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Production of medium-chain-length polyhydroxyalkanoates by activated sludge enriched under periodic feeding with nonanoic acid

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ABSTRACT

The potential use of activated sludge for the production of medium-chain-length polyhydroxyalkanoates (MCL-PHAs) was investigated. The enrichment of bacterial populations capable of producing MCL-PHAs was achieved by periodic feeding with nonanoic acid in a sequencing batch reactor (SBR). Denaturing gradient gel electrophoresis analysis revealed *Pseudomonas aeruginosa* strains to be predominant in the bacterial community during the SBR process. The composition of PHA synthesized by the enriched biomass from nonanoic acid consisted of a large concentration (>89 mol%) of MCL monomer units and a small amount of short-chain-length monomer units. Under fed-batch fermentation with continuous feeding of nonanoic acid at a flow rate of 0.225 g/L/h and a C/N ratio of 40, a maximum PHA content of 48.6% dry cell weight and a conversion yield ($Y_{p/s}$) of 0.94 g/g were achieved. These results indicate that MCL-PHA production by activated sludge is a promising alternative to typical pure culture approaches.

1. Introduction

Poly(3-hydroxyalkanoates) (PHAs) are versatile polyesters produced by numerous bacteria as an intracellular carbon and energy storage compound. PHAs have recently attracted a great deal of industrial attention as promising biomaterials capable of replacing synthetic polymers due to their excellent biodegradability, biocompatibility and capability of being produced from renewable resources (Keshavarz and Roy, 2010). These polyesters can be classified into two subgroups, short-chain-length PHAs (SCL-PHAs) and medium-chain-length PHAs (MCL-PHAs), depending on the number of carbon atoms in the monomer units. SCL-PHAs consist of 3-hydroxyalkanoates of C_3–C_5, while MCL-PHAs are composed of 3-hydroxyalkanoates of C_6–C_{14}. In general, MCL-PHAs have a significantly lower degree of crystallinity as well as lower melting and glass transition temperatures than SCL-PHAs. Accordingly, MCL-PHAs are referred to as elastomers, while SCL-PHAs are known as thermoplastics (Kim et al., 2007).

MCL-PHAs are considered to be much more suitable biomaterials for various applications based on their physical properties (Chen and Wu, 2005). Many pseudomonads belonging to the ribosomal RNA homology group I can produce MCL-PHAs when they are grown with relatively long carbon substrates such as alkanes, alkenes, and carboxylic acids. In addition, some PHA-producing organisms, such as *Pseudomonas oleovorans* and *Pseudomonas putida*, are capable of synthesizing MCL-PHAs bearing functional groups such as halogens, cyclohexyl, aromatic, and unsaturated groups in the side chains when they are grown with substrates containing corresponding chemical structures (Kim et al., 2007). Incorporation of functional groups into PHA is of great importance since it can improve the physical properties of PHA as well as allow further chemical modification of PHA, thereby extending the potential application of these biopolymers (Hazer and Steinbüchel, 2007). Despite these advantages, their widespread applications have been limited due to high production cost. Indeed, over 40% of the total operating expense of PHA production is related to the raw materials, and more than 70% of this cost is attributed to the carbon source (Salehizadeh and Loosdrecht, 2004).

There has recently been increased interest in the production of PHAs via the use of activated sludge as mixed culture. The production of PHAs by activated sludge is expected to be more economically favorable than pure culture processes since the waste sludge contains PHA-producing microorganisms within it and sterile conditions can be avoided during the synthesis process (Castilho et al., 2009; Dias et al., 2006). To increase the PHA production rate, bacteria that are capable of accumulating PHA must be enriched in the activated sludge. The enrichment of PHA-accumulating organisms can be achieved by periodic supplementation of a sequencing batch reactor (SBR) with external substrate. This SBR periodic feeding creates an alteration of excess and lack of external substrate (feast and famine conditions) that favors indigenous microorganisms with an ability to store internal carbon reservoirs, such as PHAs, during the feast phase (Serafim et al., 2004). Under these

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dynamic conditions, PHA-accumulating microorganisms have an advantage over those that do not have this ability, resulting in their becoming dominant in the complex microbial community.

To date, many studies have been conducted to evaluate the potential for PHA production by activated sludge using various external carbon substrates (Salehizadeh and Loosdrecht, 2004; Serafim et al., 2008). However, most of the data obtained from these studies have been limited to the production of SCL-PHAs, such as polyhydroxybutyrate (PHB), and its copolymer with 3-hydroxyvalerate [poly(3HB-*co*-3HV)]. Recently, Ciesielski et al. (2006) identified four distinctive MCL-PHA-producing bacterial strains based on PCR amplification of MCL-PHA synthase gene fragments from activated sludge fed with methanol as the sole carbon source. Among them, three strains were *Pseudomonas* species and the other one was *Comamonas testosterone*. Moreover, open mixed cultures enriched in glycogen accumulating organisms with fermented sugar cane molasses were shown to produce PHAs containing significant amounts of MCL monomer 3-hydroxyhexanoate (Bengtsson et al., 2010; Pisco et al., 2009). Although these studies revealed the feasibility of MCL-PHA production by activated sludge, a great deal of information regarding the fermentation strategies and eco-physiological features of activated sludge in connection with the efficient production of MCL-PHAs is still needed.

