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Production of Poly-3-hydroxybutyrate from Xylose by *Bacillus megaterium* J-65

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A microorganism capable of producing high level of poly-3-hydoxybutyrate (PHB) from xylose was isolated from soil. The isolated strain J-65 was identified as *Bacillus megaterium* based on the morphological, biochemical and molecular biological characteristics. The optimum temperature and pH for the growth of *B. megaterium* J-65 were 37°C and 8.0, respectively. The optimum medium composition for the cell growth was 2% xylose, 0.25% $(NH_4)_2SO_4$, 0.3% $Na_2HPO_4 \cdot 12H_2O$, and 0.1% KH_2PO_4. The optimum condition for PHB accumulation was same to the optimum condition for cell growth. Copolymer of β-hydroxybutyric and β-hydroxyvaleric acid was produced when propionic acid was added to shake flasks containing 20 g/l of xylose. Fermenter culture was carried out to produce the high concentration of PHB. In batch culture, cell mass was 9.82 g/l and PHB content was 35% of dry cell weight. PHB produced by *B. megaterium* J-65 was identified as homopolymer of 3-hydoxybutyric acid by GC and NMR.

Key words : Poly-3-hydoxybutyrate, xylose, *Bacillus megaterium* J-65

Introduction

Poly-3-hydroxybutyrate (PHB) is synthesized and accumulated by a number of bacteria as an energy and carbon storage molecule, under unfavorable growth conditions in the presence of excess carbon sources [14,15]. Among the variety of currently known biodegradable polymers, PHB is considered to be a particularly attractive substitute for conventional petrochemical plastics, due to the fact that it evidences mechanical properties similar to those of a variety of thermoplastics and elastomers, as well as the fact that it is completely biodegradable in a number of environments [5,7,8,18,21,25]. Although PHB has been recognized as a good candidate for a biodegradable plastic, its high price compared to conventional plastics has limited its use in a broad range of applications [13].

In an effort to ameliorate the cost of PHB production, a great deal of effort has been devoted to the development of superior bacterial strains, as well as more efficient fermentation/recovery processes [12,13]. One approach to reducing the cost of PHB production involves the use of less expensive carbon sources, for example, cane and beet molasses, cheese whey, plant oils, hydrolysates of starch (corn and tapioca), cellulose, and hemicellulose [3,15,27]. Agricultural waste materials, such as lignocellulose, are considered to be desirable as carbon sources for PHB production. Several techniques (e.g., chemical and enzymatic hydrolysis) have already been developed and new techniques are being pioneered to convert the hemicellulosic fraction of agricultural residue and wood wastes for fermentable sugars, including xylose (a major sugar component of hard woods), arabinose, glucose, and mannose [20]. Although xylose is one of the principal components of lignocellulose, few commercial products can currently be obtained from xylose. *Pseudomonas cepacia* [20,29], *P. pseudoflava* [3], and recombinant *E. coli* harboring PHB biosynthesis genes from *Ralstonia eutropha* [14] have been shown to produce PHB from xylose, but the productivity of PHB in these cases was not as high as would be desirable [26].

This report describes the isolation of a microorganism which rapidly generates PHB from xylose. The characteristics of cell growth and PHB accumulation in mineral salt media containing xylose were assessed in order to gain insight into the primary physiological characteristics of an isolated strain.

Materials and Methods

Isolation of soil bacteria for PHB production

Bacteria were isolated from soil located in Geumjeong-gu

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and Buk-gu, both locations in Busan, Korea. Appropriately diluted soil samples were spread onto YM (3 g/l of yeast extract, 3 g/l of malt extract, 5 g/l of polypeptone, 10 g/l of glucose) agar plates. The colonies were isolated after 24 hours of incubation at 30°C Several colonies were transferred to mineral salt media containing 10 g/l of xylose, and then incubated for 48 hours at 30°C. Each liter of mineral salt medium contained 10 g of xylose, 2 g of $(NH_4)_2SO_4$, 4.5 g of $Na_2HPO_4 \cdot 12H_2O$, 1.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2 \cdot 2H_2O$, and 2 ml of a trace element solution (0.3 g of H_3BO_3, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of $NaMoO_4$, 20 mg of $NiCl_2 \cdot 6H_2O$, and 10 mg of $CuSO_4 \cdot 4H_2O$ per liter).

