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High poly(β-hydroxybutyrate) production by *Pseudomonas fluorescens* A2a5 from inexpensive substrates

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Abstract

A strain of poly(β-hydroxybutyrate) (PHB)-accumulating bacterium was isolated from the soil in Alaska of USA, identified as *Pseudomonas fluorescens*, and designated as strain A2a5. The organism grew at temperatures below 30 °C, and accumulated a large amount of granules in its cells when it was cultured in the sugarcane liquor medium. The purified sample from cells was determined as PHB by gas chromatographic and nuclear magnetic resonance analysis of polyesters. The cell density of the culture in shaking bottles reached OD_{600} = 155 with PHB concentration of 31 g l^{-1}. In 5 l bioreactor, a maximum cell dry weight (CDW) of 32 g l^{-1} with a PHB concentration of 22 g l^{-1} was obtained, and the PHB content was up to 70% and productivity was 0.23 g l^{-1} h^{-1}.

Keywords: *Pseudomonas fluorescens* A2a5; Poly(β-hydroxybutyrate) (PHB); Sugarcane liquor; Gas chromatography; Nuclear magnetic resonance; Batch fermentation

1. Introduction

Polyhydroxyalkanoates (PHAs) represent a rather complex class of polyesters that are synthesized by most genera of bacteria and members of the Archaea [1]. The most studied microorganisms for PHA production are *Alcaligenes* sp. [2–4], *Azotobacter* sp. [5], *Bacillus* sp. [6] and *Pseudomonas* sp. [7–9]. Huisman et al. found that PHA formation appeared to be a common trait in a number of fluorescent pseudomonads. They are capable to synthesize PHAs during cultivation on fatty acids and related compounds [10].

Poly(β-hydroxybutyrate) (PHB), which is the simplest biopolyester of the PHA family, can be accumulated as an intracellular carbon-energy storage source by many bacteria that are challenged by nutrient limitation [11,12]. PHB is a biodegradable thermoplastic polyester that can be considered analogous to many conventional petrochemical-derived plastics currently in use [13,14]. Due to potential applications in medicine, agriculture and marine fields [15,16], the production of PHB by microorganisms has drawn much attention in recent years. However, one of the most important factors that would popularize the use of these polymers as conventional plastics is its production cost [17,18]. Therefore, less expensive substrates, improved cultivation strategies and easier downstream processing methods are required for reducing the cost [19,20].

In the present work, we isolated *Pseudomonas fluorescens* A2a5 that generated PHB from the soil in Alaska of USA, which showed that other *P. fluorescens* bacterial strains could produce PHB. Furthermore, relatively cheap sugarcane liquor was used instead of expensive yeast extract which could not be used for large scale cultivation. We designed and tested techniques for shake flask study and batch cultivation in 5 l bioreactor using the sugarcane liquor medium.

2. Material and methods

2.1. Microorganism

Poly(β-hydroxybutyrate)-accumulating strain *P. fluorescens* A2a5, which was isolated from the soil in Alaska of USA, was used in this study.

2.2. Medium

The strain was cultured aerobically at 20 °C in the sugarcane liquor medium containing 1.0 g l^{-1} KH_2PO_4, 2.5 g l^{-1} Na_2HPO_4. Relatively cheap sugarcane

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liquor was used in place of expensive yeast extract as a source of vitamins and minerals. Moreover, sugarcane liquor was also used as carbon source in concentration of 50% (v/v), while monosodium glutamate was used as nitrogen source in concentration of 1% (w/v). The pH of the medium was adjusted to 7.0 with 1 M NaOH.

In 5 l bioreactor, seed culture was prepared in a 250 ml Erlenmeyer flask containing 50 ml media. Batch cultivation was carried out at 25 °C in a 5 l bioreactor containing 3 l media. The bioreactor was sterilized at 121 °C for 20 min, cooled and then inoculated with 1% inoculum (v/v).

2.3. Identification of P. fluorescens A2a5

2.3.1. Morphological characteristics

Gram staining was performed as described by Magee et al. [21]. Morphology was examined by transmission electron microscopy. For transmission microscopy, centrifuged cell pellets were fixed with 4% glutaraldehyde and 1% osmium tetroxide. Ultra-thin sections of the sample embedded in epoxy resin were prepared with an ultramicrotome, stained with uranyl acetate and lead citrate, and examined with transmission electron microscope.

2.3.2. Biolog gram-negative (GN) assay

Biolog GN MicroPlate (Biolog Catalog #1011) was provided. The suspension of strain A2a5 cells in saline was used to inoculate Biolog GN plate (150 ml per well), and then incubated at room temperature for 24 h. Sample (10 ml) was removed from selected wells of each plate at specific times during the incubation period. The OD_{590} was measured with a turbidmeter after 4 and 24 h of incubation. For MicroPlate read at 4–6 h of incubation, the similarity index must be at least 0.75 to be considered acceptable species identification. At 16–24 h of incubation, the similarity index must be at least 0.50 to be considered acceptable. These two threshold values gave comparable levels of accuracy.

