage of slow mass transfer of gases due to the lack of aeration. It was appeared that the recycled gas culture system have higher gas mass transfer rates due to recycling of gaseous substrate. In this study to enhance the PHB productivity by increasing the mass transterin the fermentation, the head space pressurized termentor system using various carbon source was proposed.

2. Materials and methods

2.1. Description of fermentor system

Two fermentors were used to compared the data between low pressure and elevated pressure. For low pressure, 14 liter Virtis fermentor(Model 43-100 VIRTIS Co., Inc., New York, NY) was used with diluted antifoam(1:10) and for elevated pressure, the 3.8 liter stainless fermentor(Autoclave Engineers, Erie, PA) with working volume of 2 liters is used. The dimension of these two fermentors was explained to those of our previous work.[15]

2.2. Growth

This experiment was performed with *A. eutrophus*. The seed culture was not prepared. The bacteria colony from an agar plate was aseptically transferred to fermentor (first step culture) to prepare the second step culture. The first step and second step culture were explained at previous paper.[15] The experimental conditions were taken at P = 1, 6, 21 atm and T = 30, 35℃ using various oxygen composition and carbon source. The culture media are also identical to the previous work.[15]

2.3. Analytical methods

The samples were taken periodically at every 6 to 10 hours depending on the measured variable. Cell dry weights, cell numbers, pH, protein by Lowry method[16], ammonium ions using phenate method[17], reducing sugar by dinitrosalic acid[18], and PHB amounts[19] in the second culture of the fermentor broth were analyzed to see the effect of the carbon source and gas composition by analysing the cell growth and PHB productivity.

The suitable gas composition bottles(O_2+N_2) were also prepared by a week before using to make the constant gas composition in the bottle, and then the oxygen composition was analyzed three times by gas chromatography. The gas chromatography was equipped with the molecular sieve 5A column. The hydrogen and carbon dioxide bottles were obtained from the commercial products and these gases were supplied to the fermentor directly by controlling the gas flow rate.

3. Results and discussion

3.1. Carbon source effects

A hydrogen oxidizing bacterium, *A. eutrophus* has been reported to be able to grow on various organic source under heterotrophic conditions, and capable of consuming gaseous substrates, H_2, O_2, and CO_2 under the autotrophic culture conditions as well. When the fructose was served as a carbon source, the overall PHB formation did not exceed 50% by weight percent according to the previous studies.[20, 21] The fructose which is expensive compared to the counter part, glucose is a carbon source. Presently, the cost of fructose is about $0.49/lb. Thus the fructose should be changed to another economical substrate. For this reason, successful searching for a cheap carbon source is prime importance for commercialization of the process.

In the pressurized system, the mass transfer problem of gaseous substrates may not exist at all. In this experiments, we maintained the volumetric gas flow rates of 0.0075vvm for hydrogen, 0.25vvm for a mixture containing 8% oxygen, 8% carbon dioxide, and 84% nitrogen. Such flow rates are very low compared to the flow rates commonly employed in conventional fermentation system where approximately 1vvm is commonly employed.[13] But, we had observed adequate cell growth and PHB production with low flow rates.

Table 1 lists a comparative study between our data and those reported previously. Regardless of the lower usage of hydrogen for the pressurized system, the system produced better results than the previously reported data. The cell density as well as the PHB contents was far exceeding the data previously reported. Ishizaki and Tanaka[9] employed the recycled gas closed-circuit system for the study of the PHB synthesis. The investigators were able to obtain only 16% PHB in the cell dry weight and claimed that it was due to hydrogen limitation. However, the PHB contents in our fermentation system which was conducted at 6 atm were much higher even with of the low hydrogen usage of 0.0075vvm. *Fig.* 1 compares the experimental ran (a) and (b) in which two different gas mixtures were used. In experiment (a), the ratio of H_2:CO_2:O_2 was 0.0075:0.01:0.01(in vvm) and for experiments (b), the ratio was 0.0075:0.02:0.02 (vvm). The increase in the cell dry weight and the PHB accumulation are evident from the figure, while the pH changes were not seen in this study.