For PHB production, each of the strains was assessed using fluorescence microscopy after staining with Nile blue A [2] and then the PHB production strain was finally selected using gas chromatography (GC).

Identification of an isolated strain

The taxonomic characteristics of the isolate were determined in accordance with Bergey's Manual of Systematic Bacteriology [6]. The bacterial 16S rRNA gene was amplified using the universal primer set, 27F (AGAGTTTGATCMTGG CTCAG) and 1492R (GGYTACCTTGTTACGACTT) (where M is A or C, Y is C or T), and the purified PCR products directly sequenced on an ABI 3730XL capillary DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The 16S rRNA gene sequence was blasted in the NCBI database, and the closest type strains determined. The partial 16S rRNA gene sequence has the GenBank accession no. EU109501.

Cultivation

The optimum growth condition of the isolated strain J-65 was determined in shake-flask cultures. Cells (10 ml) grown in YM broth for 18 hours at 37°C were inoculated into 500 ml shake flasks containing 200 ml of the mineral salt medium, and the cells were grown for 24 hours at 37°C and 200 rpm. In order to determine the optimum condition for PHB production, a two-stage culture technique was utilized [10]. The cells were initially grown for 24 hours in mineral salt medium without nutrient limitation, after which an essential nutrient, such as N, P, S, or Mg was deleted from the medium in order to facilitate efficient PHB synthesis. Batch cultivation was conducted in a 5-L Fermenter (Biostat, B. Braun Melsungun, Germany). The culture condition was as follow: Temperature, 37°C agitation speed, 300 rpm; air flow rate, 1 vvm; working volume, 3 l; inoculum size, 6% (v/v); culture pH was controlled to 7.0 by adding NaOH or HCl.

PHB purification

The biomass harvested by centrifugation from the culture broth was stored at 4°C Cells containing PHB were treated with NaOH solution. Lyophilized biomass (100 mg) was mixed with 10 ml of chloroform for 1 hour at 40°C Residual biomass was removed by filtration. Five volumes of cold methanol were added to the PHB solution. The precipitate was then pressed to remove methanol and dried for 2 days under ambient condition [11].

Analyses

Xylose concentration was determined according to the method of Somogyi-Nelson [16,22]. The ammonium concentration was quantified using a modified Berthelot reaction [23]. Dry cell weights was determined after drying to a constant weight at 105°C after centrifuging 50 ml of culture broth for 10 min at 16,000× *g*, and washing once with deionized water.

The PHB and P (3HB-3HV) concentrations were determined according to Braunegg *et al* [4] using gas chromatography (Hewlett-Packard HP-5890A). The gas chromatographic analyzer was equipped with a flame ionization detector (FID) and a 6-foot glass packed column filled with 10% carbowax 20 M. The detection condition was as follow: initial temperature, 140°C; final temperature, 180°C, with a rate of temperature increase of 10° DEG/min. PHB powders of *R. eutropha* (ICN Biomedical Co., USA) and P(3HB-3HV) (Sigma Aldrich Co., USA) were used as the standards. The chemical structure of the PHB produced was determined by measurement of ^{1}H NMR at 300 MHz.

Results and Discussion

Screening of the strain and its PHB accumulation from xylose

The colonies were stained with Nile blue A, and the PHB-containing cells emitted a bright orange fluorescence, which could be readily distinguished from the dark background. The colonies harboring PHB granules were screened further by GC for measurements of PHB content. Among the iso-

lated strains, J-65 was selected for further detailed study. Strain J-65 evidenced catalase, gelatin hydrolysis, and starch hydrolysis activity, and also utilized a broad range of carbohydrates. In particular, this strain grew well on xylose, arabinose, and glucose, the main products of hemicellulose. According to Bergey's Manual of Systematic Bacteriology [6], this strain was identified as a *Bacillus* species. Analysis of the partial nucleotide sequence of the 16S rRNA indicated that the bacterium was similar to *Bacillus megaterium* (99.9%). Therefore, the strain was designated as *B. megaterium* J-65 (Fig. 1).

