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Biosynthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer by *Cupriavidus* sp. USMAA1020 isolated from Lake Kulim, Malaysia

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Abstract

Cupriavidus sp. USMAA1020 was isolated from Malaysian environment and able to synthesize poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), [P(3HB-*co*-4HB)] when grown on γ-butyrolactone as the sole carbon source. The polyester was purified from freeze-dried cells and analyzed by nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and ^{13}C NMR results confirmed the presence of 3HB and 4HB monomers. In a one-step cultivation process, P(3HB-*co*-4HB) accumulation by *Cupriavidus* sp. USMAA1020 was affected by carbon to nitrogen ratio (C/N). A two-step cultivation process accumulated P(3HB-*co*-4HB) copolyester with a higher 4HB fraction (53 mol%) in nitrogen-free mineral medium containing γ-butyrolactone. The biosynthesis of P(3HB-*co*-4HB) was also achieved by using 4-hydroxybutyric acid and alkanediol as 1,4-butanediol. The composition of copolyesters varied from 32 to 51 mol% 4HB, depending on the carbon sources supplied. The copolyester produced by *Cupriavidus* sp. USMAA1020 has a random sequence distribution of 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) units when analyzed by nuclear magnetic resonance (NMR) spectroscopy. When γ-butyrolactone was used as the sole carbon source, the 4HB fraction in copolyester increased from 25 to 60 mol% as the concentration of γ-butyrolactone in the culture medium increased from 2.5 g/L to 20.0 g/L.

Keywords: Polyhydroxyalkanoates; Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate); *Cupriavidus* sp.; Biosynthesis; γ-Butyrolactone

1. Introduction

Polyhydroxyalkanoate (PHA) is a biodegradable and biocompatible thermoplastic that can be synthesized in many microorganisms from almost all genera of the microbial kingdom. Many microorganisms synthesize polyhydroxyalkanoates (PHAs) as intracellular carbon and energy reserve materials (Anderson and Dawes, 1990; Doi, 1990). PHAs are usually accumulated within cells when growth is limited by nutrients such as nitrogen, oxygen, and other essential elements while in the presence of excess carbon (Reddy et al., 2003). Instead of being consumed for cellular growth, the excess carbon is taken into the cells and stored in the form of PHAs granules. Microorganisms are able to accumulate various types of PHAs in the form of homopolyesters, copolyesters, or polyester blends. More than 150 different monomer units are known to be incorporated into the polyester chain (Steinbüchel and Lütke-Eversloh, 2003). Accordingly, various copolyesters are expected when a bacterium is grown on mixtures of different precursors. One of these polyesters, poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] has been found to exhibit useful properties relative to other PHAs. Combining different monomers to form copolymers, as in P(3HB-*co*-4HB), produces a family of materials with mechanical properties that can be tailored to specific needs (Sudesh et al., 2000). Incorporation of 4HB units results in various copolymers with physical properties that range from being highly crystalline to strong elastomeric

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rubber-like material (Saito et al., 1996). Generally, P(3HB-*co*-4HB) is produced by feeding precursor carbon sources such as 4-hydroxybutyric acid, 1,4-butanediol and γ-butyrolactone. P(3HB-*co*-4HB) has desirable mechanical properties for applications in the medical and pharmaceutical fields (Williams and Martin, 2002). P(3HB-*co*-4HB) has also been identified as a polyester with therapeutic interests. This is because both 3-hydroxybutyrate and 4-hydroxybutyrate are common metabolites present in human (Sudesh et al., 2000). These makes P(3HB-*co*-4HB) one of the most valuable biopolymers among the vast number of different PHAs synthesized by microorganisms. To date, five wild-type bacteria which can produce P(3HB-*co*-4HB) have been reported. They are *Ralstonia eutropha* (Kim et al., 2005), *Alcaligenes latus* (Kang et al., 1995), *Comamonas acidovorans* (Lee et al., 2004), *C. testosteronii* (Renner et al., 1996) and *Hydrogenphaga pseudoflava* (Choi et al., 1999). Among these microorganisms, *R. eutropha* has been reported to produce P(3HB-*co*-4HB) with various 4HB fractions (0–100 mol%) and has been studied most extensively. However, not many fermentation studies have been carried out for the production of P(3HB-*co*-4HB) (Kim et al., 2005). In the present study, we report the biosynthesis of P(3HB-*co*-4HB) by *Cupriavidus* sp. USMAA1020, a novel bacterium that was isolated from Malaysian environment.