Table 1. Comparison of the growth of *A. eutrophus* H16 with limiting concentration of hydrogen

Parameter	H_2 limitation[17]	O_2 limitation[17]	H_2 limitation (this experiment)
Culture volume(mL)	640	725	2000
Cell density(g/L)	0.167	0.237	6.59
PHB contents(% dry wt)	<1	23	40
Ratio H_2/O_2	9.1	4.6	0.33
Gas flow rate(vvm)	0.47	0.414	0.25

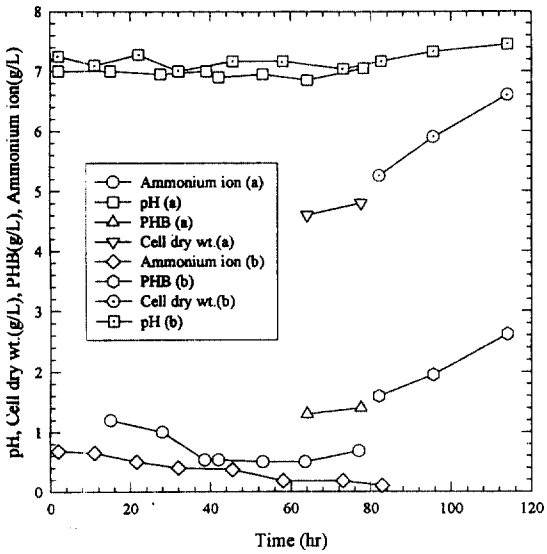

Fig. 1. A comparative study of *A. eutrophus* using two different levels of H_2:CO_2:O_2 at 6 atm and 30℃: (a) 0.0075:0.01:0.01, (b) 0.0075:0.02:0.02

This represented that higher gas compositions are needed to increase the cell dry weight and to enhance the PHB synthesis. Therefore, it may be necessary to increase the vvm of gases, or boost the operating pressure.

3.2. Gas composition effects

Oxygen composition in the inlet gas is an important factor for aerobic fermentation, especially at the elevated pressures. The fraction of oxygen in the gas phase affects the dissolved oxygen which in turn influences the growth of the cells and oxygen toxicity. Moreover, the rate of oxygen consumption may exceed the oxygen supply with the cell culture at high cell density. Therefore, finding an optimum oxygen concentration of cell culture would be an important factor when a fermentor is operated at elevated pressures. Above the critical oxygen concentration, the growth rate becomes independent of a dissolved oxygen concentration; but below the critical oxygen concentration, the cell growth was dependent upon a dissolved oxygen concentration.

With the use of 21% oxygen at 21 atm, the cells divided into smaller size cells which disappeared gradually as the fermentation proceeded. This phen omenon which was seen in *Table* 2 was possibly caus ed by hyperbaric oxygen toxicity. Dissolved oxygen at 21 atm and 30℃ is about 500ppm from the previous experimental data.[23] At 2% O_2 composition, the cell growth was better than that at 21% O_2 composition. This phenonma may be explained the toxic effect of oxygen as mentioned earlier in this paper. At 6 atm, the cell dry weight and PHB production were closely related to the oxygen composition in the inlet gas. The *Fig.* 2 shows the O_2 com position effect on cell dry weight and PHB fermentation at 30℃.

The oxygen solubility charts were prepared using the solubility factor[24] and the data reported previously using pure oxygen at 100℃.[24] The data were then analyzed by the least squares method to compare the data to Henry's Law. The collected data are shown in *Fig.* 3. *Fig.* 3 shows the results of the

Table 2. The oxygen composition effects in the high pressure condition at 30℃

Pressure (atm)	O_2 comp. (%)	pH	log cell number	cell dry wt. (g/L)	PHB (g/L)
21	21	6.38	8.70	no growth	
21	2	4.74	8.16	2.70	1.05
21	0.5	5.81	8.49	1.62	0.247
6	8		8.67	4.86	2.93
6	4		8.86	4.03	2.12
6	2		8.90	2.23	0.306

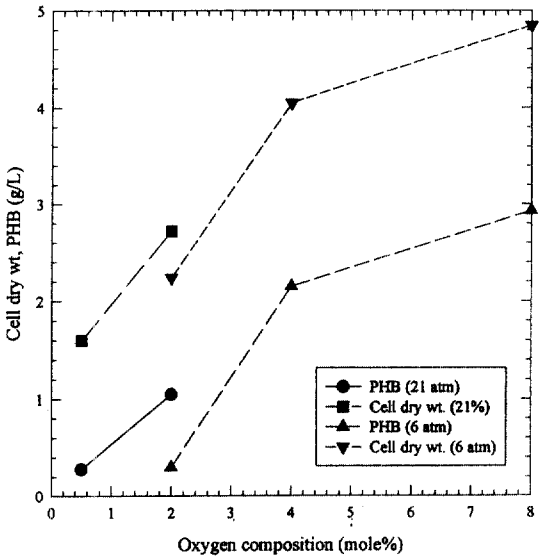

Fig. 2. O_2 composition effects on cell dry weight and PHB at 30℃.