Optimum conditions for cell growth

The optimum temperature and initial pH for the growth of *B. megaterium* J-65 were found to be 37°C and 8.0, respectively. For optimum cell growth, it is crucial that the concentrations of carbon, nitrogen, and phosphorus be controlled. In order to determine the effects of xylose concentration, test-tube cultures were conducted at a variety of xylose concentrations. The concentration of cells increased with increasing concentrations of xylose up to 20 g/l (data not shown). The cell growth observed with the various tested nitrogen sources. Maximum growth was observed on polypeptone. However $(NH_4)_2SO_4$ was suggested to be used to lower the final production cost, its optimum concentration was 2.5 g/l. We also evaluated the effects of different concentrations of other components of the mineral salt medium on growth. The optimum medium composition for the cell growth was 2% xylose, 0.25% $(NH_4)_2SO_4$, 0.3% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.003% $FeSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, and 0.4% trace element solution.

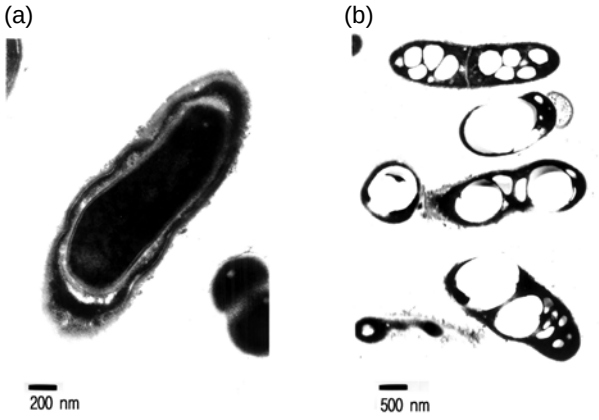

Fig. 1. Transmission electron micrograph (TEM) of *B. megaterium* J-65. (a) It was identified as a Gram-positive and endospore-forming rod-shape bacterium. (b) TEM revealed the presence of PHB granules.

The optimum condition for PHB accumulation

PHB synthesis is favored under certain unbalanced growth conditions, such as limitation of N, P, S, Mg and/or O_2 [1]. Thus, a two-stage culture technique for the production of PHB was employed. When nutrient limitation was applied, the cell concentration and PHB content decreased compared to those observed in nutrient-rich condition (control).

In order to evaluate the effect of a major cultivation variable, the C/N molar ratio, on PHB accumulation, *B. megaterium* J-65 was cultured in mineral salt medium using various concentrations of $(NH_4)_2SO_4$ ranging from 0 to 3.5 g/l, coupled with a fixed xylose concentration of 20 g/l (Fig. 2a).

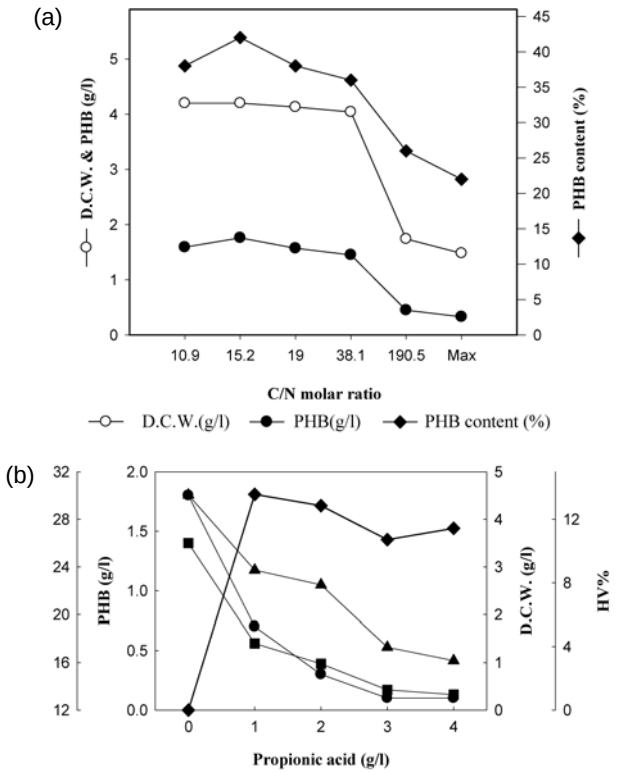

Fig. 2. Effects of C/N molar ratio and propionate concentration on PHB production of *B. megaterium* J-65. (a) The maximum PHB concentration, 1.8 g/l, was achieved at a C/N molar ratio of 15.2. (b) Cells were cultivated in optimum growth medium containing 2% of xylose as a primary carbon source and each concentration of propionic acid as a secondary carbon source. It shows the dry cell weight, concentration, and content of P (HB-co-HV), as well as the molar fraction of 3-hydroxyvalerate (3HV). *HV%: 3-hydroxyvaleric acid content in PHB/HV (w/w).

