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# Environmentally Safe Polyhydroxybutyrate Synthesis by *Alcaligenes eutrophus* in Pressurized Fermentor

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**Abstract** : A new high pressure fermentation system was employed for high density cultivation of *Alcaligenes eutrophus* (ATCC 17699) in order to improve the productivity of Poly-$\beta$-hydroxybutyrate(PHB). The effects of pressure and temperature on microbial growth and metabolism was examined. The maximum PHB yield was approximately 62% of the cellular dry weight when the cells were grown on fructose in a high pressure fermentor system at a controlled oxygen composition.

## 1. Introduction

The demand for synthetic plastics promoted a massive production of plastics during the last three decades. However, plastics has created environmental problems since it is not degraded in the ecosystem. Poly-$\beta$-hydroxybutyrate (PHB), a microbial polyester may offer a great opportunity for replacing nondegradable plastics in the field of agriculture (mulching, film), industrial products (bottles, container), commodity markets (packing, films) and medical application (drug release, surgical sutures). Since it is completely degraded to carbon dioxide and water within several months when buried in a landfill.

PHB is a straight chain aliphatic polyester and chemical synthesis has been proven to be extremely difficult. It is a thermoplastic material; meaning it can be molded to form any shape desired at a high temperature. Over 50 microorganisms were found to synthesize the polymer and accumulate it as a reserve material in a granule form. Prokaryotes and archaebacteria[1] are known to be major producers of PHB. Recently, PHB and other poly-3-hydroxyalkanoate(PHA), were found to be produced by some bacteria in a copolymer form[2]. Alcaligenes eutrophus was cultured under unbalanced growth condition, and was also reported to produce a copolymer, poly(3-hydroxybutyrate)-poly(4-hydroxybutyrate): P(3HB-4HB) [3,4].

Here we studied the effects of pressure and temperature on the productivity of the PHB by *A. eutrophus* H16 (ATCC 17699). To achieve this goal, a comparative study between normal fermentation at 1 atm and pressurized fermentation at elevated pressure were conducted. The working pressure range to be considered was from 1 to 21 atm and the working temperature range was between 30 to 40 °C.

## 2. Materials and Methods

### 2.1 High Pressure Batch Fermentation System

A 3.8-liters batch reactor (Autoclave Engineers, Erie, PA) was modified for fermentation at elevated pressures. The reactor and all the internal components was made of 316 stainless steel and can sustain pressures up to 340 atmospheres and temperatures as high as 340 °C. The dimensions of the working vessel and the impellers were identical to those of previous work[5].

### 2.2 Low Pressure Batch Fermentation

Low pressure fermentation was conducted in a 14-liters Virtis fermentor (Model 43-100 VIRTIS Co., Inc. New York, NY) containing an air sparger of the dimensions of 6.5 cm long and a 2.5 cm diameter. The sparging air was filtered through a two-stage air filter. Foam was suppressed by manual addition of sterilized 10% antifoam (Antifoam C Emulsion No. A-8011, Sigma Chemical Co.). The samples were withdrawn intermittently by a programmable Dispensing Pump (New Brunswick Scientic, Model DP200) through a sampling port attached in the head plate. The agitation speed was 150 rpm and the temperature was controlled at 30±0.1 °C, respectively. The unit was sterilized for 15 minutes at 121 °C.

### 2.3 Culture Media and Sampling

#### 2.3.1 Culture Media

We employed two-step fermentation which used two different media separately and sequentially. A nutrient-rich medium was employed in the first step to grow the cells without forming PHB. In the second step fermentation, PHB formation was induced using nitrogen-free mineral medium. The composition of each media is listed in Table 1[6,7,8,9].

#### 2.3.2. Sampling

Samples were taken from the pressurized bioreactor through a sampling port at a rate of 10 mL/min. Samples were taken at every 10 to 12 hours to record pH changes, counts of the cell numbers, concentration of ammonium ions[10] and fructose[11]. For these measurements, about 30 mL of the liquid samples were sufficient to perform the analysis. At the end of every fermentation cycles, about 100~500 mL samples were taken to determine cell dry weight and PHB[12]. Samples were collected by carefully opening the sampling valve and allowing the pressurized gas to force the liquid out at a slow trickle. Normally, a 30 mL sample of liquid was collected from the port.