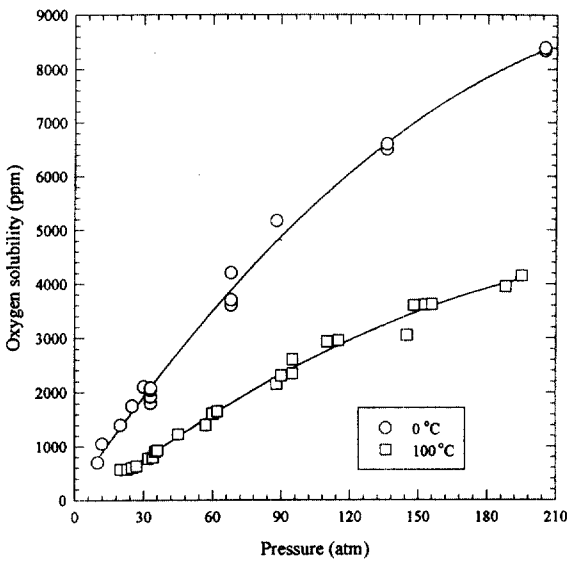

Fig. 3. Pure oxygen solubility.

oxygen solubility which can be determined at up to 30 atmosphere pressure and temperature at 100℃. Even though Henry's Law is a function of temperature and pressure, the oxygen solubilities up to 30 atm were used at various temperature ranges, because of the lack of reported data. The solubility factor is defined as the oxygen solubility of growth medium divided by oxygen solubility of pure water at given temperature and pressure. The solubility factor[24] for a growth medium was 0.67 between pres sure of 30 to 90 atmosphere when compared with pure water. By using the oxygen solubility chart in the growth medium, which is multiplied by the water solubility with a solubility factor(0.67) as shown *Fig.* 4. The appropriate oxygen fraction in gas phase is 1.6~1.8mol% for 21 atm and 6.4~7.2mol% for 6 atm by introducing a dissolved oxygen concentration of 8ppm. Comparing the experimental result at 6 atm, the optimum oxygen composition is a little higher than that of *Fig.* 4; thus the oxygen composition chart is reproduced to apply the pressurized fermentation for *A. eutrophus* as shown in *Fig.* 5. From this chart the optimum O_2 composition for 21 atm is 2.0~2.25mol% and for 6 atm is 8 to 9mol% at temperature ranges between 30 and 40℃. Using *Fig.* 5, the study of pressurized

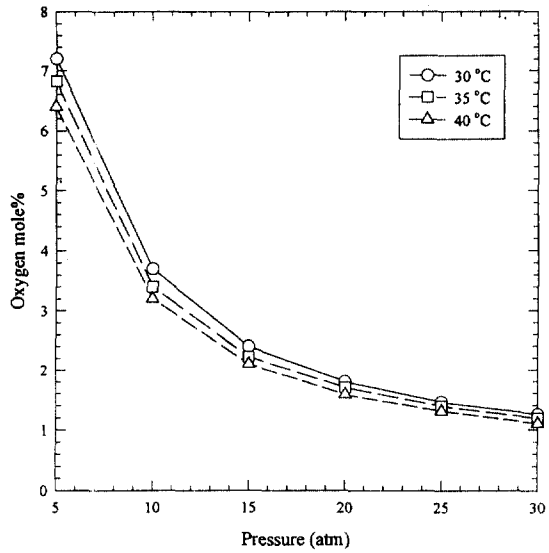

Fig. 4. Oxygen solubility chart at 8ppm using Henry's law up to 30 atm and solubility factor for growth medium.