In general, at high C/N molar ratios, the carbon source may be utilized principally for PHB accumulation due to the nitrogen limitation, whereas at a low C/N molar ratio, the carbon source may be utilized principally for cell growth, due to sufficient nitrogen availability [24]. However, this strain evidenced an increased final PHB concentration at a low C/N molar ratio. The above results show that *B. megaterium* J-65 is a growth-associated PHB producer. Some organisms, including a mutant strain of *Azotobacter vinelandii* [8,17], *Pseudomonas olevorans* [19], and certain strains of *Alcaligenes latus* [28], produced PHB in growth-related mode, regardless of nitrogen limitation conditions. The main advantage associated with growth-associated PHB producers are reduced fermentation time and easily regulated fermentation. These qualities contribute significantly to a reduction in cost.

Cultivation of *B. megaterium* J-65 under balanced-growth condition

Cell growth was activated relatively rapidly from 4 h, and the stationary phase was achieved at the 21 hr of fermentation. The dry cell weight and PHB content, measured after 21 hours, were 9.82 g/l and 35%, respectively (Fig. 3). This strain began to accumulate PHB during the early exponential growth phase, while a sufficient amount of $(NH_4)_2SO_4$ remained in the medium. The result observed from the batch fermentation of *B. megaterium* J-65 using xylose as a carbon source indicates that PHB production occurred in a growth-related mode. At the stationary phase of cell growth, PHB production was also halted, even though the carbon source remained at a concentration of 10 g/l. After stationary phase, the PHB concentration decreased as the result of cell catabolism of PHB as a carbon source. For the efficient production of PHB by a growth-related PHB-producing strain, it will be crucial to develop a fed-batch culture strategy, considering the determined balance between cell growth and PHB synthesis.

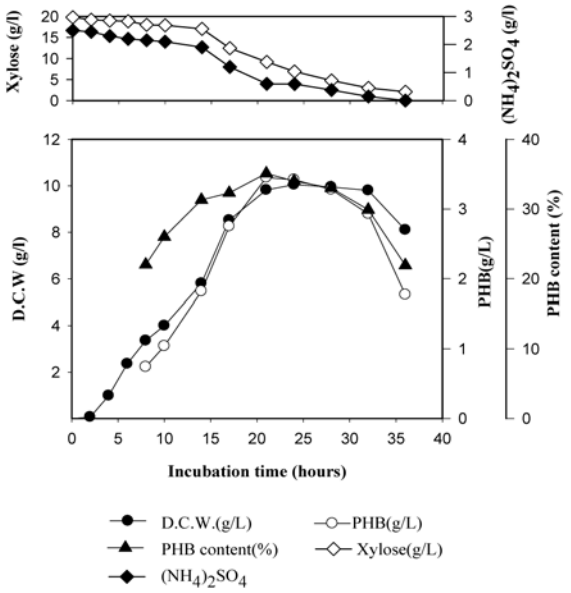

Fig. 3. Time course of batch culture of *B. megaterium* J-65. Culture was carried out in 5-L fermentor at pH 7.0, 37°C, 1 vvm and 300 rpm; 2% xylose and 0.25% $(NH_4)_2SO_4$. It shows the patterns of total cell growth, PHB accumulation for *B. megaterium* J-65 cultured in a 5-L fermenter.

P(HB-co-HV) biosynthesis of *B. megaterium* J-65

When propionic acid was added to flasks containing 20 g/l of xylose, the industrially more interesting P(HB-co-HV) copolymer was generated (Fig. 2b). As the result of the increase in the propionic acid concentration, both cell growth and PHB production were inhibited due to propionate toxicity in the medium. Propionic acid was shown to inhibit the growth of *R. eutropha* at the concentration above 1 g/l in the fermentation broth [9]. Hence, the feeding of propionic acid must be controlled precisely in the fermentation process for copolymer synthesis.