**Table 1.** Culture Media

| | |
|---|---|
| Growth Medium | |
| $(NH_4)_2SO_4$ | 5.0g/ℓ |
| Polypeptone | 10.0 |
| Yeast extract | 10.0 |
| Meat extract | 5.0 |
| Nitrogen Free Medium : 0.03M potassium phosphate buffer, pH=7.0(initial pH) | |
| Na2HPO4 · 12H2O | 3.8g/ℓ |
| $KH_3PO_4$ | 2.65 |
| $MgSO_4$ | 0.2 |
| Carbon Source | |
| Fructose | 20 |
| Butyric Acid | 20 |
| $NaHCO_3$ | 5-20 |
| $CO_2/H_2$ | |
| Microelements Solution* | 1ml |
| *Microelements Solution(grams per lite of 0.1-NHC1) | |
| FeCl3 | 10g/ℓ |
| $CaCl_2 \cdot 2H_2O$ | 7.8 |
| $CuSO_4 \cdot 5H_2O$ | 0.02 |
| $ZnSO_4 \cdot 6H_2O$ | 0.14 |
| $CoCl_2 \cdot 6H_2O$ | 0.2 |
| KI | 0.07 |

#### 2.3.3. The First Step Culture

The cells collected from agar plates was incubated in an oven (Fisher Isotemp. Oven, Model 350D) for 60 hr at 30 °C, and were transferred into 14-L Virtis fermentor containing 4.5 liter of nutrient rich growth medium as shown in Table 1. Cell growth was measured by the increase in cell numbers when they became $3\sim6\times10^8$ cells/mL or stationary phase. After the 24 hour cultivation, the culture broth

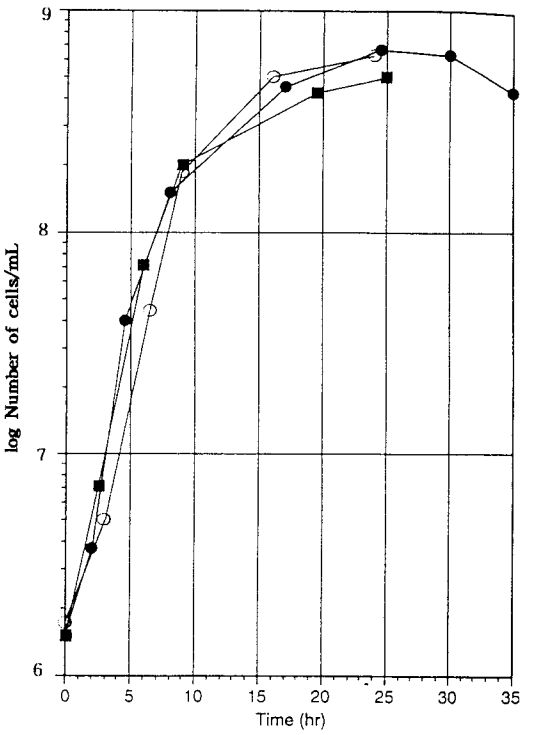



Fig. 1 Growth curve of *A. eutrophus*. These curves were from the culture broth at 30℃ in the 14-liter virtis fermentor. The rates of air and agitation were 0.5vvm and 150rpm respectively.(Each symbols are same condi-tions)

was centrifuged at 5000-g for 10 min in a Beckman J2-21 Centrifuge and the supernatant was decanted. To collect the cell in the growth rich medium, the growth curve was made as shown in Figure 1. In this growth curve, the lag phase is not observed, and the cell arrived at the stationary phase after 15 hr. In this step, the cells were grown for 24 hr at 30 °C, but PHB accumulation was not observed. The collected cells were used in second-step cultivation. The propagation of the cells in the first-step culture were plotted as in Fig. 1. Based on the data collected the specific growth rate and doubling time were calculated.