2. Methods

2.1. Isolation and characterization of P(3HB-co-4HB) producer

Samples from environment such as soil, sludge and water in Peninsular Malaysia were screened for PHA producers. Samples were enriched with mineral salts medium (MSM) containing γ-butyrolactone as the sole carbon source (0.74% v/v) but with limited nitrogen source to maintain the C/N ratio at 20. After overnight incubation, cultures were diluted in sterile distilled water and plated on solid mineral salts medium containing γ-butyrolactone and Nile red stain (5 μg/ml) (Spiekermann et al., 1999). Colonies that formed following incubation were replicated onto a fresh medium. The original plate was then exposed to ultraviolet (320 nm) illumination to identify PHA producers. In addition, biochemical characterization, 16S rRNA sequencing, DNA base composition, cellular fatty acids analysis and DNA–DNA hybridization were performed to determine the identity of the isolate, USMAA1020.

For conventional biochemical characterization, bacteria were grown on nutrient agar plates for 24–48 h and then inoculated into the test reagent. The API 20 NE (bioMerieux) was utilized according to the protocol supplied by the manufacturer. The 16S rRNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA. Genomic DNA extraction, PCR mediated amplification of the 16S rDNA and purification of the PCR product was carried out as described previously (Rainey et al., 1996). Purified PCR products were sequenced using the CEQ™ DTCS-Quick Start Kit (Beckmann Coluter) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the CEQ™ 8000 Genetic analysis System. The resulting sequence data from the strain was put into the alignment editor ae2 (Maidak et al., 1999), aligned manually and compared with the representative 16S rRNA gene sequemces of organisms belonging to the β-*Proteobacteria*. A phylogenetic tree based on the neighbour-joining method was constructed by using the PHYLIP package (Felsenstein, 1993).

For the determination of DNA base composition, DNA was enzymically degraded into nucleosides as described by Mesbah et al. (1989). The resulting deoxyribonucleosides were analyzed by HPLC (HP 1090 Liquid Chromatograph, Hewlett Packard) using a Vydac 201SP54 C18 column at 40 °C. The solvent was 0.3 M $NH_4H_2PO_4$ (pH 4.4) with 2.5% (v/v) acetonitrile. Non-methylated lambda-DNA (Sigma) was used as the calibration reference.

For fatty acids analysis, fatty acid methyl esters were obtained by saponification, methylation and extraction using the method as described by Kuykendall et al. (1988). The fatty acid methyl esters mixtures were separated using Sherlock Microbial Identification System (MIS) (Microbial ID, Newark, DE 19711 USA.) which consisted of a Hewlett-Packard Model 5980 gas chromatograph fitted with a 5% phenyl–methyl silicone capillary column (0.2 mm × 25 m), a flame ionization detector, Hewlett-Packard model 7673A automatic sampler, and a Hewlett-Packard model KAYAK XA computer (Hewlett-Packard Co., Palo Alto, California, USA). Peaks were automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID).

DNA–DNA hybridization by the initial renaturation rate was carried out under consideration of the modifications described by Huss et al. (1983) using spectrophotometer (Cary 100 Bio UV/VIS) equipped with a Peltier-thermostatted 6X6 multicell changer and a temperature controller with in situ temperature probe (Varian). The hybridization temperature was 70 °C and the reaction was carried out in 2X standard saline citrate (SSC) and 10% formamide. Each value was the mean of at least two hybridization experiments.

2.2. Bacterial strain

Cupriavidus sp. USMAA1020 (DSM 19416) used in this study was isolated from sludge in Lake Kulim, Malaysia after screening 663 isolates. To prepare inoculums, *Cupriavidus* sp. USMAA1020 was grown at 30 °C under aerobic condition in a nutrient broth (5 g of peptone, 2 g of yeast extract, 1 g of beef extract, and 5 g of NaCl in 1 L distilled water). For maintenance purpose, *Cupriavidus* sp. USMAA1020 from the exponential growth phase was stored at −20 °C in 20% (v/v) glycerol.