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# Isolation and Characterization of a Methylotroph Producing 3-hydroxybutyrate-3-hydroxyvalerate Copolymer

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A bacterial strain C-02 using methanol as a carbon source was isolated from Gumi Industrial Estate and selected based on its rapid growth and capability of poly-β-hydroxybutyrate accumulation. Characteristics of strain C-02 showed that it belongs to the *Methylococcaceae* family, Type II subgroup. Strain C-02 could incorporate valerate into the PHB chain to form 3-hydroxybutyrate and 3-hydroxyvalerate (P(3HB-co-3HV)). Among various nutrient limitation tests, the nitrogen limitation test resulted in the highest content of P(3HB-co-3HV) per dry cell weight, 50%. Under the nitrogen limited condition, the average molecular weight of P(3HB-co-3HV) obtained was determined to be approximately $2.8\times10^5$ daltons.

Increasing concern about environmental pollution caused by discarded petrochemical plastics has led to extensive research on biodegradable polymers such as poly-β-hydroxyalkanoic acid (PHA) including poly-β-hydroxybutyric acid (PHB) and its copolyesters, pullulan, and starch. PHB is one of the most promising candidates for biodegradable biopolymers, even though it has some limitations in commercial exploitation, mainly due to high production costs. Compared with polypropylene, PHB is stiffer and more brittle (1). The physical properties of PHB can be improved by incorporating 3-hydroxyvaleric acid (3HV) unit into the PHB chain (2). The resulted copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate (P(3HB-co-3HV)), also given the trade name Biopol, is tougher but less stiff than PHB.

In microbial production of P(3HB-co-3HV), one major factor determining the price of the product is the cost of the carbon source. In this regard, methanol is an attractive carbon source because of its low cost as well as high purity and abundance. In addition, the use of methanol as a carbon source reduces the risk of contamination (4, 9). There are several reports about PHB production from methanol. *Methylobacterium extorquence* was isolated by Bourque et al. from methanol contaminated soil (4). In this case, PHB was accumulated under the nitrogen-limited condition. Suzuki et al. (12) reported the mass production of PHB from methanol. They produced 149 g/l of PHB by automated fed-batch culture system. However, Ueda et al. reported the accumulation of copolymer in the *Paracoccus denitrificans* and *Methylobacterium extorquens* (13) and the fraction of HV unit were 91.5 and 38.2 mol%, respectively. In an effort to isolate methylotroph producing PHB and its copolyesters, we screened methylotroph producing P(3HB-co-3HV) among the bacterial strains isolated from Gumi Industrial Estate.

In this article, we report on the screening of methylotroph producing P(3HB-co-3HV). Further, accumulation of P(3HB-co-3HV) with isolated methylotroph was investigated under various nutrient-limited conditions.

## MATERIALS AND METHODS

### Microorganism and Medium

Microorganisms were isolated from methanol-contaminated soils in Gumi Industrial Estate. The medium used for the isolation of methanol-utilizing bacteria is summarized in Table 1 and for the isolation of PHB-accumulating bacteria, the concentration of ammonium sulfate was reduced to 0.2 g/l to lead nitrogen-limited condition.

The composition of medium used for the cultivation of methylotroph is almost identical to that used for the

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**Table 1.** Composition of medium used for isolation of methyltroph.

| Source | Concentration |
|---|---|
| Carbon | Methanol: 0.5% (v/v) |
| Nitrogen | $(NH_4)_2SO_4$: 1.0 (g/l) |
| Phosphate | $Na_2HPO_4 12H_2O$: 2.13 (g/l) |
| Mineral 1 | $KH_2PO_4$: 0.45 (g/l) |
| Mineral 2 | $CaCl_2 2H_2O$: 3.3 mg/l; $FeSO_4 7H_2O$: 1.3 mg/l |
| Mineral 3 | $MnSO_4 4H_2O$: 130 μg/l; $ZnSO_4 4H_2O$: 130 μg/l; $CuSO_4 5H_2O$: 40 μg/l; $Na_2MoO_4 4H_2O$: 40 μg/l; $CoCl_2 6H_2O$: 40 μg/l; $H_3BO_3$: 30 μg/l |

Carbon, nitrogen and phosphate, mineral 1, 2 and 3 were sterilized separately. In case of the isolation of PHB-accumulating bacteria, 0.2 g/l of ammonium sulfate was used as nitrogen source to lead the nitrogen limitation.

isolation except in the concentrations of methanol and minerals (6). The methanol concentration was increased to 1%(v/v). The mineral concentration was also increased by two fold. The initial pH of the medium was adjusted to 7.0.