**2.3.4 The Second-Step Culture**

Experimental investigations were performed

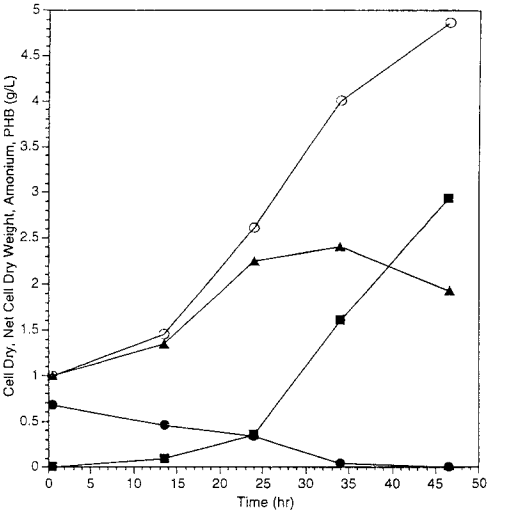



Fig. 2 Accumulation of PHB in *A. eutrophus* under second-stage culture at 6atm, 30 ℃ and 8% $O_2$,( ● : ammonium, ■ : PHB, ▲ : Net Cell Dry Weight, and ○ : Cell Dry Weight).

for both low and high pressure condition to compare the growth behavior and the product formation of *A. eutrophus*. The cells grown for 24 hours in the nutrient-rich growth media in the fermentor were harvested and aseptically transferred into the Virtis fermentor containing 4.5 liters of ammonium ion as a limiting medium as shown in Table 1. In the Virtis fermentor, the cells are cultivated at three different temperatures (30, 35 and 40 °C) and at pressures (1 to 21 atm) using various oxygen composition (2, 3.5, 4, 8 and 21%). After 24 hours, parts of the cells were transferred into a pressurized vessel (working volume 2 L) when the ammonium ion concentration dropped to almost zero. The rest of the cells (2.5 L) were kept at the same low pressure.

For redistribution of the wet cell mass collected from the first stage, the mass was dissolved in a known volume of distilled water and the volume was allocated to the low and pressurized fermentor in proportion to the working volume of the fermentor. It was found out that each fermentor contained about 0.5 to

**Table 1.** Non-growth related specific Production Rete at Various Experimental Conditions

| Pressure atm | T ℃ | $O_2$% | Cell Dry Weight(g/ℓ) | | PHBb/ℓ | Fermentation time(hr) | $\beta \times 10^2$/hr |
|---|---|---|---|---|---|---|---|
| | | | Initial | Final | | | |
| 1 | 30 | 21 | 1 | 6.06 | 2.94 | 42 | 1.155 |
| 1 | 35 | 21 | 1 | 4.29 | 1.98 | 52.5 | 0.879 |
| 1 | 40 | 21 | 0.5 | 2.80 | 0.63 | 46 | 0.489 |
| 6 | 30 | 8 | 1 | 4.86 | 2.93 | 46.5 | 1.297 |
| 6* | 30 | 8 | 1 | 6.41 | 3.94 | 42 | 1.463 |
| 6 | 35 | 2 | 1 | 4.63 | 2.32 | 43 | 1.165 |
| 21 | 30 | 2 | 0.5 | 2.7 | 1.05 | 39.5 | 0.985 |
| 21 | 35 | 2 | 0.5 | 4.6 | 1.60 | 49.5 | 0.703 |
| 21 | 40 | 2 | 0.5 | 2.6 | 0.44 | 46 | 0.368 |
| 1** | 30 | 21 | 4 | 9.4 | 3.47 | 48 | 0.769 |

* 7.5ml/min, hydrogen gas flow rate
** Data from reference[13]

1.0 grams of cells (as dry weight) per liter of the fermentor.

## 3. Results

### 3.1. Cell growth dynamics

The Fig. 1 shows the growth curve of *A. eutrophus*. These curves were obtained from the culture broth at 30 °C in the 14-liter Virtis fermentor. The rates of air flow and agitation were 0.5 vvm and 150 rpm, respectively. The doubling time was measured between 2.77 and 3.06 hr and the specific growth rate ($\mu$) was found to be from 0.126 to 0.250 $hr^{-1}$.

Fig. 2 was a plot of the time course of ammonium ions, the amount of PHB, the cell dry weight and the net dry weight of a second-stage culture system. The fermentation was conducted at 6 atm and at 30 °C, with a gas mixture containing 8% oxygen and 92% nitrogen. It is seen from the figure that residual ammonium ion is still detected. This is because it was almost impossible to remove all the ammonium ion by centrifugation. Due to the presence of nitrogen (0~20 hr), the growth of cells and the PHB synthesis was not observed. The formation of PHB was measurable when the ammonium ions were almost depleted (after 20 hr). The residual biomass (total cell mass minus PHB) appeared to have been constant and then slightly decreased toward the end of the culture. Thus, the kinetics of the PHB formation could be related to the product formation associated with non-growth related.