Nonanoic acid is a well-known carbon substrate for the efficient production of MCL-PHAs by many pseudomonads (Sun et al., 2007). With only a few exceptions, MCL-PHAs produced by microorganisms grown on nonanoic acid as the sole carbon substrate generally consist of odd-numbered 3-hydroxyalkanoates such as 3-hydroxynonanoate (3HN), 3-hydroxyheptanoate (3HHp), and 3-hydroxyundecanoate (3HU). Therefore, nonanoic acid can be an ideal external substrate for the enrichment of MCL-PHA-producing microorganisms in activated sludge and for the discrimination of PHA constituents (monomer units) synthesized from the external substrate (nonanoic acid) and various other substrates that may be present in activated sludge.

The current study describes MCL-PHA production by mixed cultures enriched by periodic feeding with nonanoic acid in a SBR starting from activated sludge. The polymer composition and yield during batch and fed-batch fermentation of the enriched biomass was evaluated. The possible application of the mixed culture for the production of MCL-PHAs bearing functional groups in the side chains was also investigated. In addition, to extend our knowledge regarding the microorganisms responsible for MCL-PHAs, molecular analysis of the bacterial community composition of the mixed culture enriched under feast and famine aerobic conditions in a SBR was conducted based on denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene segments.

2. Methods

2.1. SBR operation

A laboratory scale SBR was used to enrich and produce sludge with a higher storage response. The reactor was inoculated with activated sludge collected from the municipal wastewater treatment plant in Daejeon, Korea. Each SBR cycle consisted of 1 h of feeding, a 5 h feast phase, 18.9 h famine phase, and 0.1 h withdrawal phase. The system had a total working volume of 6 L, where 5.5 L functioned as the stationary volume and the remaining 500 mL functioned as the fill volume. During each cycle, the fill volume was supplemented with 500 mL of 6-fold concentrated E* mineral medium containing 10.8 g/L nonanoic acid as the sole carbon source. Each liter of E* mineral medium contained 0.5 g NH_4Cl, 5.8 g K_2HPO_4, 3.7 g KH_2PO_4, 0.37 g $MgSO_4{\cdot}7H_2O$, and 3 mL trace element stock solution. The trace element solution contained 0.29 g $ZnSO_4{\cdot}7H_2O$, 1.98 g $MnCl_2{\cdot}4H_2O$, 1.67 g $CaCl_2{\cdot}2H_2O$, 2.78 g $FeSO_4{\cdot}7H_2O$, 2.81 g $CoSO_4{\cdot}7H_2O$, and 0.17 g $CuCl_2{\cdot}2H_2O$ per liter of 1 N HCl. However, after the bacterial community in the SBR attained a steady state (i.e., after at least 20 cycles from the reactor inoculation), the fill volume in each cycle increased to 2 L. Simultaneously, to replace the same volume, a newly prepared 2 L of 1.5-fold concentrated E* mineral medium containing 5.4 g/L of nonanoic acid as the sole carbon source was added for continuation of the enrichment process. In this setup, the solid retention time was 3 days and equaled the hydraulic retention time. The average organic acid concentration at the beginning of each cycle was 1.8 g/L. The reactor was aerated by means of membrane compressors with an air flow rate of 0.5 vvm and stirred by a mechanical impeller at 200 rpm. The temperature and pH were automatically controlled at 30 °C and 7.0, respectively. At the end of each cycle, samples were taken at predetermined times for analysis of the PHA, biomass concentration (dry cell weight, DCW), residual carbon substrate, dissolved oxygen (DO), and nitrogen concentration.

2.2. Fermentation process

Batch fermentations for the production of MCL-PHA by the biomass selected in the SBR were conducted in a 5-L jar fermentor (Biotron Co., Korea) with a working volume of 2 L. Two liters of an activated sludge mixed liquor (approximately 3.0 g/L DCW) were withdrawn at the end of the SBR cycle, centrifuged (10,000*g*/15 min) and then resuspended into a fermentor containing 2 L of E* mineral medium amended with 1.8 g/L nonanoic acid as the sole carbon substrate. The concentration of ammonium chloride was varied to evaluate the effects of the carbon to nitrogen (C/N) ratio on cell growth and PHA accumulation. The other process parameters (temperature, pH, airflow rate, and agitation speed) were maintained the same as described for SBR operation. Cell cultivation was stopped approximately 2 h after the growth reached the stationary phase. Fed-batch fermentations were conducted using a procedure similar to that described above for the batch fermentation. The only difference was that nonanoic acid was constantly supplied to the fermentor at a flow rate of 0.225 g/L/h while varying the C/N ratio at 5–40.

2.3. Preparation of PHA

The activated sludge from the SBR or fermentor was centrifuged (10,000*g*/15 min), washed with distilled water, and then lyophilized. PHA was isolated from lyophilized cells by extracting with hot chloroform using a Soxhlet apparatus. To obtain the fine product, the extracted crude PHA was purified by repeated precipitation by the drop-wise addition to vigorously stirred cold methanol. This precipitation procedure was conducted at least three times.

2.4. Analytical methods

DCW was determined by centrifugation followed by freeze-drying of the cell pellet. Lyophilized cells and purified PHAs were subjected to methanolysis in the mixture of chloroform/methanol/sulfuric acid (1 mL/0.85 mL/0.15 mL) at 100 °C for 3.5 h. Following vigorous vortexing of the mixture with 1 mL de-ionized water, PHA-rich chloroform was finally recovered for analysis. The relative amounts of monomer units of synthesized PHAs were calculated from the area of the peaks of the methyl-esters of each monomer unit in the gas chromatograms of the methanolyzed samples. A Hewlett–Packard 6890 plus gas chromatograph equipped with a flame ionization detector and an HP-1 capillary column were used for gas chromatography analysis. The oven temperature was initially maintained at 80 °C for 4 min and then in-