**Culture Conditions**

Seed culture was prepared as follows; The cells from single colony was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of liquid medium, and cultivated for 24 hours in a rotary shaker at 30℃ and 200 rpm. The cells was subcultivated once in the liquid medium. The cells in the exponential phase were harvested by centrifugation for 10 minutes at 3600 rpm. After washing twice, the cells were used as inoculum.

For the screening of precursors forming copolyesters, the cells were inoculated into the liquid media supplemented with various precursors (0.2% (v/v)). For the nutrient limitation experiment, the cells were inoculated into the specific nutrient-deficient liquid media supplemented with valeric acid (0.2% (v/v)). The culture condition and scale of these experiments were identical to those used for preparing the seed culture.

For the oxygen limitation experiment, a 5 l jar fermentor (Korea Fermentor Co.) with a working volume of 3 l was employed to control dissolved oxygen (D.O.) concentration. The pH was controlled at 7.0 by adding 4 N NaOH. D.O. concentration was controlled by changing the agitation rate. The oxygen-deficient experiment was carried out using an Erlenmeyer flask containing the medium deprived of oxygen. In addition, a rubber stopper was used.

**Analytical Methods**

Biomass was estimated by measuring both optical density at 570 nm (Spectronic 21, Milton Loy Co., U.S.A.) and dry cell weight (DCW). Dry cell weight was measured after drying overnight at 105℃.

The concentrations of poly-β-hydroxyalkanoate (PHA) and its 3HV were estimated by the modified Braunegg method (5) using gas chromatography (GC-8A, Shimadzu, Japan) with a capillary column (CBP-1, Shimadzu, Japan). Nitrogen was used as a carrier gas.

Molecular weight of PHA was measured by gel permeation chromatography (GPC 150CV, Waters, U.S.A.) with polystyrene as a standard (1). Chloroform was used as an eluent in GPC operation.

Ammonia concentration was measured by the indophenol method (3) with ammonium sulfate as a standard.

## RESULTS AND DISCUSSION

**Isolation of Methylotroph**

Sterilized saline solution was added to each sample from methanol contaminated soil, and the mixture was vigorously stirred. After settling the soil, the supernatant was serially diluted and then plated on the agar plates containing methanol as a sole carbon source. One gram soil sample was found to contain approximately 106107 cells of methylotrophs. To obtain pure cultures of methylotroph, 21 colonies randomly selected from the agar plates were cultivated in a liquid medium, and replated on agar plates. Based on their growth rate and the fraction of PHB accumulation of the cells in the liquid medium, 5 strains were selected for further tests of their capabilities of copolyester production.

When the cells were in the stationary phase, valeric acid, a well-known precursor, was added to the cultures at a concentration of 0.2%(v/v). All of the 5 strains could incorporate valerate into the PHB chain to form P(3 HB-co-3HV). Among the 5 strains tested, the C-02 showed the highest percentage of P(3HB-co-3HV) per biomass, and was selected for further study.

**Characteristics of Strain C-02**

The characteristics of strain C-02 were determined according to Bergey's manual and summarized in Table 2.

Strain C-02 could not grow in anaerobic condition, and therefore, was identified as a strict aerobe. The color of colonies on the agar plates was pink. The shape of cells observed by light microscope (BH2, Olympus, Japan) is rod type and the cells have motility. The photograph of strain C-02 taken by transmission electron microscopy (TEM) shows that the cell has flagella, peripheral internal membrane (type II), and PHA granules (Fig. 1). The internal peripheral membrane is a characteristic of methylotroph type II. Although a major carbon source was methanol, the cells could also utilize glucose. Hence, strain C-02 is a facultative methylotroph.

Taken together, the observation made here shows that the strain C-02 belongs to *Methylococcaceae* family Type II-b subgroup (10).