According to the product formation kinetics which is commonly known as the Luedeking-Piret equation, the growth-related specific production rate ($\alpha$) should become zero, leaving a term associated with the non-growth. The Luedeking-Piret equation is

$$r_P = \alpha r_X + \beta C_X \tag{1}$$

By simplifying equation (1)

$$r_P = \beta C_x \tag{2}$$

$$\frac{dP}{dt} = \beta X \tag{3}$$

From the equation (3), the non-growth related specific production rate can be easily calculated using the product rate ($r_P$) and the measured cell mass concentration. Table 2 shows the non-growth related specific production rate at various experimental conditions. The maximum non-growth related production rate was observed at 6 atm, 30 °C using 8% $O_2$ composition(with 7.5 ml/min. hydrogen flow rate)), and the minimum value was measured at 21 atm, 40 °C using 2% $O_2$ composition. At

**Table 3.** Producing PHA in Different Culture Media

| Method | Strain | Garbon Source (g/ℓ) | Nutrient Depletion | Cell g/ℓ | PHA g/ℓ | PHA wt% | Culture time, hr | misocella. neous | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Two-step | A. *eutrophus*(ATOC 17699) | CH3COONa(20 | Ammonium | 2.6 | | 51 | 48* | Flask | 14 |
| Two-step | A. *eutrophus*(NCIB 11599) | Glucose(20) | Ammonium | | | 54 | 48 | Flask | 15 |
| Two-step | A. *eutrophus*(ATOC 17699) | CH2COONa(22) | Ammonium | 4.4 | | 53 | 48 | Flask | 16 |
| Two-step | A. *eutrophus*(ATOC 17699) | γ-butyrolactone | Ammonium | 6.2 | | 21 | 48 | 3HB-4HB | 17 |
| | | butyric acid | | 8.7 | | 58 | 48 | | |
| Two-step | A. *eutrophus*(NCIB 11699) | butyric acid | Ammonium | 7.6 | | 38 | 48 | | 2 |
| | | Acetic acid | | 5.9 | | 35 | 48 | | |
| | A. *eutrophus*(ATOC 11599) | pentanoic acid | Ammonium | 6.6 | | 46 | 48 | | |
| | | butyric acid | | 7.3 | | 48 | 48 | | |
| Two-step | A. *eutrophus*(ATOC 17699) | butyric acid | | 9.6 | | 51 | 48 | | 18 |
| | | 4-hba** | | 4.9 | | 19 | 48 | 3HB-4HB | |
| Two-step | A. *eutrophus*(ATOC 17697) | lactic acid(20) | | | 5.8 | 62 | 25 | 4.5g/ℓ tranfer | 19 |
| Two-step | A. *eutrophus*(ATOC 17699) | Fructose(20) | Ammonium | 6.41 | 3.94 | 61.5 | 48 | 1g/ℓ transfer | This study |
| Two-step | A. *eutrophus*(ATOC 17699) | 4-hba | Ammonium | 3.7 | | 30 | 48 | | 20 |
| | | 4-cha*** | | 5.1 | | 27 | 48 | 3HB-4HB | |
| Two-step | A. *eutrophus*(ATOC 17699) | Fructose(20 | Ammonium | 9.4 | | 37 | 48 | 4g/ℓ transfer | 13 |

* : Second step culture ** : 4-hydroxybutyric acid

*** : 4-chlorobutyric acid + : after the growth phase in which cell mass concentration reached(g/ℓ)

6 atm, the value greater than normal optimum growth conditions (30 °C, 1 atm) was obtained [13]. The non-growth related production rate was also calculated using previously reported data[13]. The result of the calculation showed that the non-growth related production rate was $0.769\times10^{-2}$ $hr^{-1}$. This value is much lower than the study conducted here at 6 atm and 30 and 35 °C. These results suggest a possibility of the pressurized fermentation system being employed to increased the PHB productivity. In general, at 21 atm, low $\beta$ values were obtained. This result may be due to the fact that low dissolved oxygen concentration in the growth medium at 40 °C and the low pH values prevailed in the experiment. Thus, to verify this, additional studies are required to find the optimal growth condition for increased PHB productivity.