2.3.3. 16S rRNA gene amplification

PCR amplification was performed with the conserved 16S rRNA primer pairs [22]. Each PCR consisted of 5 μl of Ex-Taq polymerase buffer, 4 μl of 2.5 mM dNTPs, 4 μl of 2.5 mM $MgCl_2$, 1 μl of 0.5 mM each of the two primers, and 1 μl Ex-Taq polymerase (1 unit), brought to 50 μl with demonized water.

Amplification conditions were subjected to the three temperature cycles. PCR involved initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were then separated on a 1% agarose gel containing 1 mM ethidium bromide for visualization on an ultraviolet light box.

2.4. Poly(β-hydroxybutyrate) extraction

The cells were harvested and treated with 10% SDS at 100 °C for 20 min. After centrifugation, the pellets were washed, dried, and extracted with chloroform at 60 °C for 1 h. The non-PHB cell matter was removed by filtration, and the dissolved PHB was separated from chloroform by evaporation, washed twice with methanol, filtered out and dried at 60–70 °C.

2.5. Analytical methods

Microbial growth was monitored by measuring the cell density of the culture at 600 nm after suitable dilution with distilled water. Residual sucrose was measured by the phenolsulphuric acid method [23]. The content of residual inorganic nitrogen in sugarcane liquor was determined according to the Kjeldahl method [24]. Organic nitrogen in the samples was estimated following its mineralization with hot sulphuric acid. Both cell dry weight (CDW) and PHB quantification were determined gravimetrically. Cell concentration was defined as cell dry weight per litre of the culture medium. The PHB content was defined as the ratio of PHB concentration to cell concentration given as percentage.

2.6. Gas chromatography (GC)

A weighed amount (about 10 mg) of the sample was combined with 6 ml acidified methanol (1% H_2SO_4), and 2 ml chloroform. Samples were heated for 1 h at 100 °C in test tubes with Teflon-lined caps. After cooling to room temperature, 4 ml of distilled water was added. After vigorous shaking and centrifugation (3000 × *g* for 5 min), the chloroform phase containing the PHB methyl ester was transferred to a GC vial.

Gas chromatographic analysis was performed on a HPSF-1890 gas chromatograph, using a 0.25 mm diameter column 30 m in length. Samples (0.6 μl) were injected and the injection was done in the splitless mode. Nitrogen was the carrier gas at a flow rate of 40 ml min^{-1}. The analysis started at 80 °C for 2 min, whereupon the temperature was increased to 195 °C at a rate of 8 °C min^{-1}. After reaching 195 °C, this temperature was maintained for 20 min before the analysis was terminated [25–27].

2.7. Nuclear magnetic resonance spectroscopy (NMR)

The nuclear magnetic resonance spectrum was recorded at on a BRUAK-400 spectrometer, using a 5 mm $^1H/^{13}C$ dual probe, and deuterated chloroform was used as solvent. The ^{13}C NMR spectrum, which was utilized to identify the structure of PHB, was recorded at 100.62 MHz. For analysis, 10 mg of sample and 1 ml of solvent were employed.

3. Results and discussion

3.1. Morphological and physiological characteristics

The morphological characteristics of strain A2a5 were mainly as described below. It was a gram-negative, bacillus-shaped, non-spore-forming bacterium. The cells occurred singly or in cluster, and could accumulate massive amounts of PHB and, not surprisingly, the cells became swollen (Fig. 1a and c). Electron micrographs of thin sections showed that the type of cell division was schizogenesis (Fig. 1b).

Strain A2a5 exhibited a doubling time of about 6 h in the sugarcane liquor medium at pH 7.0 and 20 °C. Visible colonies appeared on agar plates after 1 day of incubation. The colonies were circular (diameters, 0.5–1 mm), smooth, convex, and cream colored at the early stage of growth. After 3 days of incubation, the colonies were 5–6 mm in diameter and yellowish in color, and could produce water-soluble yellow fluorescent pigments.

The physiological characteristics of strain A2a5 were as follows. The sugarcane liquor medium was used to evaluate growth temperature and pH. The temperature range for growth was 15–30 °C, and the optimum temperature was 20–25 °C, however, the strain A2a5 could not grow over 30 °C. The pH range for growth was 5.0–8.0, and optimum growth occurred at pH 6.5–7.0.

3.2. GN MicroPlate performance characteristics

The database was designed to give identifications of all species in the database, in accordance with current standards of classical identification methods and current taxonomic nomenclature. The GN MicroPlate performance characteristics, which had been determined by establishing a database from a large collection of clinical and environmental microorganisms, indicated that the strain A2a5 was similar to the species *P. fluorescens*. The similarity index of the species was 0.77 at 24 h.