**Precursor Screening**

**Table 2.** Characteristics of methylotroph C-02.

| Characteristics | Systematics | Method |
|---|---|---|
| Rod<br>Obligate aerobe<br>Motile<br>Length: 2.5 μm<br>Diameter: 1 μm | Obilgate aerobic bacteria | Microscopic observation (light and TEM) |
| Pink colored colony | | Colony observation |
| Methanol utility: + | Familiy: *Pseudomonadaceae* or *Methylococcaceae* | Cultivation |
| Internal membrane: Plat, Staged. Peripheral to cell membrane | Family: *Methylococcaceae* Type II | TEM |
| Glucose Utility: + | Subgroup II-b | Cultivation |
| Strain C-02. | Obligate aerobic bacteria *Methylococcaceae* family Type II-b subgroup | |

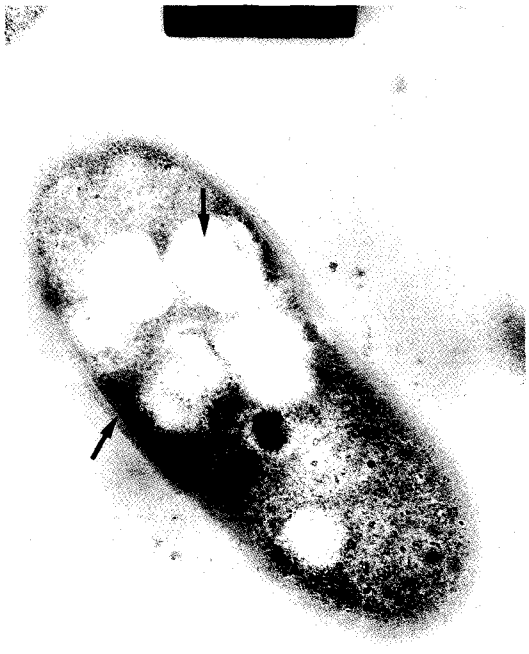

**Fig. 1.** Transmission electron microscope photogram of isolated methylotrope C-02 Arrows indicate PHB granule and internal platted membrane, respectively.

**Table 3.** Biosynthesis of PHA from various precursors.

| Kinds of Precursor | Added Precursor | Copolyester formation |
|---|---|---|
| Control | Only methanol | − |
| Acids | Propionic acid | − |
| | Butyric acid | − |
| | Iso-butyric aicd | − |
| | Valeric acid | − |
| | 5-Cl-Valeric acid | + |
| | Caprioc acid | − |
| | Heptanoic acid | − |
| | Methyl acetate | − |
| | Ethyl acetate | − |
| Alcohols | n-Popanol | − |
| | 2-Popanol | − |
| | 1-Cl-2-Propanol | − |
| | Butanol | − |
| | 1,4-Butandiol | − |
| | 1,5-Pentandiol | − |
| Alkanes | n-Ocatane | − |
| | n-Nonane | − |

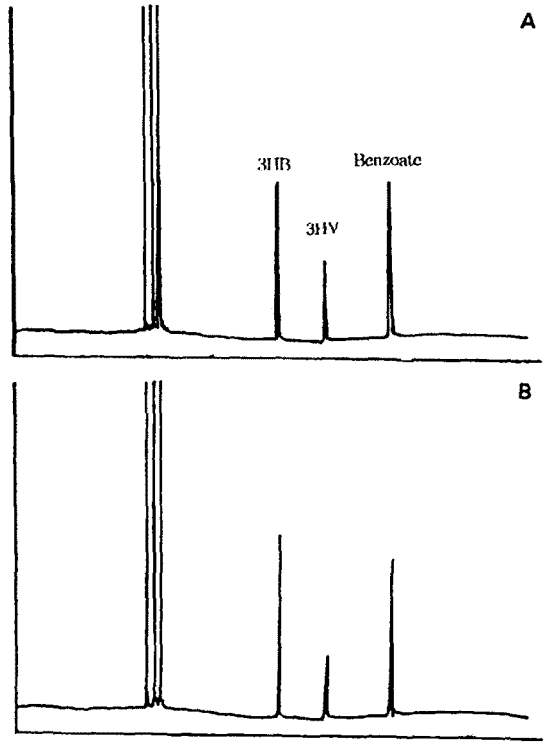


**Fig. 2.** Gas chromatogram of P(3HB-co-3HV).
A. Standard P (3HB-co-3HV) contain 24% 3HV. B. P(3HB-co-3HV) produced by methylotroph C-02.