Table 3 summarizes previous experimental results on the cell dry weight, the amount of PHB synthesized, and the culture time at various conditions to compare the result. As shown in the Table 3, the culture medium of *A. eutrophus* employed several different carbon sources. This table shows that the result of the PHB formation using *A. eutrophus* is very affirmative compared to another previously reported experimental result.

### 3.2. Pressure Effect

Increased solubility of oxygen can be either beneficial or harmful for aerobic microorganisms depending on the extent of oxygen tension in the culture medium. Most aerobes can tolerate between 10 to 50 atm of compressed air[20]. The effects of other biologically important gases, such as $O_2$, $N_2$, $CO_2$ and $H_2$ on bacterial growth under hyperbaric conditions have not been well investigated. Carbon dioxide, for instance, normally a by-product of metabolic activity, is known to be an inhibitor at elevated levels for certain organisms.

When fructose is used as a substrate for both low and high pressure fermentation for PHB production, the pH values drop move rapidly in a high pressure fermentor system than in a low pressure system. It seems the by-product carbon dioxide produced via Entner-Doudoroff

pathway from fructose causes a drastic pH change by increasing the solubility of carbon dioxide at elevated pressure.

In this experiment, the whole system was pressurized by mixed gas which was pressurized from top of the head plate. By supplying the air into top of the head space at elevated pressures, the problems of foam control and the loss of the volatile substances during the fermentation appeared to have been reduced. This may be other advantages for the pressurized fermentation systems. For instance, when organic volatile substrates such as n-octane served as a carbon source, traditional fermentation carries several inherent disadvantages associated with the loss of these volatile substrates. These substrate are usually sparingly soluble in aqueous media and due to the high vapor pressure of these substances, the loss of the substrates would be great under the condition of the aeration. This problem would be much more severe when a thermophilic bacterium, which required culture temperatures much higher than the psychrophilic bacteria, is employed in a fermentor. Preusting *et al.*[21] employed a sterilized air which was presaturated with n-octane in order to prevent stripping of n-octane in the fermentor. Luong [22] tried another technique to avoid the loss of the substrate. The exit air stream was passed through a reflux cooler in which butanol was condensed and returned to the fermentor, thus minimizing the evaporation of butanol during the course of the fermentation. So, to prevent the loss of organic materials, the high pressure system was another alternate method of supplying the air in the gas phase.

To evaluate the pressure effects at three different temperatures and oxygen compositions, some of the relevant data are shown in Table 4. The reported amount of PHB production in batch cultivation varied with time. In an early report (in the 1970's), the amount of PHB was over 60% by weight[23], compared to 37% reported by Doi *et al.*[13] in 1990. In our research we were able to obtain 48.5 wt% of PHB at 1 atm and 61.5% at 6 atm as are seen in Table 4. The PHB formation at the high pressure (6 atm) increased more than 22%, compared with the reported low pressure fermenttaion data[13]. Thus, this research and data yielded a positive and encouraging result for the PHB synthesis under the pressurized conditions. At 21 atm, the PHB production rate was increased after the pressure was applied. This effect can be explained by the oxygen solubility in the growth medium. So, the different $O_2$ composition can be used at different pressures to find the optimal PHB production condition.

**Table 4.** Results of Second-stage Culture for PHB

| Pressure (atm) | Temp. (℃) | $O_2$% | Cell dry wt(g/ℓ) | PHB (g/ℓ) | wt% |
|---|---|---|---|---|---|
| 1 | 30 | 21 | 6.06 | 2.94 | 48.5 |
| 1 | 35 | 21 | 4.29 | 1.97 | 46.1 |
| 1 | 40 | 21 | 2.85 | 0.627 | 22.0 |
| 6 | 30 | 2 | 2.23 | 0.306 | 13.7 |
| 6 | 30 | 8 | 4.86 | 2.93 | 60.3 |
| 6* | 30 | 8 | 6.41 | 3.94 | 61.5 |
| 6 | 35 | 8 | 4.63 | 2.32 | 50.1 |
| 21 | 30 | 2 | 2.70 | 1.05 | 38.9 |
| 21 | 35 | 2 | 4.6 | 1.601 | 34.8 |
| 21 | 40 | 2 | 2.6 | 0.44 | 16.9 |
| 1 | 30 | 21 | 9.4 | 3.47 | 37 |

*7.5ml/min, hydrogen gas flow rate

### 3.3. Temperature Effects

Temperature is an important factor affecting the cell metabolism. Pressure could be considered as a kind of stress factor. Because of this reason the increased temperature could be used to overcome the stress caused by the pressure. By increasing the culture temperature it may be possible to increase the rate of biochemical reaction occurring within the cells, thereby enhancing the biomass formation. In the pressurized culture system, the optimal temperature was reported[13,24] to have been shifted to a few degrees of higher, although the rough claim have never been experimentally proved.