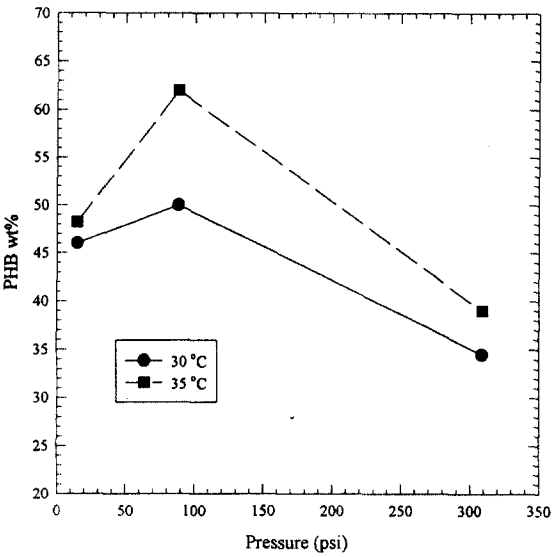

Fig. 6. Experimental conditions of optimal PHB formation at different O_2 composition (1 atm = 21%, 6 atm = 8%, and 21 atm = 2% O_2).

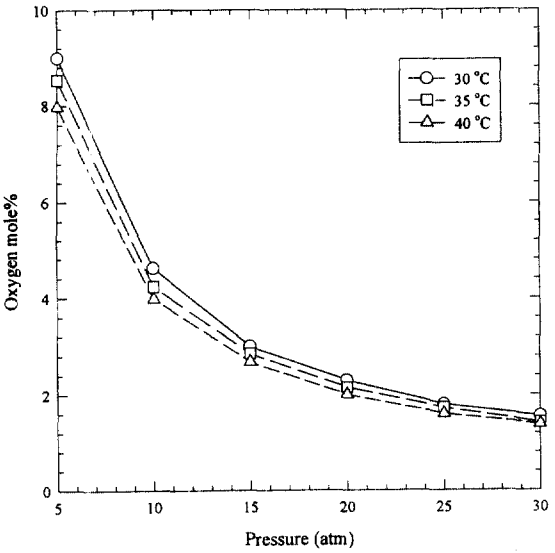

Fig. 5. Oxygen solubility chart at 10ppm using Henry's law up to 30 atm and solubility factor for growth medium.

fermentation would be done to increased the PHB productivity without the effect of O_2 toxicity.

Fig. 6 illustrates the optimal PHB formation at constant dissolved oxygen concentration. The productivity of PHB in weight percent in 21 atm was relatively low than 1 atm at working experimental conditions. This was due to the low pH value(pH = 4.74).

This procedure means an increase in productivity of *A. eutrophus* cells. Fed-batch culture was a very reliable method to increase the cell density. In fed-batch culture, an oxygen transfer rate was one of the limiting components for mass production of PHB. Suzuki *et al.*[25] controlled the dissolved oxygen in the range of 2~3ppm with a microcomputer. At 72 hr culture time of *Pseudomonas sp. K.*, dissolved oxygen was no longer able to be maintained at 2~3ppm due to the limitation of oxygen transfer rate of the bioreactor. The agitation speed and the flow rate of gas(53.3% O_2) became 1400rpm and 2.4vvm, respectively. However, the oxygen transfer rate can be easily changed by introducing the high pressure fermentation system at appropriate oxygen composition. Thus, this pressurized system can be applied on the fed-batch culture for high cell culture.

4. Conclusions

We improved the yields and productivity of PHB biosynthesis by *A. eutrophus* when fermentation was conducted in conditions of 6 atm, 8% oxygen composition and 30℃. The amount of the dissolved oxygen, for the organisms under elevated pressures of 6 atm and 2%, did not supplied enough oxygen. By increasing the oxygen from 2% to 8% (*Table* 2), we were able to obtain increased PHB synthesis. This observation was expected from the *Fig.* 4 and 5. From these figures it was evident that the level of a dissolved oxygen was far less than 8ppm which was considered to be the minimum con centration required for the cells at ambient pressure. Another possible explanation on the slow growth would be the decrease in oxygen diffusivity within the cells, as the amount of PHB accumulate in the cells. *Fig.* 4 and 5 which correlated oxygen composition, system's pressure up to 30 atm, and the solubility of oxygen was presented. These figures may provide valuable information for initial estimation of the oxygen in the pressurized fermentor in order to the experiment except for the oxygen toxicity. We were also able to culture the organisms autotrophically, using a gas mixture of hydrogen, carbon dioxide, and oxygen. The amount of PHB formed in autotrophic culture was far exceeding our anticipation.

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