To screen the precursors that can be incorporated into PHB chain, the cells were cultivated in the media supplemented with various precursors. Candidates for precursors to form PHA were selected considering their thermodynamic stability and mimicry to monomer. After 48 hours cultivation, DCW and PHA content were determined.

Among the various kinds of acid, alcohol, and alkane tested, only valeric acid could be incorporated into PHB to form the P(3HB-co-3HV) (Table 3). Fig. 2 shows the chromatogram prepared from the cells grown in the valeric acid-supplemented medium. It seemed that strain

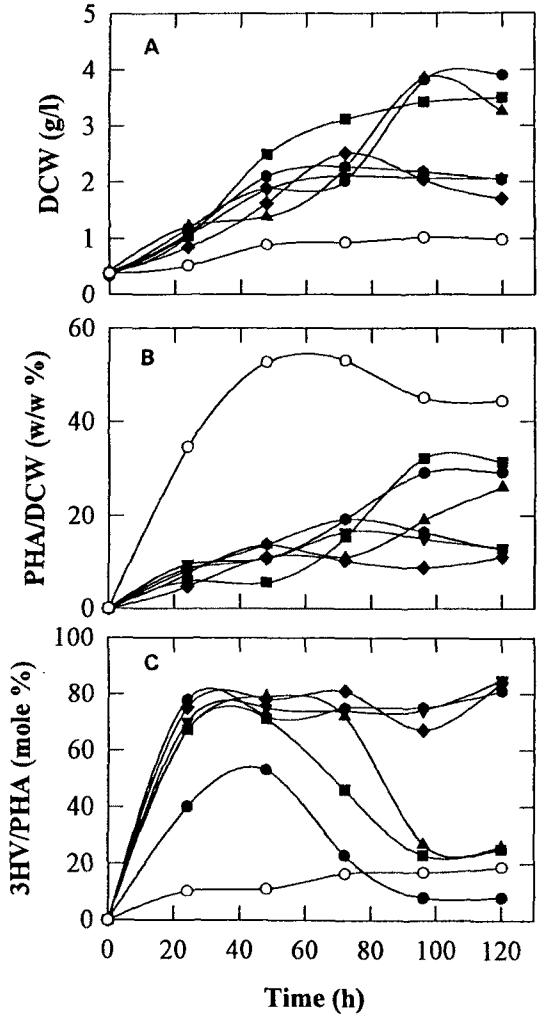


**Fig. 3.** Effect of nutrient limitation on cell growth and PHA accumulation by strain C-02.
A. Dry cell weight. B. PHA content per DCW. C. 3HV mole fraction per PHA. Nitrogen-limitation (○), Sodium (●), Magnessium (■), Potassium (▲), Sulfate (▼), Phosphate (◆), and Control (•).

C-02 could not metabolize valerate but only incorpoate into the PHB, because the increasing rate of 3-HV was same during the PHB accumulating period.

**Effect of Nutrient Limitation**

Many kinds of mineral are needed for cell growth. When one kind of mineral is limited, the cells do not show balanced growth (8). If there is an excess of carbon, the cells accumulate the carbon as reserve like PHA. To investigate the effect of nutrient limitation on P(3HB-co-3HV) accumulation, the cells were inoculated into the specific nutrient-deficient media supplemented with valeric acid. Fig. 3 shows the cell growth and P(3HB-co-3HV) production under various nutrient-limited conditions. Final values of DCW and % P(3HB-co-3HV) per DCW obtained after 48 hours cultivation are summarized in Table 4.