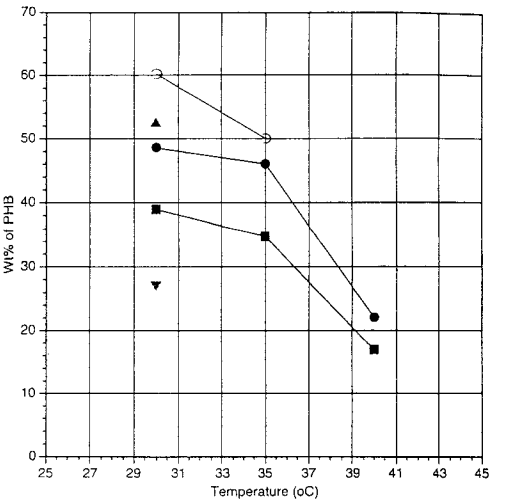
**Table 5.** Temperature Effects at Vaious Pressure Ranges

| Pressure psig | Temp. ℃ | O2 Composition % | Cell dry Wt g/ℓ | PHB g/ℓ |
|---|---|---|---|---|
| 21 | 30 | 21 | no growth | |
| 21 | 40 | 21 | no growth | |
| 21 | 30 | 2 | 2.0 | 1.05 |
| 21 | 35 | 2 | 4.6 | 1.601 |
| 21 | 40 | 2 | 2.6 | 0.44 |
| 6 | 30 | 3 | 2.5 | 0.65 |
| 6 | 35 | 3.5 | 2.64 | 0.644 |
| 6 | 35 | 3.7 | 2..7 | 0.62 |
| 6 | 35 | 4 | 4.03 | 2.12 |
| 6 | 30 | 8 | 6.41 | 3.94 |
| 1 | 30 | 21 | 4.7 | 2.02 |
| 1 | 35 | 21 | 4.29 | 1.976 |
| 1 | 40 | 21 | 2.85 | 0.627 |

At 1 atmosphere pressure, the increasing temperature decreased the cell dry weight and the formation of PHB. The temperature of 30°C appeared to be the optimum temperature for both PHB formation and the cell growth. By increasing the growth temperature it was expected that the solubility of oxygen would be decreased, thus, increased PHB production rate was anticipated. But the data in Table 5, the operating, 30, 35, 40°C at 1 atm did not support this claim. The same observation is seen for operating, 35, 40°C at 21 atm and 30 °C at 6 atm. On the contrary, at the pressure of 6 atm and 35°C , the higher oxygen composition (4%) produced the better cell dry weight and PHB productivity.

The oxygen composition appeared to have affected more seriously on the PHB formation and the cell growth than the temperatures. Fig. 3 shows the temperature effects on the PHB production when three different oxygen compositions were used: 2, 8 and 21%. For the pressure ranges, 1atm, 6 atm, and 21 atm, the PHB production rates were decreased with the increased temperatures. These results may support the explanation of the reduced oxygen solubility as the temperatures rose.

One of the problems experienced during the study was the control of pH. No commercial




– ● – 1atm(21% Oxygen) – ▲ – 6atm(4% Oxygen)
– o – 6atm(8% Oxygen) – ▼ – 6atm(2% Oxygen)
– ■ – 21atm(2% Oxygen)

**Fig. 3.** Temperature effects on PHB production

probes were available for accuracy of pressures above the 10 atmospheres. Accommodating the probe into the high pressure fermentor since the thickness of the wall was almost two inches, was very difficult, as well. Any attempt to bore the wall would create a problem of structural damage and may pose a serious problem of explosion. Therefore, it might be necessary to have a recycle loop for continuous pH monitoring. Several high pressure pH meters were designed and reported in the articles published in marine microbiology and deep sea research.