Identification and characterization of polymer

The structure of PHB was characterized via ^1H nuclear magnetic resonance (NMR) analysis using a 300 MHz spectrometer in deuterochloroform. The purified PHB was found

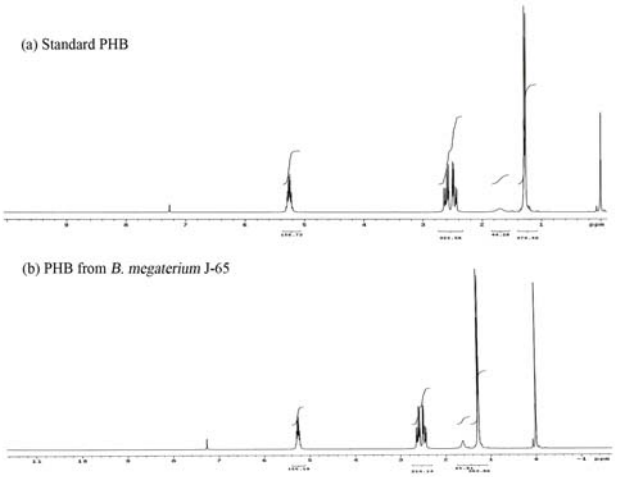

Fig. 4. 300 MHz ^1H-NMR spectrum of PHB produced by *B. megaterium* J-65.

to be identical to the standard PHB (Fig. 4). According to the results of NMR analysis, the PHB produced from xylose was identified as a homopolyester of D-3-hydroxybutyric acid.

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초록 : *Bacillus megaterium* J-65에 의한 xylose로부터 poly-3-hydroxybutyrate 생산

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본 연구는 생분해성 플라스틱인 poly-β-hydroxybutyrate (PHB)의 생산단가를 낮추기 위한 노력으로 저가의 기질로부터 PHB 대량생산을 위한 기초자료를 얻는데 그 목적을 두었다. Hemicellulose hydrolysate는 지구상에 풍부하게 존재하는 저가의 waste by-product로서 xylose가 많이 포함되어 있다. 본 연구에서는 xylose로부터 PHB를 생산할 수 있는 균주를 토양에서 분리하여, 분류학적 위치를 밝히고, 균체 생육 최적 조건, PHB 생산을 위한 최적 발효 배양 조건, PHB의 구조 확인 등을 검토 하였으며, 그 결과는 다음과 같다. 토양으로부터 분리한 균주 J-65는 형태학적, 배양적, 생화학적 및 partial 16S rRNA sequence에 근거하여 *Bacillus megaterium*로 동정하였다. *B. megaterium* J-65의 균체 생육 최적 조건은 온도 37°C, 초발 pH 8.0이었으며 2% xylose, 0.25% $(NH_4)_2SO_4$, 0.3% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4였다. PHB 축적에 영향을 미치는 요인을 검토하기 위해 균체생육 최적배지에서 37°C, 24시간 1차 배양한 후, 균체를 회수하여 각종 영양분이 결핍된 배지에 2차 배양을 실시한 결과 *B. megaterium* J-65는 균형생육조건(balanced-growth condition)에서 PHB를 합성하는 균주로 나타났다. PHB보다 물성이 향상된 PHB/HV 공중합체를 생산하기 위하여 보조기질로 propionic acid를 첨가하였을 때, 0.1% propionic acid 농도에서 HV mol%가 14%인 PHB/HV 공중합체가 합성되었다. 5 l 용량의 발효조에 *B. megaterium* J-65를 회분배양하였을 때 배양 21시간에 건조균체량 10 g/l, PHB 3.5 g/l를 얻을 수 있었고, 유가배양을 실시한 결과 배양 48시간에 건조균체량 26.52 g/l, PHB 9.28 g/l를 얻을 수 있었다. 생산된 PHB를 alkaline solution 처리와 chloroform을 이용한 유기용매 추출법을 이용하여 추출 · 정제한 후 Gas Chromatography로 정제를 확인하고 300MHz 1H-NMR을 실시한 결과 3-hydroxybutyrate의 homopolymer임을 확인하였다.