**Table 4.** Effect of nutrient limitation on cell growth and P(3HB-co-3HV) production.

| Deficiedt Nutrients | Dry cell Weight (g/l) | 3HV content in P(3HB-co-3HV) (mol%) | P(3HB-co-3HV) in DCW (%) |
|---|---|---|---|
| No limitation | 3.34 | 76.2 | 10.8 |
| $NH^{4+}$ | 1.00 | 18.2 | 50.2 |
| $Na^{+}$ | 4.12 | 75.9 | 11.4 |
| $Mg^{2+}$ | 3.00 | 76.0 | 7.7 |
| $K^{+}$ | 3.20 | 80.7 | 13.1 |
| $SO^{4-}$ | 3.21 | 81.7 | 13.1 |
| $PO^{4-}$ | 1.66 | 85.5 | 20.7 |

Nitrogen-limitation showed the most detrimental effect on cell growth (Fig.3 A), probably because nitrogen is the most required nutrient next to carbon. On the other hand, the highest P(3HB-co-3HV) per DCW was obtained in a nitrogen-limited culture (Fig. 3 B). Thus, excess carbon such as methanol and valerate appeared to be converted to PHA faster as the cell growth is more significantly inhibited. The maximum percentage of P(3HB-co-3HV) per DCW which was achieved after 48 hours of cultivation in a nitrogen-limited culture was determined to be 50.2%. This value is approximately 5 times higher than that obtained from the control culture without any nutrient limitation. The lowest mole fraction of HV per PHA was obtained in a nitrogen-limited culture because of the enhanced PHB production in a nitrogen limited culture. No significant difference in the absolute amount of HV was observed among various nutrient-limited cultures.

The average molecular weight of P(3HB-co-3HV) obtained in a nitrogen-limited culture was measured by GPC and was found to be approximately $2.8\times10^5$ daltons. When methylotroph produced only PHB in a nitrogen limited condition, its molecular weight was found to be approximately $3.7\times10^5$ (7) Accordingly, precursors like valerate may inhibit the polymerization of PHB.

Oxygen is another essential nutrient for cell metabolism. When *Azotobacter beijerinckii* was cultivated in oxygen-limited condition, its PHB content per cell increased (11). To determine the effect of oxygen limitation on strain C-02's PHB production, the cells were first cultivated in an oxygen-deficient condition. As mentioned previously, strain C-02 is an obligate aerobic bacterium. Thus, in an oxygen-deficient condition, cell growth stopped and PHB did not accumulate (data not shown). Next, when the D.O. concentration was maintained close to zero (Fig. 4), accumulation of PHB was observed (data not shown). The percentage PHB per DCW after 60 hours of cultivation was 4.6 %. On the other hand, when D.O. concentration was maintained between 30~70 %, no accumulation was observed after

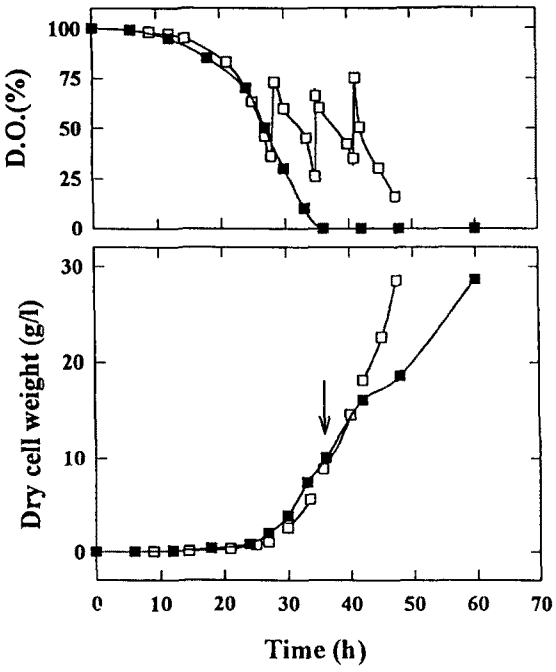


**Fig. 4.** Growth and dissolved oxygen profile with time cource by strain C-02.
D.O.-limited culture (□) and Control (■). In a control experiment, D.O. concentration was maintained about 30 to 70% of air saturation during culture time. Arrow indicates the starting time of D.O. limitation.

45 hours of cultivation. Accordingly, it was found that oxygen limitation also induced PHB accumulation in strain C-02, methylotroph. However, the enhancement in PHB accumulation under oxygen-limited condition was not as significant as that in nitrogen-limited condition.

In conclusion, the methylotroph (strain C-02) producing PHB from methanol was isolated. Strain C-02 can form P(3HB-co-3HV) by incorporating valerate into the PHB chain. In a nitrogen-limited condition, the % P(3 HB-co-3HV) per DCW was 50.2. To maximize the production of P(3HB-co-3HV), we are currently developing a strategy for fed-batch culture.

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