## 4. Conclusions

We have conducted a series experimental studies on the PHB biosynthesis by *A. eutrophus* H16 (ATCC 17699) using a pressurized fermentor. The system worked very well and had proven that it was capable of operating at pressures up to 21 atm under the continuous aeration. Higher yield of PHB was obtained

when *A. eutrophus* was cultured at elevated pressure of 6 atm and 30 °C. The dry weight was 6.41 g/l and the content of PHB was 61.5% (PHB amount/cell dry weight amount) in 42 hr. The effects of temperatures on the cell growth was not quite understood due to the difficulties involved with the relationship between the solubility of oxygen and the temperature.

## Nomenclature

| | |
|---|---|
| $C_x$ | Cell concentration |
| dP/dt | Production rate |
| $r_P$ | Production rate |
| $r_X$ | Cell growth rate |
| X | Cell mass concentration |
| $Y_{P/x}$ | Yield coefficient (=mass of products formed/mass of cells formed) |

## Greek Letters

| | |
|---|---|
| $\alpha$ | Growth related specific production rate |
| $\beta$ | Non-growth related specific production rate |

## References


1. R. Fernandez-Castillo, F. Rodriguez-Valera, J. Gonzalez-Ramos, and F. Ruiz-Berraquero, *Appl. Environ. Microbiol.*, **51**, 214 (1986).
2. Y. Doi, A. Tamaki, M. Kunioka, and K. Soga, *Appl. Microbiol. Biotechnol.*, **28**, 330 (1988).
3. M. Kunioka, Y. Nakamura, and Y. Doi, *Polm. commun.*, **29**, 174 (1988).
4. Y. Doi, A. Tamaki, Y. Nakamura, and K. Soga, *Macromolecules*, **21**, 2988 (1988)
5. K. Kim, Ph. D. Dissertation, New Mexico State Univ., Las Cruces, USA (1994).
6. Y. Kawaguchi and Y. Doi, *Macromolecules*, **25**, 2324 (1992).
7. Y. Doi, Y. Kawaguchi, Y. Nakamura, and M. Kunioka, *Appl. Environ. Microbiol.*, **55**, 2932 (1989).
8. V. Oeding and H. G. Schlegel, *Biochem., J.*, **134**, 239 (1973).
9. R. Repaske and A. C. Repaske, *Appl. Environ. Microbiol.*, **32**, 585 (1976).
10. Standard Methods for Examination of water and wastewater, 16th ed., American Health Association, Washington, D. C. (1985).
11. H. G. Schlegel, R. Laffery, and I. Krauss, *Arch. Microbiol*, **71**, 283 (1970).
12. D. H. Williamson and J. F. Wilkinson, *J. Gen. Microbiol.*, **19**, 198 (1958).
13. Y. Doi, A. Segawa, and M. Kunioka, *Int. J. Biol. Macromol.*, **12**, 106 (1990).
14. Y. Doi, A. Tamaki, M. Kunioka, and K. Soga, *J. Chem. Soc., Chem. Commun.*, 1635 (1987).
15. Y. Doi, M. Kunioka, Y. Nakamura, and K. Soga, *Macromolecules*, **19**, 2860 (1986).
16. Y. Doi, A. Segawa, and M. Kunioka, *Poly. Commun.*, **30**, 169 (1989).
17. M. Kunioka, Y. Kawaguchi, and Y. Doi, *Appl. Microbiol. Biotechnol.*, **30**, 569 (1989).
18. S. Linko, H. Vaheri, and J. Seppala, *Enzyme Micro. Technol.*, **15**, 401 (1993).
19. E. Hustede, A. Steinbuchel, and H. G. Schlegel, *Appl. Microbiol. Biotechnol.*, **39**, 87 (1993).
20. S. F. Gottleib, *Ann. Rev. Microbiol.*, **37**, 42 (1971).
21. H. Preusting, W. Hazenberg, and B. Witholt, *Enzyme Microb. Technol.*, **13**, 770 (1993).
22. J. H. T. Loung, *Biotechnol. Bioeng.*, **29**, 242 (1987).
23. H. G. Schlegel and R. M. Lafferty, *Adv. Biochem. Engin.*, **1**, 143 (1971).
24. A. Steinbuchel and H. G. Schlegel, *Appl. Microbiol.*, **31**, 168 (1989).